PHYSIOLOGICAL DYNAMICS OF DORMANCY IN APPLE BUDS GROWN IN AREAS WITH INSUFFICIENT WINTER CHILL

by

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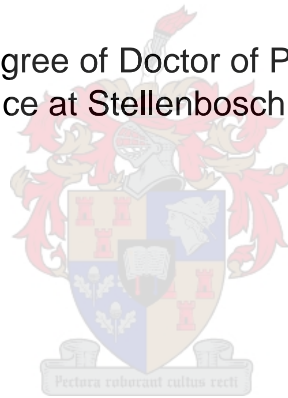

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the the authorship owner thereof (save to the extent explicitly otherwise stated) that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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DEDICATIONS

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SUMMARY

Physiological dynamics of dormancy in apple buds grown in areas with insufficient winter chill.

Apple trees, that do not fulfil their winter chill requirement, undergo inadequate dormancy release resulting in poor budbreak with irregular and delayed flowering that impact negatively on fruit production and tree architecture. Under mild winter conditions, such as most production sites in the Western Cape region of South Africa, the application of chemical rest breaking agents is standard practise to artificially release dormancy and synchronise budbreak in spring to ensure sustainable and profitable apple production. However, rest breaking chemicals can be harmful to the buds and may result in phytotoxic damage if not applied optimally, therefore a good understanding of the dormancy status of the buds is valuable and necessary to mitigate this risk when applying a rest breaking agent. Furthermore, little information is available on the physiological and biochemical dynamics of commercially produced apple buds when under these milder conditions and no information is known for the 'Cripps Pink' variety when grown under climatically contrasting South African conditions. The main aim of this study was to investigate the effect of inadequate winter chill and the application of a blend of hydrogen cyanamide and mineral oil (HCo) as a rest breaking agent on the respiration rate and lipid composition of the terminal buds of full bearing 'Cripps Pink' apple trees. Dormant apple buds from two climatic regions, Elgin (insufficient winter chill) and the Koue Bokkeveld (sufficient winter chill) were compared in terms of their respiration rate and lipid composition. Buds from the Elgin region were also tested and compared after receiving a commercial HCo treatment at budswell. Shoots were excised and given a specified amount of artificial chill in the laboratory to act as a point of reference.

Apple buds exposed to sufficient chill (orchard and laboratory) showed an early dormancy entrance, high maximum dormancy level and an early release, while buds from the milder area generally showed atypical dormancy behaviour. During winter, the total respiration rate and the rate of the main respiratory pathways (tricarboxylic acid cycle (TCA) and cytochrome C (CYT)) were reduced with the decline in temperature. Their levels increased again in the cold area at the beginning of spring to provide energy for growth resumption. In contrast, the main respiration levels remained low in the warm area and the pentose phosphate pathway (PPP) and alternative pathway (ALT) tested higher suggesting an attempt by the plant to compensate for the deficiency in energy production. The HCo treatment induced hypoxia in the buds and immediately decreased the total respiration as well as the main respiratory pathways

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(TCA and CYT). After this initial response the treated buds showed a significant increase in respiration and reached high levels towards budbreak and green-tip stage. This increase was not observed in the untreated buds from the mild winter climate, in contrast, these buds maintained a higher use of the PPP and ALT pathways. It was also seen that the HCo treatment followed by warmer spring temperatures hastened the occurrence of the green-tip stage.

Both cold and warm winter temperatures also affected the lipid composition in the apple buds at the end of the winter period towards the onset of spring. Warmer winter conditions reduced the desaturase process preventing the desaturation of linoleic acid to linolenic acid, a polyunsaturated fatty acid with three double bonds. Lower free phospholipids and higher free sterols content were also detected in buds from the warmer area and is thought to reduce the membrane fluidity and permeability hampering budbreak and growth resumption even under favourable spring conditions. The high saturated and monounsaturated fatty acids in buds from the mild winter area confirmed the results and again pointed towards less fluid and permeable membranes. The HCo treated buds from a mild winter area showed results similar to buds that received sufficient winter chill, characterised by induced desaturation and higher linolenic acid levels enabling earlier budbreak. It is suggested that the artificial rest breaking of terminal apple buds, via the use of HCo, targets similar biological pathways and physiological mechanisms as the natural accumulation of sufficient chill when inducing growth resumption.

In general, the physiological aspects under investigation in this study provided evidence that treatment with HCo, in the area with insufficient winter chill, act very similar to sufficient winter chill. It enhanced the main respiration pathways and stimulated the production of polyunsaturated fatty acid, which in turn, provides more fluid and permeable membranes that increase energy production needed for growth resumption in spring. Results from this study provide evidence that future development of artificial ways to target these same systems may improve apple cultivation in marginal production areas, especially in the Western Cape of South Africa.

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OPSOMMING

Die fisiologiese dinamika van dormansie in appelknoppe vanaf streke met onvoldoende winterkoue.

Appelbome, waarvan die koue vereiste nie beveredig is nie, ondergaan 'n onvoldoende dormansieopheffing wat lei tot swak knopbreek, onreëlmatige en vertraagde blot en het 'n negatiewe impak op vrugproduksie en boomargitektuur. Onder matige winterkondisies, soos in meeste van die produksie areas in die Wes-Kaap streek van Suid-Afrika, is die gebruik van chemiese rusbreekmiddels standaardpraktyk om dormasie kunsmatig op te hef, knopbreek in die lente te sinkroniseer en so volhoubare, winsgewende appelproduksie te verseker. Daar is egter min informasie beskikbaar rakende die fisiologiese en biochemiese dinamika van kommersieël geproduseerde appelknoppe onder hierdie matige winterkondisies en geen informasie is beskikbaar oor die 'Cripps Pink' kultivar onder klimaatkontrastrerende toestande in Suid-Afrika. Die hoof doel van hierdie studie was om die effek van onvoldoende winterkoue en die toediening van 'n mengsel van waterstofsianamied en mineral olie (HCo), as 'n rusbreekmiddel, op die respirasietempo en lipiedsamestelling van terminale knoppe van voldraende 'Cripps Pink' appelbome te ondersoek. Dormante knoppe van twee klimaatkonstrasterende gebiede, Elgin (onvoldoende winterkoue) en die Koue Bokkevled (genoegsame winterkoue), is vergelyk in terme van hul respirasietempo en lipiedsamestelling. Knoppe van die Elgin area is ook getoets en vergelyk nadat 'n kommersieële behandeling van HCo toegedien is tydens knopswel. Lote is ook gesny en 'n spesifieke hoeveelheid kunsmatige koue in die laboratorium gegee om te dien as verwysingspunt.

Appelknoppe wat bootgestel is aan genoegsame koue (boord en laboratorium) het in vroeë ingang in dormansie, 'n diep maksimum dormasievlak en 'n vroeë uitgangspatroon getoon terwyl knoppe van die matige area oor die algemeen atipiese dormansiegedrag gehad het. Gedurende die winter het die totale respirasietempo en die tempo van die hoof respirasiepadweë (trikarboksielsuur sikus (TCA) en sitokroom C (CYT)) verlaag met die daling in temperatuur. In die koue area het hul vlakke weer aan die begin van die lente gestyg om so energie te verskaf vir die aanvang van groei. Daarteenoor, het die hoof respirasievlakke van die knoppe in die warmer area laag gebly en die pentosefosfaat padweg (PPP) en alternatiewe padweg (ALT) het hoër getoets wat 'n poging deur die plant impliseer om te kompenseer vir die tekortkoming in energieproduksie. Die HCo behandeling het moontlik hipoksia in die knoppe geïnduseer wat 'n onmiddelike afname in die totale repirasie asook die hoof respirasiepadweë (TCA en CYT) veroorsaak het. Na hierdie aanvanklike reaksie het die behandelde knoppe 'n merkbare toename en hoë vlakke van respirasie getoon naby knopbreek en tydens die groenpunt stadium. Hierdie

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toename was nie waargeneem in die kontrole (onbehandelde) knoppe van die matige klimaatstreek nie, daarteenoor, het hierdie knoppe hoër vlakke van die PPP en ALT padweë gehandhaaf. Daar is ook gevind dat die HCo behandeling wat deur warmer lente kondisies gevolg is die voorkoms van die groepunt stadium verhaas het.

Beide koue en warm winter temperature het ook die lipiedsamestelling in die appelknoppe aan die einde van die winterperiode en begin van die lente beïnvloed. Warm winterkondisies verlaag die desaturasie-proses wat die onversadiging van linolïensuur na linoleniese suur, 'n polionversadigde vetsuur met drie dubbelbindings, verhoed. Lae vry fosfolipied en hoë vry sterool inhoud is ook waargeneem in knoppe van die warmer area en daar word vermoed dat dit die vloeibaarheid en deurlaarbaarheid van die membrane verminder en so knopbreek en die aanvang van groei vertraag selfs onder gewensde lente toestande. Die hoë versadigde en mono-onversadigde vetsure in die knoppe van die matige winterstreek bevestig die resultate en verwys weer na 'n laer vloeibaarheid en deurlaatbaarheid in die membrane. HCo behandelde appelknoppe van matige winterstreke toon dieselfde resultate as knoppe wat genoegsaam winterkoue ontvang het en is gekarakteriseer deur geïnduseerde desaturasie en hoë linoleniese suur vlakke wat vroeë knopbreek tot gevolg gehad het. Dit stel voor dat die kunsmatige rusbreking van terminale appelknoppe, deur die gebruik van HCo, dieselfde biologiese padweë en fisiologiese meganismes as die natuurlike akkumulasie van genoegsame winterkoue gebruik om groei te induseer. Oor die algemeen bied die fisiologiese aspekte, wat deur die studie ondersoek is, bewyse dat die behandeling van HCo dieselfde meganismes gebruik as genoegsame winterkoue deur die hoof respirasiepadweë en die produksie van poli-onversadigde vetsure te stimuleer wat tot gevolg het dat die membrane meer vloeibaar en deurlaatbaar word en sodoende die energie produksie, wat nodig is vir die hervatting van groei in die lente, aanmoedig.

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NOTE

This dissertation presents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters, therefore, has been unavoidable.

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GENERAL INTRODUCTION AND SCOPE

Commercial apple production, like production of other temperate fruit, partly depends on sufficient chill accumulation during the winter period. With the onset of colder environmental conditions, the tree enters a phase of rest/dormancy. This phase change occurs naturally and developed as a survival mechanism against harmful low temperatures and to synchronise growth resumption in the following spring when the environment warms up again. The shoot apical meristem is known to control this dormancy cycle (Van der Schoot and Rinne, 2011). Although the bud dormancy cycle is regulated simultaneously by numerous internal and external factors, low temperature is reported to be the critical factor that induces the establishment of dormancy and drives its maintenance and release in apple and pear (Heide and Prestrud, 2005).

Lang *et al.* (1987) defined three types bud dormancy: 1) ecodormancy, that is the absence of growth due to unfavourable conditions outside of the plant (i.e. low temperatures), 2) paradormancy, or the inability to grow due to inhibitory signals from within the plant but outside of meristem, and 3) endodormancy, where the meristem is dormant due to a physiological block inside the meristem itself. Generally, bud dormancy establishment suppresses cell division and growth (Heide, 2003; Heide and Prestrud, 2005). Typically, dormancy acquisition coincides with the hardening of the buds, which is accelerated by leaf fall and the accumulation of chill (Cooke *et al.*, 2012). A maximum dormancy level is reached when a specific amount of chill is accumulated by the tree (Lang *et al.*, 1987; Faust *et al.*, 1997; Rohde and Bhalerao, 2007).

The chill requirement (CR) is a genetic trait that differs, not only among plants or cultivars, but also among species, types of meristems and geographic location (Hauagge and Cummins, 1991; Campoy *et al.*, 2011). Fuchigami and Nee (1987) showed that the dormancy level decreases as the CR is fulfilled and environmental conditions become conducive for growth in the spring. At such time, the meristems are released from their growth restrictions and budbreak is induced resulting in resumption of cell division and ultimately visible growth.

Under mild climatic conditions, there is insufficient accumulation of chill and the chilling requirement of deciduous fruit trees are often not met (Midgley and Lötze, 2011). Cook and Jacobs (1999 and 2000) reported differences in the dormancy progression of apple buds in two climatically contrasting production areas in South Africa. Several studies have shown that inadequate winter chilling delays dormancy release and causes unsynchronised budbreak that negatively impacts on fruit set and quality, and alters

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the natural tree architecture (Blommaert, 1963; Cook and Jacobs, 1999 and 2000; Petri and Leite, 2004; Atkinson *et al.*, 2013). These symptoms have practical implications for commercial fruit production and reduce profitability. Although progress has been made to breed cultivars that can respond to insufficient chilling, it is time consuming and often the fruit from such cultivars do not have the desired storability for countries that are far removed from their export markets. The only current option in marginal production areas is the use of chemical rest breaking agents together with practical tree manipulation techniques to solicit efficient budbreak in spring (Cook, 2010). Currently, the spray application of hydrogen cyanamide combined with mineral oil (HCo) is widely used to partially compensate for the lack of chill and ensure acceptable levels of budbreak needed to maintain profitable yields in areas with warm winters (Erez, 1995, Faust *et al.*, 1997), such as South Africa (North, 2003). Despite of their efficacy, these chemicals can cause phytotoxicity injury to buds resulting in poor fruitset when not applied optimally. Knowledge on the physiological status of the bud during insufficient chill can assist in mitigating this risk as it has been shown that many compounds can be successful in breaking dormancy when the chilling requirements were not fulfilled (Erez et al., 2008).

The rest breaking capabilities of both adequate winter chill and hydrogen cyanamide (HC) have been ascribed to their ability to increase oxidative stress by inhibiting catalase activity (Nir and Lavee, 1993; Beauvieux *et al.*, 2018) and alter respiratory pathways (Vergara *et al.*, 2012; Amberger, 2013) in such a way that it leads to dormancy release and budbreak. It has been shown that the inhibition of catalase, as well as hypoxic conditions, have a short and long term effect on the bud physiology that ultimately regulates plant development (Vergara *et al.*, 2012). Bud lipid studies of perennial temperate fruit species reported changes in the fatty acid composition, free sterol and phospholipid concentrations under conditions of sufficient winter chill and showed that such changes increase the membrane fluidity and permeability (Wang and Faust, 1988, 1990a and 1990b; Izadyar and Wang, 1999).

This information on the physiological dynamics brought about by sufficient chill assists researchers by revealing the physiological mechanisms and biochemical systems that are targeted by chilling temperatures to release dormancy and aids in the development of artificial ways to target these same systems to mimic the physiological outcomes (Beauvieux *et al.*, 2018). This project aimed to contribute to this pool of knowledge and understanding of the physiological dynamic within dormant apple buds cultivated under sufficient and insufficient winter conditions and to compare it to the effects of an HCo application in the beginning of spring. Findings may assist in the future development of products and procedures to improve apple cultivation in marginal production areas (region) by improving productivity and profitability, especially in the Western Cape of South Africa.

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The dissertation commences with a literature review on "*Bud dormancy progression and the effect of winter chill*". The review contains a discussion on bud status, the pattern of bud dormancy progression, its measurement during seasonal progression and how different winter climates influence budbreak. These sections serve as a backdrop for more focussed discussions on the dynamics of respiratory pathways and lipid composition which are the two physiological aspects investigated in the research chapters. Excluded from the literature review are aspects such as seed dormancy, the influence of photoperiod, cold hardening, gene expression and molecular approaches to dormancy. Although such topics are relevant, the functional complexity of the physiological behaviour of perennial plants during dormancy necessitated narrowing the scope to two aspects that are fundamentally involved in the budbreak process, namely changes in respiration and lipid composition.

The research experiments were conducted on 'Cripps Pink' apple buds in two climatically contrasting apple production areas of the Western Cape, South Africa. In Paper 1, the respiration rate and respiration pathways were studied under sufficient and insufficient winter conditions as the effect of temperature changes on these mechanisms influences the availability of ATP production necessary for budbreak and growth in the following spring. In Paper 3, changes in bud lipid composition were investigated to indicate the status of stored energy and membrane flexibility and permeability during dormancy and budbreak. These two aspects were extended in Paper 2 and Paper 4, respectively, to include results from buds exposed to hydrogen cyanamide and mineral oil (HCo) to study the mechanisms of rest breaking in buds that received insufficient winter chill. Findings from two dormancy cycles and two commercial scale HCo applications (2015 and 2016) are presented. A breakdown of the physiological aspects and the flow of the research papers are illustrated in Figure 1.

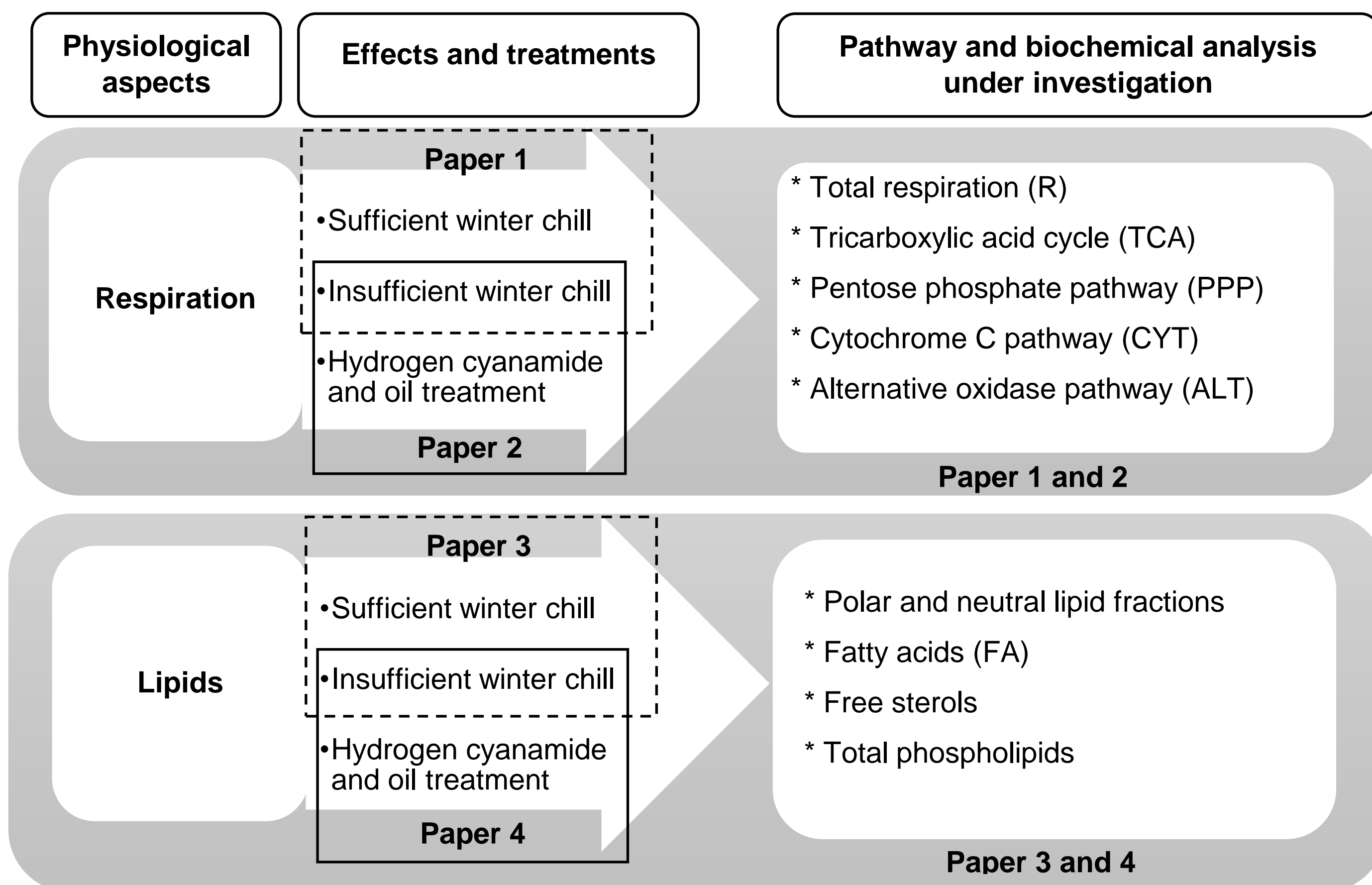

Figure. 1. Research framework of this dissertation.

References

Atkinson, C.J., Brennan R.M. and Jones, H.G. (2013). Declining chilling and its impact on temperate perennial crops. *Environmental and Experimental Botany*. **91**, 48-62.

Beauvieux, R., Wenden, B. and Dirlewanger, E. (2018). Bud dormancy in perennial fruit tree species: A pivotal role for oxidative cues. *Frontiers in Plant Science*. **9**, 657.

Blommaert, K. L.J. (1963). Winter rest of deciduous fruit trees in relation to the problem of delayed foliation. *South African Journal Science*. **59** (7), 316–319.

Cook, N.C. and Jacobs, G. (1999). Sub-optimal winter chilling impedes development of acrotony in apple shoots. *HortScience*. **34**, 1213-1216.

Cook, N.C. and Jacobs, G. (2000). Progression of apple (*Malus x domestica* Borkh.) bud dormancy in two mild winter climates. *Journal of Horticultural Science*. **75**, 233-236.

Cook, N.C. (2010). Apple Production under Conditions of Sub-Optimal Winter Chilling in South Africa. *Acta Horticulturae*. **872**, 199-204.

Cooke, J.E.K., Eriksson, M.E. and Junttila, O. (2012). The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. *Plant Cell Environment*. **35**, 1707-1728.

Campoy J.A., Ruiz D., Cook N., Allderman L. and Egea J. (2011). Clinal variation of dormancy progression in apricot. *South African Journal of Botany*, **77**, 618–630.

Erez, A. (1995). Means to compensate for insufficient chilling to improve bloom and leafing. Acta *Horticulturae*, **395**, 81-95.

Erez, A., Yablowitz, Z., Aronovitz, A. and Hadar, A. (2008). Dormancy breaking chemicals – efficiency with reduced phytotoxicity. Acta *Horticulturae*, 772, 105 –112.

Faust, M., Erez, A., Rowland, L.J., Wang, S.Y. and Norman, H.A. (1997). Bud dormancy in perennial fruit trees: Physiological basis for dormancy induction, maintenance and release. *HortScience*. **32**(4), 623-629.

Fuchigami, L.H. and Nee, C.C. (1987). Degree growth stage model and rest-breaking mechanisms in temperate woody perennials. *HortScience*, **22**, 836–845.

Hauagge, R. and J.N. Cummins. (1991b). Phenotypic variation of length of bud dormancy in apple cultivars and related *Malus* species. *Journal of the American Society for Horticultural Science*. **116**,100-106.

Heide, O.M. (2003). High autumn temperature delays spring budburst in boreal trees, counter balancing the effect of climatic warning. *Tree Physiology*. **23**, 931–936.

Heide, O.M. and Prestrud, A.K. (2005). Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. *Tree Physiology*. **25**, 109 –114.

Izadyar, A.B. and Wang, S.W. (1999). Changes of lipid components during dormancy in 'Hull Thornless' and 'Triple Crown' blackbery cultivars. *Scientia Horticulturae.* 82, 243-254.

Lang, G.A. (1987). Dormancy: a new universal terminology. *HortScience*. **22**, 817–820.

Lang, G.A., Early, J.D., Martin, G.C. and Darnell, R.L. (1987). Endo-, para- and eco-dormancy physiological terminology and classification for dormancy research. *HortScience*. **22**, 371-377.

Midgley, S.J.E. and Lötze, E. (2011). Climate change in the western cape of South Africa: trends, projections and implications for chill unit accumulation. *Acta Horticulturae*. **903**, 1127-1134.

Nir, G. and S. Lavee. (1993). Metabolic changes during cyanamide induced dormancy release in grapevines. Acta *Horticulturae*. **329**, 271-274.

North, M.S. (2003). Alternative rest-breaking agents evaluated on 'Golden Delicious' apple trees. *South African Journal of Plant and Soil*. **20**, 59-63.

Petri, J.L. and Leite, G.B. (2004) Consequences of insufficient winter chilling on apple tree budbreak. Acta *Horticulturae*. **662**, 53-60

Rohde, A. and Bhalerao R.P. (2007). Plant dormancy in the perennial context. *Trends Plant Science*. **12**, 217-223

van der Schoot, C. and Rinne, P.L.H. (2011). Dormancy cycling at the shoot apical meristem: transitioning between self-organization and self-arrest. *Plant Science* **180**, 120-131.

Vergara, R., Rubio, S. and Pérez, F.J. (2012). Hypoxia and hydrogen cyanamide induce bud-break and up-regulate hypoxic responsive genes (*HRG*) and *VvFT* in grapevine-buds. *Plant Molecular Biology*. **79**, 171–178.

Wang, S.Y. and Faust, M. (1988). Changes of fatty acids and sterols in apple buds during budbreak induced by a plant bioregulator, Thidiazuron. *Physiologia Plantarum*. **72**, 115–120.

Wang, S.Y. and Faust, M. (1990a). Seasonal changes of membrane lipids in apple shoots. *Journal of the American Society for Horticultural Science*. **115**, 462–467.

Wang, S.Y. and Faust, M. (1990b). Changes in membrane lipids in apple buds during dormancy and budbreak. *Journal of the American Society for Horticultural Science*. **115**, 803–808.

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1. Introduction

1.1. Apple tree origin and economic benefits for South Africa

The domesticated apple (*Malus x domestica* Borkh.) is a deciduous, perennial tree. It belongs to the Rosaceae family, which includes many genera and species that are divided into three subfamilies, of which the Spiraoideae and Rosoideae are known to include the economically important species. Apple trees are part of the Spiraoideae subfamily, which also includes *Pyrus* (pear) and *Prunus* (peach, cherry, almond and apricot) (Hummer and Janick, 2009). The domesticated apple cultivars known today all originate from native species found in the Tien Shan mountain range (42°02′N; 80°07′E) on the border between China and Kazakhstan (Dzhangaliev, 2003; Panyushkina *et al.*, 2017) with expansion along this latitude and areas with similar climatic conditions. Containing phytochemicals, the apple fruit is a rich source of antioxidants and may reduce the risk for contracting illnesses such as asthma, diabetes and cancer (Boyer and Liu, 2004).

Apple trees are commercially cultivated on a large scale in many countries including South Africa (SA) (Hortgro, 2018). In terms of hectares under cultivation, number of cultivars produced and tonnage of fruit sold on international markets, apples are among the most important deciduous fruits grown in SA, with the main production area being the Western Cape Province. The SA apple industry is economically profitable, generates foreign exchange earnings and creates employment. Annual reports show that the area under cultivation is steadily growing and increased from 22 167 ha in 2012 to 24 156 ha in 2017 (Hortgro, 2018). After grapes, apples are the second-most cultivated deciduous fruit in SA (GAIN Report, 2017) and apple production is estimated to grow by two percent annually. Producing 777 741 tonnes per annum, SA is estimated to be the world's 16th largest apple producer. The country produced approximately one percent of the world's apples in 2014 (GAIN Report, 2016). SA exports about 44% of its total apple production and the largest markets are the Far East and Asia (31%), the rest of Africa (30%) and the United Kingdom (18%). It also supplies the local apple market (25% of production) while the remaining 31% of the crop is processed (Hortgro, 2018).

1.2. Growth and dormancy cycles

Like other temperate deciduous trees, apple trees go through two important annual stages, i.e. the growth and the dormant cycles. The growth cycle normally starts in spring with budswell under favourable growing conditions and stops with leaf fall during autumn. The rate of tissue growth is faster during spring and gradually slows towards autumn (Cooke *et al.*, 2012). The dormant period naturally starts during autumn and goes through different phases before it ends at the beginning

of spring. During winter, apple trees suspend all visible signs of growth. This is important for the tree's survival during harsh winter conditions and also presents an opportunity to synchronise growth for the following season (Van der Schoot and Rinne, 2011; Cooke *et al.*, 2012). Low temperature is one of the most important environmental factors that induce and maintain dormancy until the end of the winter (Cooke *et al.*, 2012). During the dormant period, buds are governed by internal and external factors that influence plant growth and development (Rohde and Bhalerao, 2007). Dormancy gradually increases in depth, but after the maximum level is attained, the growth inhibition is gradually released by a period of low temperature or CR that is crop, cultivar and species specific (Hauagge and Cummins, 1991a; Dennis, 1994; Cooke *et al.*, 2012).

1.3. Scope

This literature review describes bud status during bud dormancy and its release. It discusses the pattern of bud dormancy development, its measurement and seasonal progression. It aims to illustrate how insufficient winter temperatures influence budburst, especially in the recent past, when warmer winters were experienced, also in the Western Cape. The literature review discusses some of the main physiological changes that occur during bud dormancy in cold and warm winter climates as well as some of the methods used to overcome dormancy. This review will not include, but may refer to, seed dormancy. Apple trees are not responsive to day length (Heide and Prestrud, 2005), thus this review will not discuss the influence of a reduced photoperiod (short days). Cold hardening is the process whereby growing plant parts undergo physiological changes to avoid, or mitigate cellular injuries caused by sub-zero temperatures and precedes the winter dormancy period (Kuroda *et al.*, 1990). This complex physiological behaviour and state will not be discussed in this review. Gene expression and molecular approaches to investigating bud dormancy are also excluded, as this is a field of rapid growth and has been reviewed recently (Cooke *et al.*, 2012; Preston and Sandve, 2013; Wisniewski *et al.*, 2015).

2. Bud dormancy progression

2.1. Definition, terminology and progression

Dormancy is a vast and complex topic. It is the native rest cycle that follows the active cycle in the annual vegetative activity of deciduous fruit trees. It is seasonal and requires the interaction of a number of factors (Crabbé and Barnola, 1996). Dormancy starts with the cessation of cell division, which is mainly induced by environmental conditions (cold/low temperature, or drought) although genetic factors in the plant also play a part. Generally, dormancy is established once

growth has stopped (Rohde and Bhalerao, 2007). During dormancy, the meristem is maintained in a state incapable of growth due to a combination of factors and growth is initiated again when favourable conditions occur. Lang *et al.* (1987) formulated the first scientifically recognised definition of dormancy as “a temporary suspension of visible growth of any plant structure containing a meristem”. According to this definition, dormancy occurs when the meristem stops growing yet has the capacity to grow again once favourable conditions have been met. Lang *et al.* (1987) also organised dormancy into three phases namely para- endo and ecodormancy, which is still widely used today to describe the seasonal growth and dormancy cycle of perennial fruit trees. Rohde and Bhalerao (2007) revised this definition as the “inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favourable conditions”. This definition implies the absence of morphological changes during dormancy, even though its progression can be detected physiologically and molecularly (Horvath *et al.*, 2003; Rohde and Bhalerao, 2007). The definition of Rohde and Bhalerao (2007) focusses more on the plant’s ability to initiate growth whereas Lang *et al.* (1987) is concerned with the plant’s ability to terminate growth. As both of these processes (initiation and termination of growth) are important in the life cycle of perennials the two definitions complement each other and serve as a more complete characterisation of the dormant period of a plant. Both definitions are acceptable and used interchangeably in literature.

During paradormancy, growth is inhibited from a location within the plant other than the meristem itself (Lang *et al.*, 1987). This type of dormancy is seen during apical dominance, where auxin (IAA) synthesised in the terminal bud inhibits the growth of subtending buds (Bangerth *et al.*, 2000). Chao *et al.* (2007) state that basipetal auxin transport (from terminal to lateral buds) is known as the key signal regulating paradormancy. Traditionally, the plant hormones cytokinin (CK) and gibberillic acid (GA) have been identified as the stimulators of growth in the lateral buds and IAA, abscisic acid (ABA) repressing growth (Bangerth *et al.*, 2000), but during the last decade strigolactones (SL) were identified as also playing a pivotal role in limiting the outgrowth of lateral buds (Cheng *et al.*, 2013). SL are thought to act antagonistically to CK by stimulating the BRC1 transcription factor that inhibits branching (Dun *et al.*, 2012) and at the same time increase the competition between the main IAA stream and the subtending bud by reducing (“dampening”) the polar auxin transport in the main stream (Crawford *et al.*, 2010).

Shoot growth appears to be governed by the ratio of growth promoting hormones, i.e. IAA and GA, to growth inhibitors such as ABA. Terminal bud set is marked by increased ABA (and other inhibitors) levels and a concurrent decrease in promoters such as CK, IAA and GA (Salisbury and Marinos, 1985; Seeley, 1990) until a promoter-inhibitor threshold is ultimately reached where ABA

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is able to inhibit the elongation of internodes by GA (Borkowska, 1981). Borchert (1991) provides a comprehensive summary of the possible factors giving rise to growth periodicity. Once formed, the terminal buds are paradormant due to several inhibiting factors outside of the bud, i.e. strong ABA influence from mature leaves (Singha and Powell, 1978) and/or competition between growing points (shoots, leaves and/or fruit) for nutrients (Notodimedjo *et al.*, 1981). Such paradormant effects can be alleviated through defoliation, pruning or harvesting and is then often followed by regrowth of the terminal bud (flushing). Environmental effects such as drought conditions and high temperature can increase the competition for nutrients, water and hormones and further enhance the paradormancy level of the terminal buds (Borchert, 1991). If this paradormant state, in both the lateral and terminal buds, is not released through methods discussed above, the terminal and lateral buds eventually become endodormant.

During endodormancy, growth is inhibited from within the meristem itself (Lang *et al.*, 1987). Also called true, deep or winter dormancy, this rest phase is an effect of internal physiological modifications that inhibit growth. It occurs in early winter for perennialfruit trees and protects vegetative buds by preventing the meristem from resuming growth until favourable environmental conditions (i.e. warmer spring temperatures) are achieved. ABA plays a key role in the induction of endodormancy and could act as a metabolic inhibitor of meristem activity and bud respiration. ABA is reported to decrease again when growth resumes with the increase in CK and GA levels in spring (Tanino, 2004; Rinne *et al.*, 2011; Zheng *et al.*, 2015; Parada *et al.*, 2016). Endodormancy reaches a maximum level and is then released when the required amount of chill or chilling units (CUs) have accumulated. Rest breaking agents (RBAs) or other agents, typically used to release endodormancy during mild winters or in more temperate areas, can only be effective once a specific number of CUs has accumulated (Fuchigami and Nee, 1987; Faust *et al.*, 1997).

Towards the end of winter when temperatures get warmer, bud hardiness decreases, buds exit endodormancy and budbreak occurs when temperatures permit (Powell, 1986; Hauagge and Cummins, 1991; Naor *et al.*, 2003). If this state coincides with conditions that are warm and favourable for growth, synchronised budbreak takes place (Rohde and Bhalerao, 2007; Horvath, 2009). If sufficient chill was accumulated during the winter, the requirement for warm temperatures is reduced and relatively cool spring temperatures will allow resumption of growth and budbreak (Scalabrelli and Couvillon, 1986). If the spring temperatures do not result in sufficient heat units, budbreak will be inhibited, putting the bud in a state of ecodormancy. Thus, during ecodormancy, growth inhibition is determined by the environmental conditions outside of the plant and not by biochemical regulation (Lang *et al.*, 1987). Unlike para- and endodormancy,

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mechanisms involved in ecodormancy repress the rate of metabolism and therefore the production of energy needed for growth. It is also thought that ABA is still perceived during this phase and blocks growth development and cell division (Horvath *et al.*, 2003).

In summary, lateral buds may be paradormant due to apical dominance before the cessation of shoot growth. The terminal bud follows suit due to correlative inhibition established by ABA production and/or a decrease in CK and GA (Bangerth *et al.*, 2000; Guak and Fuchigami, 2001; Rinne *et al.*, 2011). Lower temperatures can hasten this process and initiate cold hardening through the lignification of the shoot and the formation of bud scales. A state of endodormancy starts to develop in the buds and once the required chill accumulation has occurred, the same low temperatures break down the physiological block within the buds, alleviating endodormancy and leaving the bud ecodormant. The buds themselves now have a favourable growth status but await sufficient warm spring temperatures to stimulate the energy production needed for budbreak. Although it is difficult to see which part of the plant is responsible for dormancy, the growth-inhibition factor shifts from the outside of the meristem to within and subsequently to environmental conditions (Faust *et al.*, 1997). The process is regulated, from induction to release, through different factors, including environmental, genetic and physiological signals within the plant (Horvath *et al.*, 2003, Rohde and Bhalerao, 2007). Although factors influencing dormancy induction vary and depend on species and ecotype (Rohde and Bhalerao, 2007), the gradual release of endodormancy, on the other hand, depends mainly on low temperatures while the rate of dormancy release increases relative to the preceding chill exposure period of the plant.

2.2. Evaluation of dormancy levels

Dormancy is a continuous annual process that progresses from paradormancy through endodormancy to ecodormancy. There are various biological, biochemical and anatomical approaches by which dormancy progression can be measured and evaluated.

2.2.1. Biological approaches

To date, many researchers have adopted and adapted biological methods that are widely used to evaluate dormancy dynamics in perennial fruit-tree buds. The two original methods that were developed are the floral bud dormancy test developed by Tabuenca (1964) and the single-node cutting test of Erez *et al.* (1979a).

Tabuenca's test (described in Lalanne-Tisné *et al.*, 2017) involves the forcing of single buds during the endodormancy period. This method measures the evolution of floral bud weight by comparing the weight of the buds immediately after field collection and again after forcing them

at optimum growth conditions. A considerable increase in fresh weight of floral primordia of peach and apple cultivars indicated the ability to grow, hence endodormancy release (Lalanne-Tisné *et al.*, 2017). Although this method has been used successfully in apples, it was not effective in determining the end of endodormancy (Albuquerque *et al.*, 2017). However, it did indicate that cold deprivation results in low and stable fresh weight of peach buds and an absence of dormancy release (Bonhomme *et al.*, 2005). Warm winters or unforced conditions delayed the increase in fresh and dry weight of floral primordia and resulted in phenological changes and uneven budbreak (Yaacoubi *et al.*, 2016). Tabuenca's test appears to be useful in the absence of more accurate methods, but this is not a perfect tool.

The single-node cuttings method is used to evaluate the intensity of endodormancy and budbreak of temperate fruit tree buds. It involves the collection of one-year old shoots from field conditions, cutting them into single nodes and stimulating them to sprout by putting them at optimum growth conditions in a growth chamber. During this process, the time to budbreak is recorded and interpreted as the depth of dormancy. It is assumed that the longer the bud takes to start sprouting under ideal conditions, the deeper the level of dormancy. According to Dennis (2003), this test was first used in a series of rooted cutting experiments on peach to measure temperature effects on budbreak (Erez *et al.*, 1979a). The single-node cutting test was used in early studies to determine growth capacity or budbreak through the three phases of dormancy (Herter *et al.*, 1988; Bonhomme *et al.*, 2005). It was reported to provide good results of the depth of dormancy; Balandier *et al.* (1993) monitored budbreak through this method and highlighted that the decrease in the intensity of dormancy was faster in cold than in warm temperature conditions and also that dormancy exit depends on the chilling requirements of the cultivars in use. The single-node cutting method is still in use today and Schmitz *et al.* (2015) recently showed the influence of bud position and water content on dormancy release in the low chilling apple cultivar 'Eva' grown under warm winter conditions. However, the shoot cuttings method (multiple nodes) developed on sweet cherry (*Prunus avium* L.) by Arias and Crabbé (1975) (cited by Dennis, 2003) was also widely tested and proposed to be precise by many scientists (Cook and Jacobs, 2000; Trejo-Martínez *et al.*, 2009). Dennis (2003) suggests that the longer the cutting, the more accurate the results. It was also recommended that the shoot cuttings should be of similar length and age when cut and should be collected from a similar position in the tree. Additional criteria, such as bud stage, time of observation, temperature and light conditions, should be agreed on and standardised before the experiment is conducted to reduce the variability and subjectivity of the method (Dennis, 2003). In the absence of more accurate/practical methods, the single-node and shoot cutting methods are the most widely used to evaluate dormancy dynamics in perennial tree fruit buds.

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2.2.2. Anatomical approaches

During dormancy evolution, anatomical changes happen within the floral organs and the buds become hardy and accumulate winter chill towards flowering (Arora *et al.*, 1992; Viti *et al.*, 2013). Anatomical differentiation of xylem vessel elements have been used to determine bud dormancy level. Some studies measured the gradual xylem vessel development in excised buds of apricot cultivars over time using an optical microscope (Bartolini and Giorgelli, 1994; Andreini *et al.*, 2012). The acropetal progression of primary xylem differentiation along the flower bud axis was identified through five different well defined end points: stage 1 (at the basis of the axis); stage 2 (at 1/2 of the axis); stage 3 (at 3/4 of the axis); stage 4 (at the base of the ovary); stage 5 (into the pistil) (Andreini *et al.*, 2012). Adequate chilling advanced xylem vessel development while mild climates showed irregular progress of xylem differentiation. The authors also showed that xylem vessel element development depended not only on plant cultivars or seasons but also on the chilling accumulation. Ito *et al.* (2013) showed that xylem content was very low in trees with inadequate chilling compared to trees that received sufficient chilling. Under mild dormancy conditions, it was found that 'Currot' apricot buds known for having a very low CR, synchronised xylem development and endodormancy release while 'Stark Early Orange' with very high CR had no relationship between xylem development and dormancy resumption, and showed poor adaptability and performance (Andreini *et al.*, 2012).

Nuclear magnetic resonance imaging (NMRI) techniques have been used to assess water status in buds during dormancy induction, maintenance and release. This method showed that when buds initiate growth, water content increases significantly (Liu *et al.*, 1993). NMRI also revealed that free water in the lateral buds increases when the terminal buds are pruned and that the water content in buds of the upper part of the shoot, either pruned or thidiazuron (TDZ)-treated, always increases and probably prevents growth of the buds of the lower part of the shoot (Liu *et al.*, 1993). This indicates that water is bound (vitrified) in endodormant buds and upon endodormancy release, water becomes more free (less bound). NMRI also allows the monitoring of plant growth under long and short days and provides a link between water content and development of endodormancy (Fennell and Line, 2001). Sugiura (1995) indicates that changes in water status are associated with budbreak in peach buds and consequently associated with physiological changes. This technique revealed that water potential and content of apple buds collected from warm winter areas remain constant while water in sufficiently chilled buds from cold winter areas increases during dormancy release.

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2.2.3. Biochemical approaches

Developed by Gendraud (1975), one of the very first biochemical approaches to determine dormancy levels involved the use of GA in dormant Jerusalem artichoke tubers kept at 4ºC and 24ºC. Changes of non-adenylic nucleotides (NTPs) to adenosine triphosphate (ATPs) were tracked throughout an induced germination process. Both GA treatment and cold-induced germination increased ATP production, while higher levels of NTPs were found in dormant tubers.

Others have since adapted Gendraud's original approach. Lavarenne *et al.* (1982), for instance, used a similar method in their "nucleotide test", which monitored the plant tissue's ability to convert ATPs to NTPs. They found that dormant tissue showed insignificant conversion and affected the metabolic reactions that require energy. However, conversion ability recovered once endodormancy was released.

Another biochemical approach described by Dennis (1994) comprised incubating plant tissue in 5,5-dimethyl-oxazolidine-2,4-dione (DMO), a weak acid and measuring its permeability through the cell membrane. The author indicated that the non-dissociated form of DMO passes through the cell membrane in dormant tubers and then dissociates. The degree of dissociation is dependent on the pH of the intracellular environment and when comparing the amount of DMO inside the cell to that in the intercellular spaces one could estimate the nutrient movement into the cell. Using this method, it was illustrated that dormant 'Jerusalem' artichoke tubers have a lower amount of DMO moving into the submerismatic tissue compared to non-dormant tubers (Gendraud and Lafleuriel, 1983). This led the authors to believe that dormancy impeded the movement of nutrients to the bud tissue and once dormancy was broken the block was removed and growth resumed. This measurement could thus be used as an indicator of the dormancy status of the tissue.

Among diverse proteins used to detect dormancy evolution and release, a 19-kDa protein has been suggested as a usable marker to measure the level of floral bud dormancy in Japanese pear (*Pyrus pyrifolia* Nakai) (Tamura *et al.*, 1998). These authors showed that this protein increases during chilling accumulation or after buds are exposed to high temperatures (45 ºC for hours). The accumulation of a 19-kDa protein as well as a 60-kDa is associated with cold acclimation in deciduous trees (Arora *et al.*, 1992).

2.2.4. Molecular approaches

Analysis of global patterns of gene expression and metabolite levels shows that the time for flowering in Populus depends on the interaction between genetic pathways and environmental factors such as photoperiod and cold (Chen *et al.*, 2002; Cooke *et al.*, 2012; Preston and Sandve, 2013). Phytochrome genes (*PHYA*, *PHYB1* and *PHYB2*) have been mapped in Populus and *PHYB2* was shown as a candidate gene for the dormancy-related quantitative trait locus for budset and bud flush (Chen *et al.*, 2002; Allona *et al.*, 2008). Kumar *et al.* (2017) indicate that chilling temperature regulates phytohormone-related pathways and post-embryonic development during budbreak in apple. Although genetic factors are outside of the scope of this review, it is, however, important to highlight that *MADS-box* genes (also called dormancy-associated *MADS-box* or *DAM* genes) expression plays an important role in the regulation of plant growth and dormancy (Cooke *et al.*, 2012; Preston and Sandve, 2013; Beauvieux *et al.*, 2018). Wisniewski *et al.* (2015) reported three putative *DAM* genes associated with the onset and release of dormancy in apples. *MADS-box* genes have shown several *MdMADS* and most of them were assigned putative functions to control bud dormancy and regulate flowering time in apple (Wisniewski *et al.* 2015). This evidence shows a connection between gene expression and the different physiological responses due to environmental factors that regulate dormancy progression.

3. The effects of temperature on dormancy progression

Environmental factors including temperature, water status, photoperiod, nutrient and oxygen availability and the combined outcome of these factors can affect dormancy progression in apical buds (Häkkinen *et al.*, 1998; Anderson *et al.*, 2001; Heide, 2011; Cooke *et al.*, 2012). Apart from this combined effect, photoperiod and temperature are believed to be crucial individual factors that influence dormancy. However, apples are not sensitive to photoperiod/day length (Heide and Prestrud, 2005) and therefore the current discussion is limited to the importance of temperature. It has been shown that there is a strong dependence between dormancy development and temperature in temperate trees, particularly apple and pear (Heide and Prestrud, 2005; Heide, 2011; Cooke *et al.*, 2012). To overcome endodormancy, deciduous fruit trees need prolonged exposure to chilling temperatures. At temperatures between 1.5 ^{o}C and 12.5 ^{o}C, trees accumulate cold in terms of CUs (Richardson *et al.*, 1974). Budbreak takes place when the required number of CUs has been met, usually following long-term exposure to chilling. Although the chill itself does not induce growth, it creates conditions that favour growth resumption. Sufficient chilling favours endodormancy induction and a rapid exit from dormancy in cold winters, while warmer

winter climates that do not provide the amount of chill that is required contribute to the extension of endodormancy (Naor *et al*., 2003; Rohde and Bhalerao, 2007).

The following two sections discuss the effect of temperature, especially low temperature, on dormancy progression followed by discussions on the nature of the chill requirements in apple trees (3.3) and the quantification of chill accumulation (3.4). Section 4 focuses on the effects of insufficient winter chill and ways to overcome it.

3.1. Effects of temperature on dormancy induction (paradormancy)

Terminal bud formation of shoots is induced in autumn when paradormancy in the terminal bud prevents cell division and apical elongation (Rohde and Bhalerao, 2007). Growth cessation takes place in the meristem or vascular cambium and induces the formation of bud scales (Rohde and Bhalerao, 2007; Van der Schoot and Rinne, 2011; Cooke *et al*., 2012). Note that lateral bud formation occurs long before terminal bud formation as an effect of apical dominance. Although growth cessation of spurs occur much sooner (sometimes in spring shortly after growth resumption), shoot growth cessation is a state that occurs in late summer, and it is directly connected to endodormancy (Heide and Prestrud, 2005). Buds develop in size depending on the number of primordia they contain (Bergh, 1985b). Shoot growth cessation is believed to be the main event in dormancy induction of terminal buds and is under the influence of correlative inhibition, also known as “summer dormancy” (Heide, 2011). As mentioned, this phase occurs in response to various conditions that lead to an increase in ABA and/or a reduction in growth reducing hormones such as auxin, GA and CK (Borchert, 1991; Guak and Fuchigami, 2001, Li *et al*., 2018). This is often synchronised with the start of acclimation to low temperatures and freezing stress (Anzanello *et al*., 2014). For instance in woody plants, growth cessation induces the development of cold hardiness and this state strongly depends on the local climatic conditions (Horvath *et al*., 2003). Exposure to low temperatures (<9 °C for *Sorbus* genotypes and <12 °C for pomefruit trees) leads to the cessation of growth (Heide and Prestrud, 2005; Heide, 2011).

Once growth cessation is complete, bud development and dormancy establishment intensify and develop over a number of weeks during which temperature can play an important role, although its mechanism is not yet clear. Several studies suggest that the duration of this induction period varies and depends on low autumn temperatures (Heide, 2003; Heide and Prestrud, 2005; Cooke *et al*., 2012). Others observe the induction of dormancy, albeit delayed, without considerable chilling in warmer conditions (Cook and Jacobs, 2000). Under South African conditions shoots from ‘Golden Delicious’ and ‘Granny Smith’ apples grown in warm winter areas reached maximum dormancy at 600 Utah CUs, while those from colder winter areas reached maximum dormancy

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already at 100 Utah CUs (Cook and Jacobs, 2000). These observations led to Cook and Jacobs (2000) questioning the role of chill during dormancy induction or the appropriateness of the Utah chill model to indicate dormancy progression in warm winter areas.

Under dormancy induction and intensification, perennial trees go through different stages, including leaf senescence, which might be a sign of induction development (Van der Schoot and Rinne, 2011). During this period, dormancy acquisition intensifies and can be seen as an extension of bud hardening induced throughout autumn (Cooke *et al.*, 2012). The intensity of dormancy progressively increases with the acquisition of chill until maximum dormancy is reached (Lang *et al.*, 1987; Dennis, 1994; Rohde and Bhalerao, 2007).

Dennis (2014) found that low autumn temperatures may intensify dormancy acquisition and Cook *et al.* (2005) demonstrated that low temperatures (even in autumn) significantly enhance dormancy induction. Cook *et al.* (2005) also suggested that the perception of induction factors occurs within the buds themselves. Cook and Jacobs (2000) suggested that sub-zero night time temperatures may also hasten dormancy induction. Results such as these suggest that temperature is not necessarily the driver for dormancy induction, but it can hasten and intensify the process.

3.2. Effects of temperature on dormancy maintenance and release (endo- and eco-dormancy)

The critical environmental factor controlling the exit of dormancy is low temperature. Once the maximum depth of dormancy is reached dormancy stays static before release (Faust *et al*, 1997; Rohde and Bhalerao, 2007). This dormancy maintenance is known as an internal condition during which the apical meristem within the bud is kept in an inactive state of growth, even if the bud is subjected to factors that otherwise favour growth. Although the mechanisms that maintain this equilibrium are not yet well known, it is seen as a stable state under which factors that lead to growth renewal are in balance with factors that prevent growth (Dennis, 1994; Horvath *et al.*, 2003; Rohde and Bhalerao, 2007; Horvath, 2009). It is therefore possible that the mechanisms could involve the control of the cell cycle.

Low temperature can intensify endodormancy. Specific temperature ranges have been reported to be more effective in temperate trees. Winter temperatures between 0 °C and 7 °C result in successful chill accumulation, leading to high budbreak percentages in perennial fruit trees (Naor *et al.*, 2003) according to Figure 1. Arora *et al.* (2003) and Naor *et al.* (2003) indicate that there is little difference in budbreak of shoots chilled between 0°C and 7.5 °C (Fig 1), implying that this temperature range is most suitable for the chilling of apple buds. Their research showed a

decrease in budbreak at temperatures ranging from 7.5 ⁰C to 12.5 ⁰C, while no budbreak occurred between 12.5 ⁰C and 15 °C. They also indicated that diurnal cycles in temperature (6 °C night temperatures and 14 °C day temperatures) are better suited to breaking dormancy than to a continuous 6 ⁰C treatment. These authors, however, found that diurnal cycles with temperatures higher than 17 ⁰C leads to chill negation and decreased budbreak.

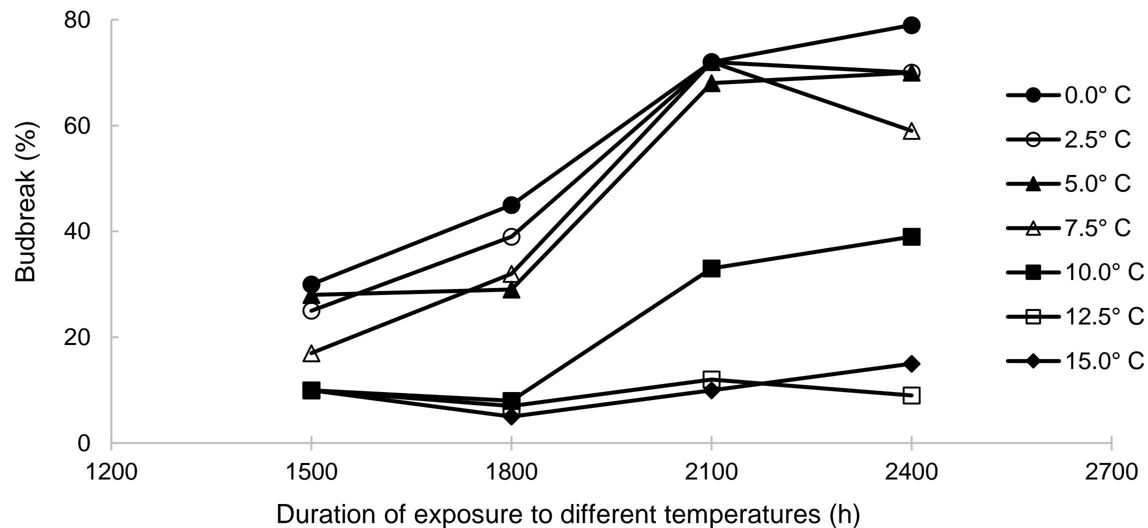

Figure 1. Vegetative budbreak (percentage) of one-year-old 'Golden Delicious' apple shoots as a function of the duration of exposure to various temperatures in 2000. Budbreak was determined after ≈21, ≈31 and ≈40 days of forcing in a chamber temperature at 22 ⁰C. Trees were placed in a horizontal position during forcing. (Redrawn from Naor *et al.*, 2003).

Moreover, in a study conducted by Dokoozlian (1999) all grapevine cuttings exposed to temperatures between 0 and 10 °C for 200 hours showed similar rates of budbreak and were considered commercially acceptable. Heide (2011) indicated that a minimum of nine weeks at 9 °C is efficient to break dormancy in woody Rosaceae species such as *Sorbus* genotypes (*S. aucuparia*, *S. commixta* and *S. intermedia*). Chilling apple and pear shoots at temperatures between 6 and 9 °C for six weeks (approximately 1 000 hours) induces budbreak and the resumption of growth (Heide and Prestrud, 2005). Hawerroth *et al.* (2013) report temperatures of 15 ⁰C and 5 to 10 ⁰C as ideal for budbreak in 'Castel Gala' and 'Royal Gala' apple trees respectively. According to Dennis (2003), temperatures below 0 ᵒC contribute to freezing acclimation but do not have an effect on dormancy release. Arora *et al.* (2003) also reported that temperatures below or above 0 to 7 ⁰C are not believed to contribute toward chill-unit accumulation. However, Cook *et al.* (2005) suggested that the sub-zero temperatures could play an important role in dormancy induction of apple buds. This was based on other studies that highlighted that freezing temperatures may enhance the progression of dormancy (Seeley *et al.* 1998; Cook and Jacobs, 2000; Jacobs *et al.*, 2002). Cook *et al.* (2017) suggested that the

occurrence of sub-zero temperatures depends on the altitude and that areas with low elevation rarely show such temperatures when compared to the areas with high elevation.

In most warm winter conditions, the high day temperatures negate the effects of CUs accumulated at night, resulting in deficient chill accumulation to release dormancy in deciduous fruit trees (Erez *et al.*, 1979b). It has been suggested that the most effective range of chilling temperatures in apples is from 0.6 °C to 16.5 °C and the optimum temperature is 7.2 °C (Shaltout and Unrath, 1983); temperatures below or above this range do not seem to contribute to chill accumulation (Erez *et al.*, 1979b).

Towards the end of winter when temperatures get warmer, bud hardiness decreases, buds exit endodormancy and budbreak occurs if temperatures permit (Powell, 1986; Hauagge and Cummins, 1991; Naor *et al.*, 2003). This state coincides with conditions that are warm (sufficient heat units) and favourable for growth and synchronisation of budburst in spring (Rohde and Bhalerao, 2007; Horvath, 2009). If the spring temperatures do not provide sufficient heat units, budbreak will be inhibited, putting the bud in a state of ecodormancy. In this state, growth is prevented by environmental factors only and as soon as conditions warm up, buds will resume growth thus exiting ecodormancy. As previously mentioned, a sufficiently cold winter will decrease the temperatures at which buds can become active in spring (Scalabrelli and Couvillon, 1986). This could be problematic in the case where a low chill variety is planted in a high chill area as the terminal buds will resume growth at relatively low temperatures and cause the subtending lateral buds to remain paradormant. Such trees thus develop delayed foliation symptoms even though they had sufficient winter chill.

3.3. Chilling requirements

Under natural conditions, a specific amount of chilling is required to release dormancy (Naor *et al.*, 2003; Rohde and Bhalerao, 2007). This critical minimum amount of chill is referred to as the chill requirement (CR) of that specific cultivar and it depends on both temperature and the duration of exposure (Dokoozlian, 1999; Rohde and Bhalerao, 2007). It is a genetic trait that varies not only among plants but also among cultivars, species, types of bud and location (Hauagge and Cummins, 1991b; Labuschagné *et al.*, 2002b). Fulfilment of CRs results in the synchronisation of bud development, deep dormancy, dormancy release, flowering and fruit set and also a yield increase (Fuchigami and Nee, 1987). By comparison failure to acquire sufficient chilling negatively affects tree branching and budbreak, production, fruit quality and marketability (Campoy *et al.,* 2011; Atkinson *et al.*, 2013; Maguylo, *et al.*, 2014). For these reasons, it is important that reported CR values are reliable and accurate.

In the literature, several different values have been documented for apple trees in general and for individual cultivars (Table 2). Diverse CRs have been identified under different measurements. Different chill models can also lead to the misclassification of a cultivar as having both a low and a high CR, which leaves the user confused and complicates comparisons across cultivars.

Table 2. Documented chilling requirement for commercial apple cultivars that are cultivated in SA compared to other tree fruits.

Fruit type	Cultivar	CR	PCUs / CH / CU	Source
Pome fruit in general	Various cultivars	High	PCU, >1000	Sheard, 2001
		Medium	PCU, 600 – 1000	Sheard, 2001
		Low	PCU, <600	Sheard, 2001
Apples	'Braeburn'	High	PCU, 800–1000	ARC-Infruitec, 1997 as per Tharaga, 2014
			CU, 1141–1320	Ghariani, 1993
			CU, ±1100	Hauagge and Cummins, 1991b
			PCU, 800	Sheard, 2001
			CH, 700– 900	Powell *et al.*, 1999
	'Fuji'	High	PCU, 800–1000	Sheard, 2001
			CU, 1040	Ghariani, 1993
			CH, 550	Adams, 2006
			CH, 700–900	Powell *et al.*, 1999
	'Top Red'/ 'Starking'	High	CU, 1400	Ogundeji and Jordaan, 2017
			CU, 1320.5	Ghariani, 1993
	'Golden Delicious'	High	PCU, 800–1000	Costa *et al.*, 2004; ARC-Infruitec, 1997 as per Tharaga, 2014
			CU, 1025 shoots	Finetto, 1997 and 2014
			CU, 1095 spurs	Finetto, 1997 and 2014
			PCU, 800-1000+	Sheard, 2001
			CU, 900–1100	Powell *et al.*, 1999
			CU, 1277	Ghariani, 1993
			CU, 1400	Ogundeji and Jordaan, 2017
	'Granny Smith'	Medium to low	PCU, <800	Costa *et al.*, 2004; ARC-Infruitec, 1997 as per Tharaga, 2014
			CU, 1064	Hauagge and Cummins, 1991b
			PCU, 600	Sheard, 2001
			CH, 700–900	Powell *et al.*, 1999
			CU, 1040	Ghariani, 1993
	'Royal Gala'	High	PCU, 800–1000+	ARC-Infruitec, 1997 as per Tharaga, 2014
			CU, 1200	Ogundeji and Jordaan, 2017
			CU, 1064	Hauagge and Cummins, 1991b
			CU, 1151	Ghariani, 1993
			PCU, 800–1000+	Sheard, 2001
	'Gala' cultivars	Medium and low chilling	CH, 600	Labuschagné *et al.*, 2002
			CH, 700– 900	Powell *et al.*, 1999
			CH, 550	Adams, 2006
			CU, 1155	Ghariani, 1993
			CH, 300	Anzanello *et al.*, 2014
	'Cripps Pink'	Medium	CH, 600 – 1000	Halgryn *et al.*, 2001
			PCU, 450–800	ARC-Infruitec, 1997 as per Tharaga, 2014
	'African Carmine'	Low	CU, 300 – 500	www.culdevco.co.za

Note: CH: chilling hours, **CU**: chill units, **PCU**: daily positive Utah, **CR**: chilling requirement. The differences between positive chill unit (PCU), chill hour (CH) and chill unit (CU) are described below in winter chill quantification models (3.4)

This indicates that cultivars behave differently in different environments and/or the way that the CR is measured is inconsistent. Finetto (2014) noted that shoots and spurs of one plant can have different CRs. Anzanello *et al.* (2014) found that apple lateral buds are inhibited by apical buds (apical dominance), while Finetto (2014) showed that spurs required more chilling than shoots in all apple cultivars. Terminal vegetative buds in peaches have a shorter CR, while both lateral vegetative and flower buds have a similar longer CR (Scalabrelli and Couvillon, 1986). CR differences between apple genotypes depend on their environment (Hauagge and Cummins, 1991a).

Climate change can affect chill and heat accumulation, which could result in inadequate fulfilment of chilling or heating requirements in perennial fruit trees. Several regions have observed warm winter conditions, which decrease chill accumulation in trees and may result in failure to reach the CR for some varieties (Luedeling *et al.*, 2009; Darbyshire *et al.*, 2011; Luedeling *et al.*, 2011; Rai *et al.*, 2015). In turn, this failure leads to inadequate physiological chilling and incomplete dormancy release (McPherson *et al.*, 1997; Rai *et al.*, 2015). Buds remain dormant or take longer to exit the endodormant state, which affects tree behaviour and architecture (Cook and Jacobs, 1999; Cook and Jacobs, 2000). There are several symptoms that indicate inadequate fulfilment of CRs (Erez, 1995; Cook and Jacobs, 1999); these are discussed in section 4.

3.4. Winter chill quantification models

Chilling temperatures during winter have been studied since the 1900s. Accumulated chill in temperate and sub-tropical zones can be quantified according to a number of models that have been developed to assess CR, monitor phenological stages and predict dormancy completion. All these models hold that chilling hours, CUs or chill portions are accumulated in temperatures ranging between 0 and 7 °C (Rai *et al.*, 2015) and also 1.5 and 12.4 °C (Richardson *et al.*, 1974). These models have other structural resemblances; they calculate and estimate the amount of winter chill accumulated at hourly intervals and predict phenological development of buds and budbreak timing. However, they differ in terms of how accurate they account for temperature variability (Sheard, 2001; Luedeling *et al.*, 2009 and 2011; Tharaga, 2014; Rai *et al.*, 2015). Another area of uncertainty is how well these models are suited to predict budbreak performance (percentage and duration) in warmer climates. Literature on this topic is scarce and no direct studies have been done under South African conditions. The following section discusses some of the most important models used to determine chill perception.

3.4.1. Chilling hours model

The oldest known model, the chilling hours model (Chandler, 1942) is still in use today. According to this model, temperatures between 0 and 7.2 °C are efficient for budbreak and each hour that passes within this temperature range is counted as a chilling hour. Chilling hours can be totalled for a certain period. This model is simple but does not consider chill accumulation outside 0 and 7.2 °C, the chilling negation by high temperatures or the positive effect of alternating low and mild temperatures. It has also been argued that it is poor at predicting budbreak (Pérez *et al.*, 2008; Luedeling *et al.*, 2011; Tharaga, 2014; Rai *et al.*, 2015).

3.4.2. Utah model

Richardson *et al.* (1974) developed the Utah model for peaches under cold winter conditions and it is currently the most widely used model for deciduous fruit trees. This model's CUs, also called Richardson units, are determined according to time (an hour) exposure at specific temperatures. Typically, a single CU is best fulfilled within a range of 2.5 and 9.2 °C and temperatures below 1.5 ^{0}C or above 12.5 °C do not contribute to the chill accumulation. Unlike the chilling hours model, the Utah model includes the negation effect, but only at temperatures above 16 °C. Negative chill is acquired when temperatures exceed 16 °C and that neutralises/negates previous positive chill that was accumulated. The model's measurements were seen as reliable in cool and cold, temperate regions, but it has been seen as less efficient for areas with warm winters. Effective chill accumulation cannot be negated by high temperatures that occur after 24 hours of the chill event (Erez *et al.,* 1979). This means that the chill that takes place earlier than 24 hours to a period with negating temperatures should not be subtracted as the Utah model implies. Since the Utah model allows for the subtraction of negative units from chill that accumulated more than 24 hours before, this chill model can underestimate chill accumulation in warmer regions (Erez *et al.,* 1979).

3.4.3. Daily positive Utah / Infruitec model

Using nectarine cultivars, Linsley-Noakes *et al.* (1994) modified the Utah model by taking in account the above mentioned limit on chill negation and thereby making the model more accurate for SA conditions where day temperatures are often (>20 °C) after preceding low night temperatures. This model, which is known as the daily positive Utah (PCU) or Infruitec model, calculates values based on daily mean temperatures and tallies the total daily CUs. If the sum of total daily CUs is negative, it is then calculated as zero to prevent negation (within 24 h of chill). This adjustment to the Utah model typically leads to the recording of higher chill accumulation

values (due to less negation), especially in warm winter areas where the likeliness of having warm day temperatures within 24h after a chill accumulation event is more common. Thus, although this new revised model allows for chill negation, it limits it to only chill accumulated in the preceding 24 hours.

3.4.4. Dynamic model

In the 1980's, Fishman *et al.* (1987) quantified chill accumulation using a mathematical analysis of a two-step dormancy breaking model. This model assumes that chill accumulation is a temperature-regulated, two-step process of which the 'first-step' involves the production of an unstable "dormancy breaking factor" or precursor that requires a "second-step" to irreversibly transform this factor into a more stable "dormancy breaking factor". According to the Dynamic model, the optimum winter chill temperatures follow a bell shape with a maximum efficiency at 6 °C and zero effect at -2 °C and 14 °C, high temperatures act to negate previously accumulated chill and moderate temperatures can enhance chill accumulation (Linsley-Noakes *et al.*, 1994 and Darbyshire *et al.*, 2011).

At the heart of the model is the assumption that as soon as a certain quantity of the precursor has accumulated, it is irreversibly transformed into a Chill Portion (CP), which can no longer be destroyed. This process is promoted by short periods of moderate (e.g. 15 °C) temperatures, thus taking into account the positive effects of alternating low and mild temperatures typical of warm winter areas. In the early 1990's, Erez *et al.* (1990) developed and explained this model for the warm winters in Israel and it is thought to be the most realistic model to use in milder climates, temperate and sub-tropical regions (Sheard, 2001; Pérez *et al.*, 2008; Tharaga, 2014; Rai *et al.*, 2015).

3.5. The use of chill models in South Africa

In SA, two chill models, Utah and Infruitec, are widely used to quantify winter-chill accumulation and both models are based on hourly temperature (Midgley and Lötze, 2011; Luedeling, 2012). However, SA deciduous fruit growers are more familiar with the Utah model and use it despite evidence that it performs poorly under the warm winter conditions that are common in most of the production areas (Midgley and Lötze, 2011). In SA, the period of chill-accumulation monitoring is determined by a calendar date (beginning of May until the end of August) and not necessarily aligned with tree phenology. Orchards are then evaluated in terms of their ability or chance of meeting the CR by the end of this period based on historical data. Due to fear of the adverse effects of delayed foliation, apple producers in SA will apply a RBA irrespective of the amount of

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chill units measured for the preceding winter. In the milder production regions, the use of RBA's is completely justified since severe delayed foliation would otherwise make commercial apple production impossible. In colder regions where the chill requirements of cultivars are met in most years, producers perceive to obtain more synchronised budbreak and flowering as well as a gradual increase in bearing positions in response to RBA applications. The chill models and their recorded units are used as broad predictors of the coming growing season and are used by the producer to determine the aggressiveness of the rest breaking programme for that specific spring instead of an indication whether RBA should be used.

4. Insufficient chilling and artificial rest breaking

Under mild winter conditions, when the buds do not accumulate enough chill, they remain dormant or release from dormancy poorly and develop physiological and phenological symptoms (Blommaert, 1963; Naor *et al.*, 2003; Atkinson *et al.*, 2013; Melke, 2015) referred to as "delayed foliation". This is characterised by delayed and poor budbreak, poor spur formation, an abnormally high rate of bud abscission, unbranched shoots, a decrease in vigour, early tree maturation and delayed flowering (Blommaert, 1963). These conditions result in poor fruit set, reduced leaf surface and uneven development and ripening of fruit (Blommaert, 1963; Petri and Leite, 2004; Luedeling *et al.,* 2011). Atkinson *et al.* (2013) indicate that although the symptoms vary between fruit species, they all negatively affect yield. The following two sections discuss the impacts of insufficient chilling.

4.1. Impact of insufficient chilling on dormancy

Chilling deficiency results in abnormal tree development and budbreak. Growth, vigour and development are weakened when CRs are not fulfilled, resulting in poor and late shooting (Petri and Leite, 2004; Luedeling *et al.,* 2009; Maguylo *et al.*, 2014; Atkinson *et al.*, 2013; Melke, 2015). It has been observed that partial chill deprivation favours basal dominance, while complete chill deprivation prevents budbreak and endodormancy release in peach trees (Leite *et al.*, 2004). Fu *et al.* (2014) and Heide (2003) also reported that warm temperatures during autumn affect growth traits such as leaf senescence and flushing in temperate tree species. Erez (1995) suggested that the main symptoms of insufficient chilling are low and erratic budbreak, which delays leaf and bloom development in spring. All these growth, development and budbreak symptoms negatively impact fruit set and development, yield and fruit quality (Petri and Leite, 2004).

Generally, sufficient chill accumulation promotes pollination, fruit set and development while inadequate chilling reduces flowering synchrony and results in small fruit size, incomplete or

uneven ripening, which implies reduced production and fruit quality. Warm winters mainly result in the abscission of flower buds, which can affect fruit yield, albeit to a lesser extent in cultivars with lower CRs (Petri and Leite, 2004; Luedeling *et al.*, 2009; Atkinson *et al.*, 2013). It was also found that insufficient chilling in peaches extends the number of days to full bloom, and have a negative effect on fruit shape and fruit tip lengths (Li *et al.,* 2016). Similarly, in apples, it was found that budbreak, flowering and fruit set were also delayed when insufficient chilling occurred (Wang, 2010). It was also found that insufficient chilling in peaches extends the number of days to full bloom, and subsequently negatively impacts on fruit shape and fruit tip lengths (Li *et al.,* 2016). Similarly, in apples, it was found that budbreak, flowering and fruit set were also delayed when insufficient chilling occurred (Wang, 2010). All these findings appear to indicate an interaction between ambient temperatures and blooming, which complicates horticultural practices such as fruit thinning, spray applications, etc. Table 4 provides an overview of the symptoms pointing to inadequate winter chilling in a variety of fruit trees and shrubs. In summary, inadequate exposure to chilling affects two aspects, namely tree development and budbreak and flowering and fructification.

Table 4. The impact of low winter chill on different aspects of perennial fruit plants (Redrawn from Atkinson *et al.*, 2013).

Commodity	Aspects which are affected by low winter chilling									
	Vegetative budbreak[a]	Floral budbreak[a]	Bud abscission[b]	Flower abscission[c]	Flower quality[d]	Reproductive morphology[e]	Fruit set[f]	Vegetative growth[g]	Crop yield[h]	Product quality[i]
Apple	+	+		+	+			+	+	+
Pear				+		+			+	
Cherry			+		+	+	+			
Plum			+							
Peach			+		+	+		+		
Nectarine			+		+					
Apricots			+		+					
Almond			+				+			
Raspberry	+	+								
Blackberry	+									
Blackcurrant	+	+					+		+	+
Strawberry	+						+	+	+	+

[a] Delayed, erratic or uneven budbreak (column 1 vegetative and column 2 floral); [b] Abscission of entire flower buds; [c] Abscission of single flowers within a cluster; [d] Reduction in flower quality; [e] Changes in reproductive morphology; [f] Reduction in fruit set or increased run-off; [g] Changes in vegetative growth, apical dominance, etc.; [h] Reduction in crop yield; and [i] Changes in crop/product quality.

Insufficient winter chill accumulation in the Western Cape region is associated with the disturbance of apple bud dormancy (Cook and Jacobs, 2000). Cook and Jacobs (2000) found that 'Granny Smith' and 'Golden Delicious' apple trees grown in the milder winter conditions of the Elgin region took longer to enter and exit dormancy resulting in slower and erratic budbreak compared to the colder Ceres region. In the Elgin region, deficient chill accumulation also impedes the development of the normal acrotonic branching tendency and favours a basitonic growth pattern, giving rise to an atypical basal dominant tree shape (Cook and Jacobs, 1999). Maguylo *et al.* (2014) reported that the majority of differences found in the architectural development of 'Golden Delicious' and 'Granny Smith' apple trees grown in warm and cool areas of the Western Cape were related to the loss of acrotonic bud break and shoot development tendencies. In apples grown in the Elgin district of SA, floral organs such as carpels, sepals, petals and pollen sacs develop slowly during winter (Bergh, 1985a). Carpels grow during bud swell, sepals and petals elongate while pollen sacs develop in the anthers and a rapid development of the ovule follows bud development from green-tip stage until budburst at the end of the winter period (Bergh, 1985a and 1985b). During bud development under the warm autumn and winter periods of the Elgin area, the cells in the cortex of developing terminal flowers increase slowly, but from late winter until full bloom, the increase is considerable (Bergh, 1985b). Sheard (2008) reports that warm winter conditions in sweet cherry can induce premature ovule abortions and consequently affect fruit set. The same author mentions that warm winter temperatures reduce ovule viability after pollination. Also, high temperatures (above 25 °C) interfere with the development of reproductive organs, resulting in poor fruit set in peaches (Kozai *et al.*, 2004).

A recent study conducted across all the apple producing areas in SA examined the time to budbreak of forced apple shoots ('Granny Smith' and 'Royal Gala') collected from 24 farms (mainly from the Western Cape, but also from the Eastern Cape and high altitude, low latitude Highveld region) during five consecutive seasons (Cook *et al.*, 2017). The authors found that most apple producing areas in SA are accumulating Utah CUs below the average CR, resulting in a high variability in dormancy progression curves. Location and specifically latitude and altitude, seems to affect the onset of dormancy. When all the farms were clustered according to the maximum dormancy level and rate of dormancy release, it was found that the farms at a lower altitude had shallower dormancy levels and were associated with warm winter conditions. In the case of warm winter, RBAs are used as part of standard orchard management practices in order to synchronise budbreak with the arrival of spring and ensure a commercially acceptable yield and sustainable production (Cook and Jacobs, 2000; Costa *et al.*, 2004; Sagredo, 2008).

4.2. Overcoming delays in dormancy release

When exposed to natural conditions of sufficient chill, buds enter and maintain a deep dormancy during winter, followed by dormancy release and flowering when spring conditions are favourable. Under insufficient chill accumulation, the dormant phase persists and budbreak is irregular. Dokoozlian (1999) considers the CR as a facultative factor rather than an absolute requirement for growth, although a part of chilling cannot be completely substituted by other means. In other words, chilling is not the only factor that can release budbreak. The use of certain artificial rest breaking agents or techniques in warm winter areas could augment the natural chilling. Some of the techniques that are used to overcome delayed foliation are discussed in the next section.

4.2.1. Non-chemical artificial budbreak

In warm winter areas, practices such as bending and notching of branches may disrupt the polar auxin stream, weaken the apical dominance, overcome paradormancy and result in higher percentages of budbreak and also accelerate budbreak resulting in better branch and flower development (Ito *et al.*, 1999). In a similar fashion, delayed heading of newly planted trees is used as a horticultural practice to overcome paradormancy and stimulate branch formation (Cook, 2010).

Other non-chemical rest breaking techniques include triggers such as a hot water treatment (35 – 50 ºC) of endodormant buds. It was successful in breaking dormancy of potted berries in warmer regions and grapevine cuttings (Orffer and Goussard, 1980). Similarly, artificial chilling (Nir *et al.,* 1986) and high temperatures (50 ºC for 1 h) (Tan *et al.*, 2013) can induce budbreak in apples. Often young trees from nurseries in warm winter areas are placed in cold-room facilities (2 – 7 °C) during the winter prior to planting to ensure sufficient chilling and resulted in good budbreak and branching in spring (Ito *et al.*, 2013).

Even though budbreak and acrotonic development can be improved with certain horticultural practices, persistent warm winters and prolonged endodormancy periods continue to result in some degree of delayed foliation and flowering, which cannot be rectified without sufficient chill accumulation or newly developed low-chill cultivars.

Breeding new cultivars that are better adapted to low-chill environments can help solve the challenges of prolonged rest. Breeding programmes for low-chill cultivars have been successful in producing apple cultivars, such as 'Anna' (500 CH), that perform well in warmer climates such as Israel (Holland *et al.*, 2006). 'Eva' (IAPAR 75) is another low-chill apple cultivar, which was released by a Brazilian breeding programme (Holland *et al.*, 2006); it grows successfully in

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regions with less than 450 Utah CU. The local Agricultural Research Council (ARC) of South Africa also has a breeding programme that has released some low chill apple cultivars that are produced in non-traditional apple regions in SA (Labuschagné *et al.*, 2002b). Though low-chill cultivars grow successfully in warmer regions, some problems still remain (Lyrene, 2005). For instance, varieties such as 'Anna' are known in SA for their low storability and low post-harvest fruit quality. Some of the other low-chill cultivars are also known for poor resistance to disease. Although progress has been made in breeding low-chill cultivars, Labuschagné *et al.* (2002b) suggested that more research is needed on genetic variability and adaptability within populations bred for a low CR.

4.2.2. Chemical artificial budbreak

It has been shown that artificial RBAs increase budbreak, improve and synchronise foliation and flowering, enhancing fruit set and development in commercially cultivated deciduous fruit trees in warm winter areas (Fuchigami and Nee, 1987; Erez, 1995). RBAs lead to the resumption of growth, in this way countering the lack of sufficient chilling in cultivars with a high CR (Powell, 1986; Fuchigami and Nee, 1987; Lang *et al.*, 1987; Dennis, 1994).

Dormancy breaking chemicals that have been tested include dinitro-ortho-cresol (DNOC), thiourea, cyanamides, potassium nitrates, oils and growth regulators (Shulman *et al.*, 1983; Zhang *et al.,* 2007; Pérez *et al.*, 2009; Amberger, 2013). Although they can compensate for the lack of chilling and activate metabolic reactions, most of them have phytotoxic or toxic side effects (Baker, 1970; Fuchigami and Nee, 1987; Siller-Cepeda, 1992; Erez, 1995; Zhang *et al.,* 2007; Sagredo, 2008; Amberger, 2013). Among these chemicals, only a few have been commercially efficient and registered.

Mineral oils, also called "horticultural" or "winter" oils, are non-phytotoxic. They directly affect plant respiration and create a physical barrier by generating hypoxic conditions. Hypoxic or anaerobic conditions interfere with cell respiration and in turn hasten budbreak and flowering (Or *et al.*, 2000; Pérez *et al.*, 2009). In warm winter areas, mineral oils are mostly used in combination with other chemicals such as DNOC, hydrogen cyanamide (HC) or TDZ to boost the dormancy breaking effect (Baker, 1970; Zhang *et al.,* 2007; Sagredo, 2008).

For more than 50 years, DNOC was used successfully on commercial farms to overcome delayed budbreak on deciduous fruit, mostly apples and pears. A mitochondrial uncoupler that creates anoxia in the cell by accelerating the electron transport chain causing oxygen depletion within the cell, DNOC has been particularly effective applied in combination with mineral oils. This

combination hastens budbreak and synchronises blooming and foliation (Shulman *et al.*, 1983; Cutting *et al.*, 1991) but can lose its effectiveness if applied at low temperatures (Zhang *et al.,* 2007). DNOC has, however, been withdrawn from commercial use in South Africa since April 2001 due to its extreme toxicity to humans and the environment (Costa *et al.*, 2004; Zhang *et al.*, 2007; Sagredo, 2008).

Cyanamides have been evaluated since the 1980s as useful fertilisers but have also been effective as herbicides, fungicides, insecticides and defoliants, especially calcium cyanamide (Güthner and Mertschenk, 2012). Today, hydrogen cyanamide (HC) has largely replaced DNOC as the preferred chemical RBA in areas with insufficient chilling accumulation. At first, HC (trading as Dormex™) was tested as a growth regulator and RBA to compensate for the lack of chilling and enhanced budbreak mainly in grapes (Shulman *et al.*, 1983). Beside Shulman *et al.* (1983), others who have reported HC's efficiency on dormancy release and budbreak include Pérez and Lira (2005), Trejo-Martínez *et al* (2009), Vergara *et al.* (2012) and Rubio *et al.* (2014). Other crops in which HC has proven effective include kiwifruit (Walton *et al.*, 1991; McPherson *et al.*, 2001), figs (Shulman *et al.*, 1986), peaches and nectarines (Dozier *et al.*, 1990), Japanese pear (Zanol *et al.*, 2010) and apple (Jackson and Bepete, 1995; Carvajal-Millán *et al.*, 2007; Mohamed, 2008; El-Yazal and Rady, 2012; Amberger, 2013; Ghrab and Mimoun, 2014, Petri and Stuker, 1995; North, 2003 and 2008; Pasa *et al.*, 2018). The HC and mineral oil combination also showed positive results in pistachio (Rahemi and Asghari, 2004) and red raspberry (Snir, 1988). The combination can even improve budbreak in grape in regions accumulating fewer than 400 Utah CU (Avenant and Avenant, 2004).

Although HC treatment is widely used, its mode of action is poorly understood. It has been shown to inhibit enzymes associated with oxidative stress and affect respiration mechanisms in many crops including Japanese pear (Zanol *et al.*, 2010) and grapes (Pérez *et al.*, 2008; Amberger, 2013; Sudawan *et al.*, 2016). As a direct effect of HC treatment the catalase enzyme activity is reduced and oxidation is induced, resulting in the accumulation of peroxidases (H_2O_2) and harmful oxidative substances (O_2^-) that lead to a sequence of oxidative stress and metabolic disorders associated with bud-dormancy release (Shulman *et al.*, 1986; Nir and Lavee, 1993; Or *et al.*, 2002; Pérez and Lira, 2005; Amberger, 2013, Sudawan *et al.*, 2016). This oxidative stress leads to a decline in the tricarboxylic acid cycle (TCA) activity and energy production (Vergara *et al.*, 2012; Sudawan *et al.*, 2016). The inhibition of another enzyme by HC, cytochrome C (CYT), results in a decline in the electron transport between complexes III and IV of the electron transport system, reduces the H^+ gradient across the mitochondrial membrane and indirectly reduces the production of nicotinamide adenine dinucleotide (NADH) and flavin NADH ($FADH_2$) in the TCA

cycle, which ultimately reduces ATP synthesis (Fernie *et al.*, 2004; Taiz and Zeiger, 2010; Sweetlove *et al.*, 2010; Toro and Pinto, 2015; Beauvieux *et al.*, 2018). Furthermore, since the CYT pathway is the main and terminal pathway of oxidative phosphorylation, its inhibition reduces the election transfer and H^+ proton pumping, this induces oxidative stress and affects the whole respiration process (Taiz and Zeiger, 2010). Ben Mohamed *et al.* (2012) showed that the ascorbate-peroxidase activity declines in the presence of HC while the peroxidase activity increases and coinciding with the accumulation of putrescine and proline, which decline after the budbreak period. In turn, the decline in TCA and CYT induces the activity of the pentose phosphate pathway (PPP), alternative pathway (ALT), and glycolysis (Fernie *et al.*, 2004; Tan *et al.*, 2013). Ultimately a HC application leads to a build-up of ethanol as it increases fermentation through a simultaneous induction of both pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) transcripts (Or *et al.*, 2000). It is very likely that this increased production of ethanol can also stimulate budbreak as it has been proved to be effective in breaking seed dormancy (Cohn and Hilhorst, 2000). HC action is immediate and reaches full activity after 48 hours, after which it is quickly decomposed and metabolism returns to normal (Amberger, 2013). This temporary stress situation created by HC treatment is thought to accelerate energy production and releases buds from dormancy (Shulman *et al.*, 1986).

Potassium nitrate (KNO_3), thiourea sodium azide (NaN_3) and the synthetic cytokinin, TDZ, also proved effective in dormancy release, as Zhang *et al.* (2007) noted, but to a lesser degree than HC and DNOC. In SA, KNO_3 and urea are sometimes added to tank mixes of HC and oil to bolster the rest breaking effect. It is not fully understood how these RBAs work, but they are associated with oxidative ability (Shulman *et al.*, 1886). These products are also used as defoliation agents and herbicides (Sagredo, 2008). The high cost of TDZ makes it an expensive option (Zhang *et al.*, 2007) usually limited to high value cultivars.

Although RBAs differ greatly, they all possess the capacity to break endodormancy and their effectiveness generally depends on the dosage, time of application, crop type and cultivar. This makes it particularly complicated as CRs can differ between cultivars, species and locality (Fuchigami and Nee, 1987; Erez, 1995; Faust *et al.*, 1997; Dokoozlian, 1999; Labuschagné *et al.*, 2002b; Amberger, 2013). In order to optimise their efficiency in mild winter areas, RBAs are used in combination with each other. These combinations work in synergy to enhance budbreak, synchronise bloom and improve yield (Costa *et al.*, 2004; Erez, 1995). These agents are also combined with a variety of non-chemical interventions as mentioned above.

5. Physiological and biochemical changes during dormancy

In general, dormancy involves inhibition of some of the essential physiological and biochemical processes, such as photosynthesis, respiration, enzyme activity, oxidative stress, desaturation of membrane lipids, water content modification and endogenous hormone changes (Nir *et al.*, 1983 and 1986; Pérez and Lira, 2005; Beauvieux *et al.*, 2018). As endodormancy is overcome, hydrolytic enzymes are induced, cell organisation changes and metabolic reactions and mobilisation of reserves rise (Faust *et al.*, 1997; Beauvieux *et al.*, 2018). Understandably, it is the genetic make-up of a plant that is the major factor driving these changes and to understand endodormancy's role in the case of perennial fruit trees, one could turn to the advanced and useful tools of molecular genetics and genomic technologies. Many studies have identified gene networks regulating physiological mechanisms and thus laid bare the genetic basis for important phenological traits (Anderson *et al.,* 2001; Horvath *et al.*, 2003; Preston and Sandve, 2013). Considerable amounts of research have been conducted on gene expression and molecular events involved in the establishment, maintenance and release of dormancy in many kinds of deciduous fruit trees. Some of the studies have highlighted the correlation between specific genetic traits and key events during dormancy, including timing of bloom and perception and transduction of budbreak; other studies have also highlighted the key genes regulating budbreak (Chen *et al.*, 2002; Allona *et al.*, 2008; Cooke *et al.*, 2012; Shim *et al.*, 2014; Porto *et al.*, 2015; Aihua *et al.*, 2018; Beauvieux *et al.*, 2018). A more extensive discussion of the involvement of genetics in dormancy release is beyond the scope of this dissertation.

Dormancy induction and release in perennial fruit trees involves the interaction of several internal and external factors. Faust *et al.* (1997) reviewed dormancy on a physiological level and suggested that it is affected by the interaction between integrated factors that have a long-term result on the timing of budbreak. These factors generally include hormones, the plasma membrane, carbohydrate metabolism, mitochondrial respiration and oxidative stress (Figure 2) (Beauvieux *et al.*, 2018). Other factors include cell membrane composition, the state of water within the cells and the anabolic potential of the buds (Beauvieux *et al.*, 2018).

For the purposes of this review and in preparation for the research papers that follow we have limited our discussion of physiological changes during dormancy to oxidative stress, respiration, membrane lipids and sterols.

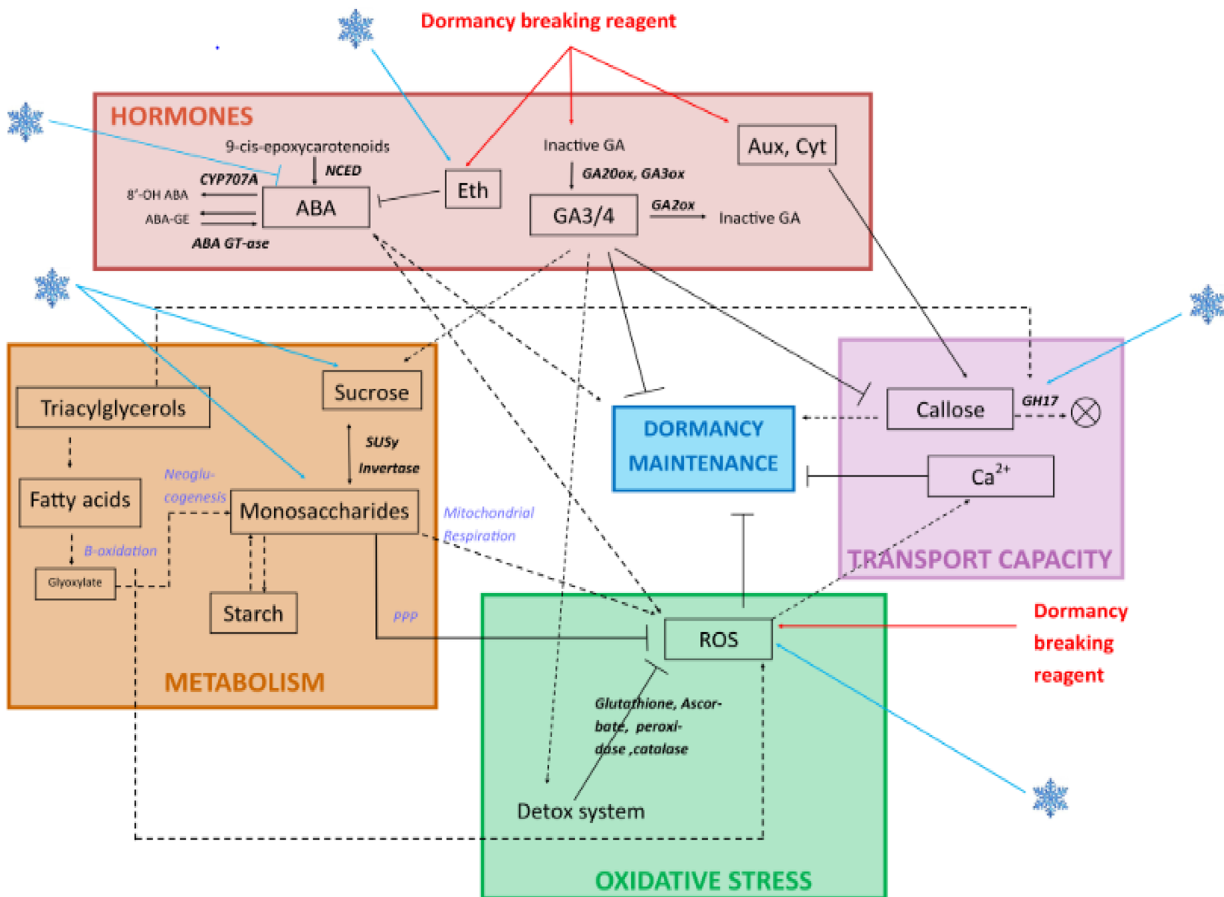

Figure 2. Dormancy pathways and their interactions. Several pathways have been shown to play a role in dormancy and relate to each other. Hormones: Abscisic acid (ABA) turnover is regulated through action of 9-cis-epoxycarotenoid dioxygenases (NCED) genes, which are repressed by the action of cold temperatures and are involved in dormancy maintenance and reactive oxygen species (ROS) generation. Ethylene (Eth) production is controlled by cold temperatures (blue flake) and dormancy-breaking reagents and ethylene diminishes ABA. GA20ox and GA3ox produce active GAs that are involved in dormancy alleviation and favour the detoxification system; GA2ox inactivates them. Auxins and cytokinins act through enhancing callose deposition at plasmodesmata. Transport capacity: callose deposition at plasmodesmata is involved in dormancy maintenance; glycoside hydrolases 17 (GH17) are involved in digesting callose. Cold temperatures enhance GH17 expression. Calcium channels inhibit dormancy maintenance. Metabolism: cold temperatures enhance sucrose and monosaccharide concentration. Monosaccharides are also produced from the beta-oxidation and gluconeogenesis from fatty acids and these monosaccharides produceROS via mitochondrial respiration or are oxidised via the pentose phosphate pathway (PPP) and participate in ROS detoxification. Beta-oxidation produces ROS. Oxidative stress: cold temperatures (blue flake) and dormancy-breaking reagents enhance ROS production and ROS production inhibits dormancy maintenance (With permission from Beauvieux *et al.*, 2018).

5.1. Oxidative stress

The oxidative stress level in plant cells seems to be one of the key factors controlling the physiology of dormancy in conditions of environmental stress. Oxidative stress reflects an imbalance between the systematic manifestation of ROS and the biological system's ability to

detoxify or repair the reactive intermediates; it also reflects the resulting damage. When growth is normal, ROS are always produced in low levels and kept at a steady-state level in cells (Mittler, 2002; Scandalios 2005; Noctor *et al.,* 1998). Many stresses – including chilling, exposure to low temperature, RBAs and oxidative and respiratory stresses – enhance the production of ROS above the steady-state level, which could release buds from dormancy or be harmful (lead to cell wall loosening/breakage) and result in cell death (Tiwari *et al.,* 2002; Theocharis *et al.,* 2012; Beauvieux *et al.*, 2018). Several review articles have indicated that some ROS, such as H_2O_2, are not just detrimental to a plants physiology, but acts as a secondary messenger that triggers growth and developmental changes that are crucial to the plants survival (Ismail *et al.* (2015); Quan *et al.*, (2008); Smirnoff and Arnaud (2019)) .

From this it is understandable that the importance of ROS scavenging is imperative. Figure 3 illustrates the major enzymes involved in ROS scavenging. High light stress energises oxygen (O_2) into singlet oxygen ($^1O_2$), which is a biologically important ROS. Oxygen (O_2) can also be reduced into superoxide radical (O_2^-), which then dismutates to hydrogen peroxide (H_2O_2) (see Fig 3A) and further to the hydroxyl radical (HO) (Apel and Hirt, 2004; Scandalios 2005; Noctor *et al.,* 2015). The most common and harmful ROS is H_2O_2, can be scavenged by one of three pathways important in the process of dormancy: 1) CAT (Fig 3B), 2) the ascorbate-glutathione cycle (Fig 3C) and 3) the glutathione-peroxidase cycle (Fig 3D). Of these three pathways, CAT is the only pathway not using nicotinamide NADH phosphate (NADP) as a substrate (Mittler, 2002; Apel and Hirt, 2004). Enzymes such as CAT, as well as antioxidants such as glutathione and ascorbate are often measured as an indication of oxidative stress within a plant as they are important in detoxifying and catalysing peroxidase (H_2O_2) into water (H_2O) and oxygen (O_2) (Noctor *et al.*, 1998 and 2015; Das and Roychoudhury, 2014; Beauvieux *et al.*, 2018).

Nir and Lavee (1993) indicate that while low temperature induces grapevine budbreak, it also decreases the activity of CAT. They suggest that this inhibition of CAT causes a rise in H_2O_2 content, resulting in a state of oxidative stress in the bud. H_2O_2 can then potentially act as a secondary messenger causing budbreak. Similar responses were reported under induced low winter temperatures in dormant grapevine buds (Nir *et al.*, 1986). Similarly, Pérez and Lira (2005) showed that cold winter conditions reduce CAT and activate H_2O_2, with the latter peaking at the end of endodormancy coinciding with the onset of budbreak in grapevine (*Vitis vinifera* L.) buds.

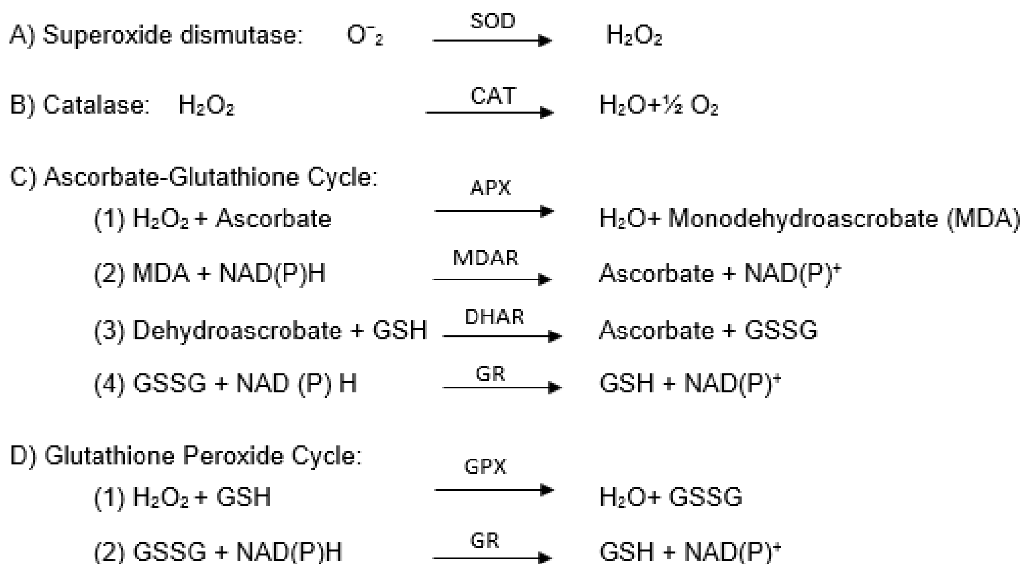

Figure 3. The principal modes of enzymatic ROS scavenging by superoxide dismutase (SOD), catalase (CAT), the ascorbate-glutathione cycle and the glutathione peroxidase (GPX) cycle. Ascorbate peroxidase (APX), glutathione reductase (GR), MDA reductase (MDAR), dehydroascorbate DHA reductase (DHAR), ascorbic acid, reduced form of glutathione, oxidised glutathione (GSSG) (adapted from Apel and Hirt, 2004).

An interesting study by Perez *et al.,* (2007) done on grapevines from two climatically contrasting areas in Chile showed that H_2O_2 levels increase similarly during the endodormant period in both the warm and cold winter area. However, at the end of the endodormant period, the H_2O_2 levels in the cold winter area decrease drastically as mitochondrial respirational capacity increases towards spring. In the warm winter areas, this drop in H_2O_2 is not observed. The H_2O_2 levels remain high and the authors suggest that this is due to an over-reduced ubiquinone pool resulting from the decreased activity of cytochrome C oxidase and alternative oxidase activity brought about by the increased winter temperatures. This prolonged state of increased H_2O_2 then causes an ongoing inhibition of the cell cycle (Clopton and Saltman, 1995) and induces the continued growth arrest associated with erratic budbreak in warm winter areas. Such increased peroxidase levels at the end of endodormancy can also cause NADP to stay in its oxidised form, which could lead to the activation of the PPP (Nir and Lavee, 1993).

HC-treated buds in warm winter regions exhibit an early occurrence of a H_2O_2 peak due to the inhibition of CAT and also an advanced peak that coincides with budbreak (Pérez and Lira, 2005). HC treatment results in CAT inhibition in dormant buds of grapevine (Shulman *et al.,* 1986; Pérez and Lira, 2005; Or *et al.*, 2002) and peaches (Bregoli *et al.*, 2006). Wang *et al.* (1991) indicate that CAT levels increase progressively during budbreak initiated by TDZ and decrease again as the buds start to grow. They also found high levels of peroxidase activity during dormancy and

indicate that it declines during budswell, increases again at bloom and then declines as the buds start expanding.

Antioxidants have also been associated with dormancy release and budbreak when RBA's are used. Wang *et al.* (1991) found that TDZ treatment increased ascorbic acid and reduced glutathione, while reducing dehydroascorbate-oxidised glutathione (GSSG). The treatment also increased the ascorbic acid:dehydroascorbate and glutathione:GSSG ratios. They subsequently proposed a scheme of an ascorbate-glutathione cycle associated with budbreak for removing free radicals in apples. Similarly, TDZ treatment of apple trees increases ascorbic-acid oxidase activity, which peaks in treated buds during bud expansion and budbreak (Wang and Faust, 1992). In the case of peach buds and cherry seeds, Siller-Cepeda *et al.* (1992) found that glutathione levels increase during the endodormant state until the CR is met and then start to decrease. The changes induced by low temperatures or the application of RBAs result in a temporary state of oxidative stress and metabolic disorder and this might be part of the short-term mechanism that leads to budbreak as a long-term effect (Pérez *et al.*, 2007 and 2008).

5.2. Respiration

5.2.1. Aerobic and anaerobic respiration

Oxygen is the central life element for the metabolic events of plant growth and survival. Naturally, plants sense atmospheric oxygen in order to complete their respiratory cycles. A decline in oxygen results in changes of physiological mechanisms for adaption and a reduction in the amount of energy produced (Taiz and Zeiger, 2010). Typically, plants are subjected to normal oxygen accessibility (normoxia). Under sub-lethal stresses initiated by chemicals, freeze, drought and toxic substances, plants go through inadequate oxygenation or low oxygen (hypoxia) to complete oxygen deprivation (anoxia), and this might induce budbreak (de Sousa and Sodek, 2002). Oxygen availability can be sensed directly or indirectly depending on metabolic events, developmental stage, temperature, severity of stress and environmental conditions (de Sousa and Sodek, 2002; Pucciariello and Perata, 2012).

Respiration involves a sequence of multifaceted reactions including glycolysis, the TCA cycle and oxidative phosphorylation that are all catalysed by specific enzymes (Taiz and Zeiger, 2010). Figure 4 provides a graphic overview of the respiration process. Aerobic respiration takes place when the organic compound glucose is mobilised during the glycolysis process and utilised in a controlled way to release and store energy such as adenosine triphosphate (ATP), which is used for cell growth and development (Taiz and Zeiger, 2010). The energy production involves two high-energy intermediates: $FADH_2$ and nicotinamide NADH. This oxidation of sugars takes place

in a series of reactions, of which the TCA cycle and the PPP are perhaps best known (Fernie *et al.,* 2004; Taiz and Zeiger, 2010). All these pathways are sensitive to temperature changes and slow down at low temperatures, thus it is understandable that respiration (and therefore growth) slows down during dormancy.

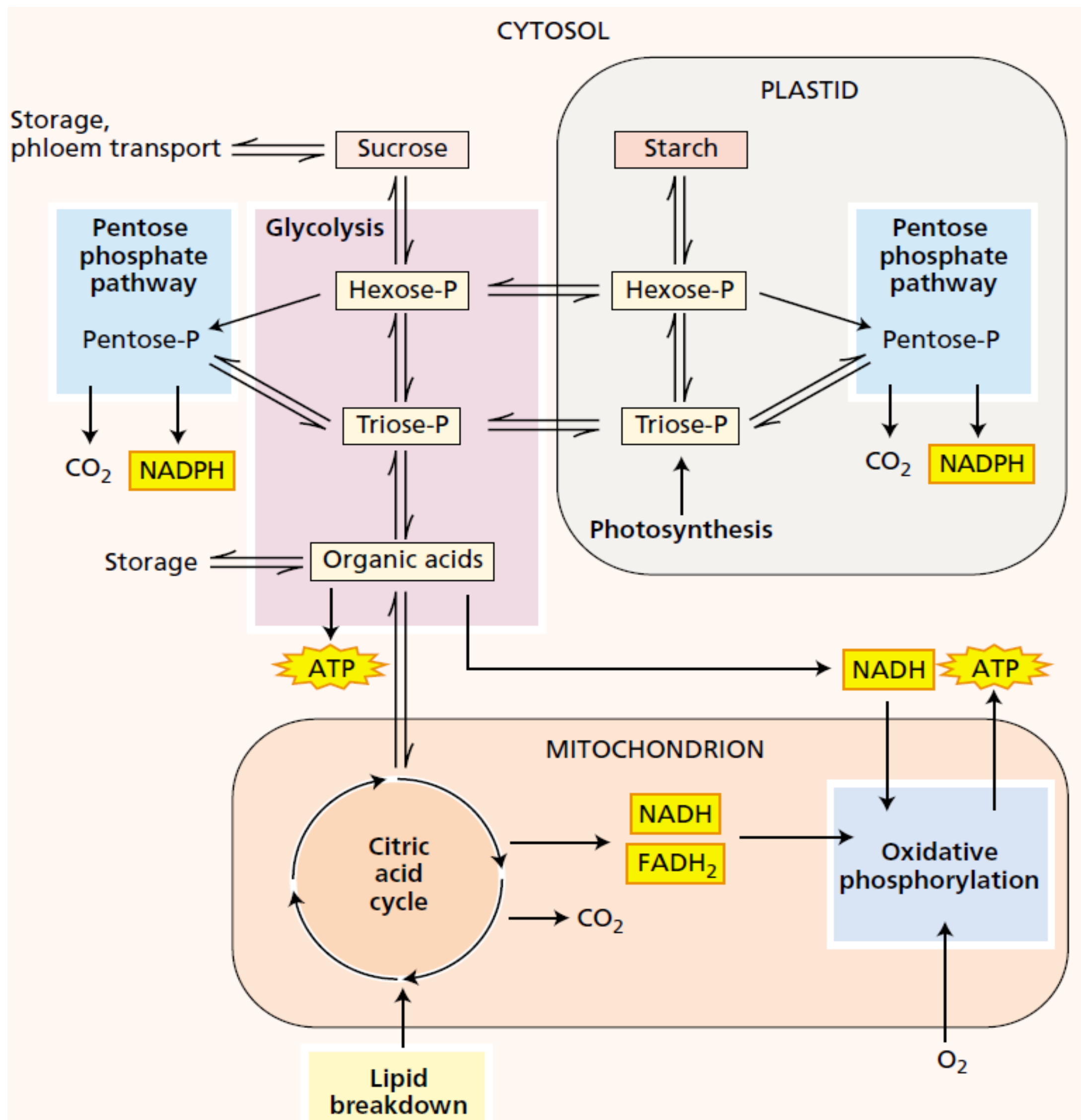

Figure 4. Overview of respiration. Substrates for respiration are generated by other cellular processes and enter the respiratory pathways. Glycolysis and the pentose phosphate pathways in the cytosol and plastid convert sugars to organic acids, via hexose phosphates and triose phosphates, generating adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) and adenylic nucleotides (ATP). The organic acids are oxidised in the mitochondrial citric acid cycle and the NADH and flavin NADH ($FADH_2$) produced provide the energy for ATP synthesis by the electron-transport chain and ATP synthase in oxidative phosphorylation. In gluconeogenesis, lipid breakdown produces carbon in the glyoxysomes, which is metabolised in the citric acid cycle (also known as the TCA cycle) and then used to synthesise sugars in the cytosol by reverse glycolysis (With permission from Taiz and Zeiger, 2010).

Oxygen shortage (hypoxia) leads to a switch from aerobic to anaerobic glycolysis. The shortage of oxygen, which is the terminal electron acceptor, leads to a decline of mitochondrial respiration (mainly oxidative phosphorylation) and NADH production, which in turn results in the decline of ATP generation. Therefore, the glycolysis reaction increases and, together with fermentation, is responsible for energy production. Reserves are mobilised to facilitate the increase of carbohydrates and ATP production in the absence of oxygen. In such situations, plants produce little energy for survival (de Sousa and Sodek, 2002; Toro and Pinto, 2015; Beauvieux *et al.*, 2018). Species or organs that can switch between aerobic and anaerobic metabolism can accumulate ROS, which are then detoxified by antioxidant enzymes by converting peroxidases into water (Taiz and Zeiger, 2010).

Oxygen deprivation is a critical survival state for deciduous fruits under low or freezing temperatures. Hypoxia can prevent plant growth and development and initiate oxidative stress (Pérez *et al.*, 2012). Hypoxia tolerance depends on many factors and the levels differ depending on species, cultivars and temperature levels. Hypoxia, which occurs during dormancy, is induced naturally by exposure to chilling or artificially, as a reaction to RBA application. This temporary switch can influence budbreak, foliation and production (Rubio *et al.,* 2014).

5.2.2. Glycolysis

Glycolysis is an anaerobic pathway of respiration (Fig. 4). Through oxidation, it breaks sucrose down into two molecules of pyruvate, ATP and a reductant (NADH), in ten distinct reactions (Taiz and Zeiger, 2010). Soluble enzymes that continuously occur in the cytosol regulate glycolysis. Of these, pyruvate kinase catalyses pyruvate oxidation and is considered to be the most important in glycolysis regulation. A glycolysis reaction of one molecule of glucose generates two molecules of ATP, two molecules of NADH and two acetyl-coenzyme A (CoA), a substrate of the TCA cycle (Taiz and Zeiger, 2010).

The regulation of glycolysis (through phosphofructokinase and pyruvate kinase) has been connected to dormancy progression and release. Gai *et al.* (2013) reported the activation of the main glycolysis enzymes at the end of endodormancy in *Paeonia ostia* under chill treatment. Similar increases in glycolysis enzymes have been reported in grapevine buds treated with HC (Sudawan *et al.,* 2016). Increases in levels of glycolysis enzymes, pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase have been reported during budbreak in apple buds treated with TDZ (Wang *et al.,* 1991). Similarly, heat-shock, HC-induced fermentation enzymes (pyruvate decarboxylase and dehydrogenase) were found to act in parallel with glycolysis enzymes in grape buds (Halaly *et al.,* 2008).

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5.2.3. Fermentation pathway

Generally, under insufficient supply of O_2 (anaerobic conditions), glycolysis cannot continue and plants carry out a series of fermentative reaction to continue the metabolism of pyruvate and uphold glycolysis. Such hypoxic (low O_2) or anoxic (zero O_2) conditions will increase the activity of pyruvate decarboxylase and alcohol dehydrogenase that will act on pyruvate and ultimately produce ethanol and CO_2 whilst oxidising NADH to NAD^+ (Taiz and Zeiger, 2010). As both the downstream TCA cycle and oxidative phosphorylation pathways are not functional, the plant uses the newly formed NAD^+ to further maintain glycolysis and thus continue to produce ATP required for cell survival. In a dormancy breaking trial, Pérez *et al.* (2009) showed that the O_2 uptake in the mitochondria of sodium azide treated grape buds was inhibited and this increased glycolysis and ethanolic fermentation which result in ROS production causing budbreak. Similar studies also reporting the stimulation of fermentative metabolism in dormant buds under chilling or RBA treatments include that of Or *et al.* (2000) and Halaly *et al.* (2008).

5.2.4. Pentose phosphate pathway (PPP)

An alternative to glycolysis, the PPP channels glucose oxidation without producing or consuming ATPs directly (Fig. 4). This pathway is located in the cytoplasm (cytosol and plastids) and is the major source of nicotinamide adenine dinucleotide phosphate (NADPH). It produces the pentose molecules needed for DNA and RNA biosynthesis, generates 12 NADPH from one glucose molecule and detoxifies H_2O_2 in cells under oxidative stress (Taiz and Zeiger, 2010). PPP enzymes (glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH)) are important for both growth and dormant conditions. Tian *et al.* (1998) suggest that the inhibition of G6PDH result in cell-growth inhibition, the production of ROS (H_2O_2) and a decline in NADPH production. Vanlerberghe (2013) notes that during oxidative stress, PPP is the main supplier of NADPH oxidase for plasma membranes. PPP and NADPH are stimulated during scavenging of H_2O_2 in the ascorbate-glutathione cycle and this is associated with the detoxification of the high level of H_2O_2 during dormancy release (Salvemini *et al.,* 1999).

PPP enzymes have been associated with bud and seed dormancy release. Swamy and Sandhyarani (1986) report high levels of PPP enzymes (G6PDH and 6PGDH) during dormancy release in peanut seeds. Similarly, an increase in PPP enzymes has been reported in *Corylus avellana* seeds during dormancy breakage (Gosling and Ross, 1980). However, Sagisaka (1972, 1974) reported a shift of the G6PGDH metabolism to glycolysis in poplar twigs in early spring and vice versa in autumn. Wang *et al.* (1991) have shown that the changes in enzyme activity of the glycolytic and pentose-phosphate cycles appear to be synchronised with budbreak and growth

resumption. They found higher activity of PPP enzymes (G6PDH and 6PGDH) in dormant buds than those in the active growing phase; this activity decreases during budbreak and resumption of growth. Walton *et al.* (1991) suggested that PPP activity increases during dormancy release in kiwifruit buds. Treatment with RBAs increases the level of proline associated with PPP increase and results in high levels of budbreak (Walton *et al.*, 1991). Similarly, short-day treatment in nectarine buds during dormancy induces a rise in G6PDH activity (Li *et al.,* 2011). The onset of bud endodormancy is associated with a switch in the respiration pathway from the predominant TCA pathway in growing buds to the PPP in dormant buds and a reverse upon dormancy release, provided that the CR has been met (Panneerselvam, *et al.*, 2007; Tan *et al.,* 2010). This means that insufficient chilling exposure (warm winter conditions) prevents the transition of respiratory mode and the buds will remain in a high-level PPP and low-level TCA (Tan *et al.,* 2010).

5.2.5. Tricarboxylic acid cycle (TCA)

Located in the mitochondrial matrix, the TCA cycle (also known as the citric acid or Krebs cycle) is an essential metabolic pathway that consists of a cyclic flux of nine biochemical reactions regulated by a number of enzymes (Taiz and Zeiger, 2010). The TCA cycle begins with the conversion of pyruvate into acetyl-CoA from the cytoplasm and into the mitochondrial matrix. Mitochondrial pyruvate dehydrogenase and multi-enzymes catalyse this reaction. For each molecule of pyruvate metabolised and intermediates of the flux that are oxidised, the TCA cycle yields molecules of $FADH_2$, generates molecules of NADH and produces ATP. These intermediates are used for oxidative phosphorylation (Fernie *et al.*, 2004; Taiz and Zeiger, 2010; Sweetlove *et al.*, 2010).

Tan *et al.* (2010) indicated that chilling exposure affects the TCA pathway activity of nectarine buds. The authors showed that sufficient chilling increases TCA activity prior to dormancy release, while deficient chilling keeps buds in a dormant state. This increase has been associated with energy production. Under chill treatment, gene transcription of TCA enzymes was induced at the end of endodormancy in tree peonies (*Paeonia ostia*) (Gai *et al.*, 2013). Similarly TCA related transcription were found in ‘Royal Gala’ apple buds treated with low temperatures to induce budbreak (Porto *et al.,* 2015). The use of TDZ initiates changes in the TCA cycle in apples when promoting budbreak. The activity of TCA cycle enzymes (isocitrate dehydrogenase) increases, peaks during green-tip periods and then declines thereafter (Wang *et al.,* 1991). These pathways seem to be connected and behave differently in warm and cold winter areas.

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5.2.6. Electron transport chain and oxidative phosphorylation

The electron transport chain (ETC) occurs within the inner mitochondrial membrane. Figure 5 shows the organisation of the ETC and ATP synthesis in the inner mitochondrial membrane. Once the activity of the TCA pathway and glycolysis increase and produce NADH and $FADH_2$, the mitochondrial respiratory chain is activated and the electrons move through complex I, III and IV and flow between two main respiration pathways: the CYT and the alternative (ALT) pathway (Fig 5) (Taiz and Zeiger, 2010). The H^+ proton gradient is generated across the membrane, which in turn uses oxygen and produces the necessary energy, in the form of ATP, through oxidative phosphorylation. During this sequence of redox reactions, one NADH molecule yields three ATPs, while an FADH molecule produces two (Taiz and Zeiger, 2010). Beauvieux *et al.* (2018) mention that at the final steps of this process of aerobic conditioning, the bulk of ATP is generated through the TCA cycle, the oxidative phosphorylation electron chain and alternative oxidase – these are the carriers and channels that provide the substrates and co-factors from the cytosol.

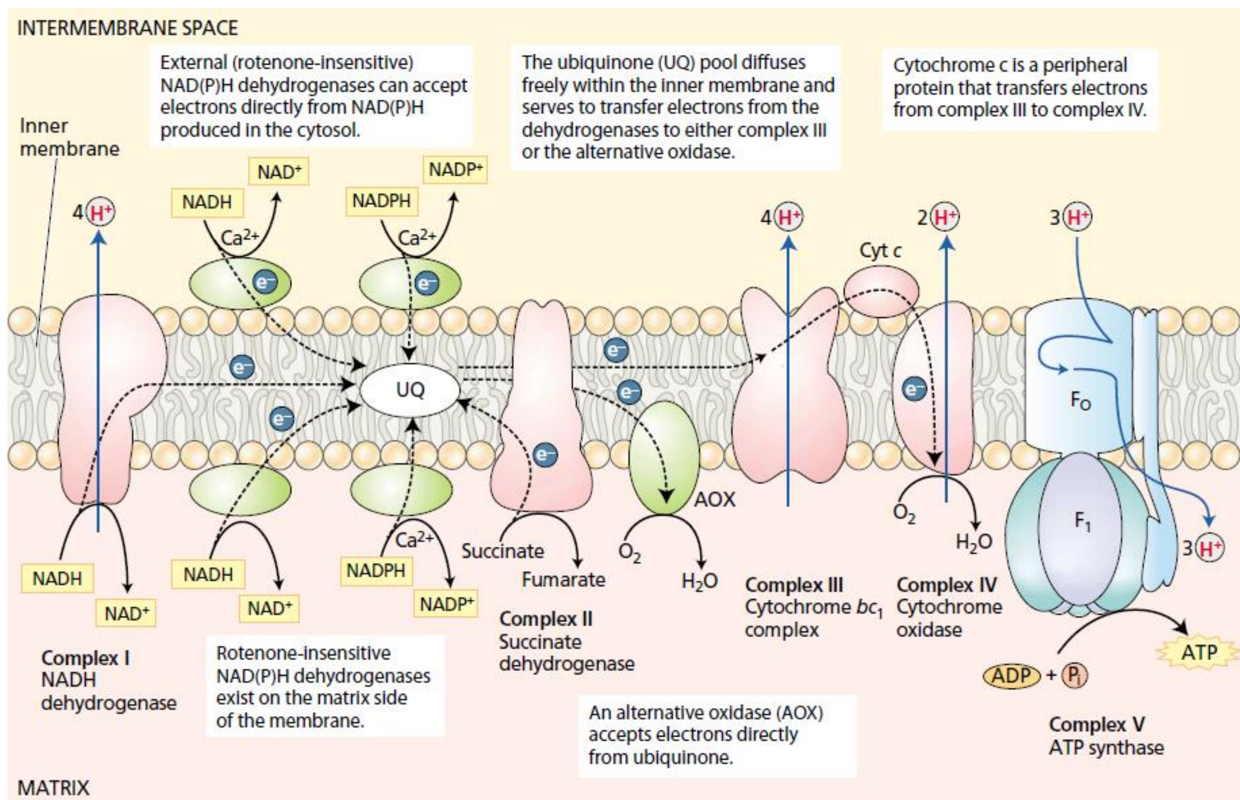

Figure 5. Organisation of the electron transport chain (ETC) and adenylic nucleotides synthesis in the inner membrane of plant mitochondria. In addition to the five standard protein complexes found in nearly all other mitochondria, the electron transport chain of plant mitochondria contains five additional enzymes, marked in green. None of these additional enzymes pumps protons. Specific inhibitors (rotenone for complex I, antimycin for complex III, cyanide for complex IV and salicylhydroxamic acid for the alternative oxidase) are important tools to investigate the ETC of plant mitochondria (With permission from Taiz and Zeiger, 2010).

The entire respiratory pathway may depend on sufficient supply of oxygen. Consequently, a decline in oxygen (hypoxia or anoxia) can result in a shift from aerobic respiration to anaerobic glycolysis and fermentation. Some studies report that mitochondrial respiration could be associated with the centre role of sensing the release of dormancy (Pérez *et al.*, 2007 and 2008; Zanol *et al.,* 2010). Pérez *et al.* (2009) found that the use of NaN_3 (a mitochondrial inhibitor of complex IV) and HC on isolated bud mitochondria, results in oxygen deprivation and dormancy release. Under chilling conditions or HC treatment, mitochondrial activities are disturbed and oxidative phosphorylation is activated during budbreak in grapevines (Pérez *et al.*, 2007) and tree peony (*Paeonia ostia)* (Gai *et al.,* 2013). The mitochondrial disruption under different stimuli (HC, hypoxia, chilling, NaN_3), results in oxidative stress with a rapid accumulation of ROS and the decline of other activities, such as ATP production and the TCA cycle (Sudawan *et al.,* 2016). To survive this temporary energy crisis, other cycles/ pathways are activated; these include glycolysis, pyruvate metabolism and anaerobic respiration or fermentation.

The two mitochondrial respiration pathways (ALT and CYT) are important and seem to act in opposite ways during budbreak. HC or other stimuli such as high temperature, water stress, were found to be inhibitors of CYT c oxidase activity, which coincides with the activation of the ALT (Ribas-Carbo *et al*., 2005; Amberger, 2013; Tan *et al*., 2013). The inhibition of the CYT pathway results in mitochondrial disturbance as well as oxidative stress in the whole respiration system. Shi *et al.* (2013) also associate a decrease in total respiration, CYT and ALT pathways, with ROS accumulation in chilled tomato. To survive this decrease in ATP production and oxygen levels, alternatives pathways – such as glycolysis, PPP and fermentation – are induced (Toro and Pinto, 2015).

5.3. Membrane lipids

Known as a boundary separating cells, the cell membrane regulates selective permeability of the outer cell wall and is involved in the signalling process of external stimuli (Taiz and Zeiger, 2010; Beauvieux *et al*., 2018). The major constituents of the cell membrane are lipids, sterols, proteins, phospholipids and carbohydrates and their composition varies from one cell membrane to another. Lipids are mainly made up of polar and neutral lipid fractions which are quite different and are involved in different metabolic processes of cells (Quinn and William, 1978; Dulf *et al.*, 2013).

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5.3.1. Lipid structure, composition and function

The presence of lipids in the membranes facilitates energy storage and offers selective permeability of sugars, salts, substrates, etc. by maintaining appropriate fluidity. Polar lipids are known as barrier membranes that introduce an amphipathic property, which means they form layers in both hydrophobic and hydrophilic aqueous form and reflect an arrangement of lipids in two thick layers of molecules (Ohlrogge and Browse, 1995). This is possible because a typical polar lipid is made up of a hydrophilic head-group and a hydrophobic tail-group. Polar lipids carry a charge in their head-group, hence polar, and is divided into two groups, phospholipids and glycolipids, based on the constitution of their head-groups. The phospholipids typically contain a phosphate head-group with choline, serine or ethanolamine attached, while glycolipids contain a sugars head-group, which can be glucose or galactose. Both types have two fatty acid (FA) chains that form the neutral tail-group. The FA tails can exist as saturated (no double bonds) or unsaturated (contains one or more double bonds) depending on the status of the bonds between the molecules that make up the carbon chain (Ohlrogge and Browse, 1995). Polar lipids can act as signal molecules for membrane biogenesis and can also change the fluidity of the membrane depending on the environmental condition (Schmid and Ohlrogge, 2002).

Lipids also occur in a neutral form; major neutral lipids include triacylglycerols and sterol- and wax esters (Athenstaedt and Daum, 2006). Neutral lipids are the major storage form of carbon and energy for growth and development (Chapman *et al.*, 2012). Sterol esters are essential membrane components for the phospholipid bilayer and provide rigidity to the membrane. Wax esters store energy and can generate FAs and long-chain alcohols that can be further metabolised. Both sterol esters and triacylglycerols form cytosolic lipid droplets or lipid particles as a special form of storage within phospholipid monolayers (Schmid and Ohlrogge, 2002; Chapman *et al.*, 2012). These neutral lipids have a similar FA tail-group to polar lipids but lack the polar head-group, hence neutral. The FA chains can also be saturated or unsaturated and, in the case of the tricylglycerols, contain three FA molecules. In both polar and neutral lipids, the FAs chains are predominantly 16 or 18 carbons long but can extend up to a chain length of 24 carbons (Ohlrogge and Browse, 1995). The saturation can vary but is typically found to be C18:1, C18:2, C18:3, C16:0 and, in some species, C16:3. The first number in the notation indicates the length of the FA chain (number of acyl groups) and the number after the semi colon indicates the amount of double bonds it contains.

5.3.2. Biosynthesis, elongation, de- or unsaturation of fatty acids

Lipids are synthesised in different locations and with different specialisations. The plastids are a group of different organelles (plasma membrane, nucleus, cytosol, tonoplast, thylakoid, chloroplast) containing similar genetic material and serve as the general storage sites for lipids. Acetyl-CoA synthetase is believed to transport the lipids from the plastids to the cytosol. Phospholipids are, however, mostly found in the endoplasmic reticulum and most of the FA desaturation (membrane lipids and triacylglycerols) occurs in the endoplasm. The mitochondrial membranes also mainly synthesise FAs from the endoplasmic reticulum and plastids. The peroxisomal system in plants (FA oxidation) appears to be another source of FAs (Ohlrogge and Browse, 1995; Schmid and Ohlrogge, 2002).

Biosynthesis of FAs is important for cell growth. FA biosynthesis is a stepwise gathering of acetyl-CoA units (substrates derived from the glycolytic pathway), which ends with palmitic acid (C16:0), a saturated 16 acyl fatty acid chain (SFA). From the C16:0 FA, the biosynthesis can continue in two ways: it increases the number of *cis*-double bonds that causes bends (kinks) in the FA chain or it increases the chain length. In the case of membrane lipids, the acyl chain length as well as the number and position of double bonds influence fluidity, permeability and stability of membranes (Quinn *et al.*, 1989; Ohlrogge and Browse, 1995).

In this way the palmitic acid (C16:0) is then lengthened into stearic acid (C18:0), another SFA. The generation of plant SFAs (16- or 18-carbon) from acetyl-CoA and melonyl-CoA is a complex pathway that requires at least 30 enzymatic reactions (Ohlrogge and Browse, 1995). This SFA can undergo a gel-to-liquid-crystalline phase transition above 40°C and tends to form dense, hydrophobic membrane lipids impermeable to ions. This induces membrane rigidification, with a decline in permeability and mobility. At lower temperatures, FAs form *cis*-double bonds that enhance membrane fluidity (Los and Murata, 2004). Contrary to the SFA biosynthesis, the unsaturated fatty acids (UFAs) synthesis seems to be a simpler process with only three related genes: *fabA, fabB* and *fabF* involved. *FabA* is reported to code for the main desaturase enzyme that introduces the *cis*-double bonds, while *fabB* and *fabF* are involved in chain elongation. The distribution of the *cis*-double bond in the FA chains is a regulated process that occurs in a specific manner and in a certain order (Ohlrogge and Browse, 1995). FA desaturation takes place after chain elongation. There are three known groups of desaturase enzymes: the most common, acyl-lipid desaturases, are specific to FAs esterified to glycerolipids; acyl-[acyl-carrier-protein] desaturase (acyl-ACP desaturases) occur in the stroma of plastids; and acyl-CoA desaturases are not common. It was reported that changes in temperature of acyl-lipid desaturases and glycerol-3-phosphate acyltransferase induce variations in the degree of unsaturation of FAs

(Ohlrogge and Browse, 1995; Nishida and Murata, 1996). During FA desaturation, both elongation of C16:0 and desaturation of C18:0 to 18:1 occur in plastids and involve acyl-ACPs desaturase (Nishida and Murata, 1996). Other desaturations, from C18:1 to the very long-chain FAs (C20:0 and above), are lipid-bound forms, although they also involve acyl-lipid desaturases (Cassaone *et al.*, 1994).

Generally, at low temperature or under certain biological stresses, stearic acid is desaturated into oleic acid (C18:1), an 18 acyl group FA containing one double bond (monounsaturated FA, MUFA) to preserve membrane fluidity. Oleic acid is desaturated into linoleic acid (C18:2), which has two *cis*-double bonds (polyunsaturated FAs, PUFAs); this desaturation introduces "kinks" or bends into a FA chain that assist in maintaining the membrane stability and fluidity. For better maintenance of membrane fluidity and permeability, linoleic acid (C18:2) is desaturated to linolenic acid (C18:3), a FA containing three *cis*-double bonds and is referred to as a high PUFA (Quinn, 1981; Ohlrogge and Browse, 1995; Nishida and Murata, 1996; Los and Murata 2004). UFAs, such as MUFAs and PUFAs, are related to different degrees of membrane fluidity and permeability. In brief, linolenic acid (C18:3) is synthesised from stearic acid (C18:0) by a series of desaturase enzymes. Unsaturation levels might lead to an increase in membrane stability and in most plant tissue, above 75% of the FAs are UFA (Ohlrogge and Browse, 1995). Depending on the abiotic or biotic stress, membrane lipids change in UFA levels by realising linolenic acid (C18:3) (Upchurch, 2008).

5.3.3. Fatty acids and dormancy release

De- or unsaturation of FAs seems to play an important role in dormancy release. Sufficient cold temperatures or chemical RBA such as TDZ influences the desaturation process of FAs, which increase the degree of unsaturation and alter the lipid composition (Wang and Faust, 1988 and 1990). In a similar fashion, Bregoli *et al.* (2006) also indicate that a HC and oil combination might affect membrane permeability, cell lipid composition and content.

Wang and Faust (1990a, b) indicated that the FA of phosphatidylcholine in 'Delicious' apple buds exposed to sufficient winter chill show an increase in linoleic (C18:2) and a simultaneous decrease in oleic acid (C18:1) during the winter period. As soon as the chill requirement is satisfied, linoleic acid (C18:2) decreased drastically and linolenic acid (C18:3) increases towards bud break. Other studies also indicated this trend of increasing UFAs as well as an increase in phospholipid content in membrane lipids associated with budbreak and growth resumption (Pomeroy and Raison, 1981; Wang and Faust, 1988; Faust *et al.*, 1997; Bregoli *et al.*, 2006).

Similar to membrane lipids, FAs in lipid reserves (neutral lipids) increase their number of double bonds (unsaturation levels), preferentially at position ω-3, in order to meet high energy required for growth (Athenstaedt and Daum, 2006; Meï *et al*., 2015). All this shows that lipid structure, fluidity and permeability are partly regulated by the desaturation level, which in turn influences dormancy release. Further studies show that linolenic acid (C18:3) is also desaturated into jasmonic acid (JA), a stress hormone that triggers a plant's defences against pathogens by influencing its growth functions (Creelman and Mullet, 1997; Taiz and Zeiger, 2010). JA is connected to dormancy release in seeds and may react in the opposite way to ABA (Creelman and Mullet, 1997) to stimulate growth.

5.3.4. Sterols and dormancy release

For the most part, sterols are known as integral components of membrane-lipid rigidity and are essential for plant growth and development (Schaller, 2003; Dufourc, 2008). Sterols have been associated with membrane fluidity, permeability and enzyme activity and their amounts vary according to tissue and species (Grunwald, 1970 and 1971). Changes in sterol levels and composition have been associated with endodormancy release and budbreak. Sufficient chilling, TDZ and nitroguanidines induced a sterol decrease in apple buds prior to budbreak and growth resumption (Wang and Faust, 1988, 1990b and 1993). Piispanen and Saranpää (2004) found that free sterols decline during spring and early summer neutral lipids in silver birch (*Betula pendula*). Sterols also act as essential signals in the plant cell membrane (Lindsey *et al*., 2003). Wang and Faust (1990b) found that the sterol: phospholipid ratio in apple buds decreases rapidly during dormancy in both terminal and axillary buds, indicating an increase in membrane fluidity and cell metabolism during cold months and as the period of budbreak and growth approaches. The interaction between sterols and phospholipids is also important, as it may influence membrane fluidity. The more sterols embedded in the membrane, the more viscous and less fluid it becomes.

In summary, changes in the physical state of the membrane lipids play an important role in plant tissue from dormancy development until budbreak in spring. Low temperatures or plant growth regulators increase the degree of desaturation of FAs, change the sterols levels and composition and lead to cell membranes fluidity and permeability.

6. Conclusion

The deciduous fruit tree dormancy cycle is a complex process governed by a network of environmental, genetic and endogenous factors. Different biochemical, molecular and physiological studies have been conducted on the induction, maintenance and release of

dormancy and some interesting findings have been reported. From this review, it is tempting to conclude that temperature might be the key factor influencing the dormancy cycle throughout the season. The review has highlighted some of the changes that low temperatures bring about in dormant buds during the winter season. It has also noted the consequences of insufficient chilling in deciduous fruit trees and the importance of RBAs in areas with warm winter conditions.

It was noted that most of the physiological studies are based on induced cold stimuli and little attention has been given to studying the dormancy period under field conditions. There is also limited information on the total respiration rate and pathways as well as on the lipid composition of apple bud membranes when exposed to insufficient chill and none available for the South African mild winter production areas. Although HCo treatments showed the capability to break endodormancy and stimulate growth, its ability to affect the respiration dynamics and lipid composition during budburst under South African mild winter conditions has not been described to date. It is not known whether HCo application affects lipid composition in a similar way as natural winter chill and how this compares to buds that did not receive adequate chill. This is why it is important to understand the physiological changes brought about by the South African climate to aid future development of products and procedures to improve apple cultivation in marginal production areas.

From the current findings, it is worth suggesting that further research should consider climatic changes, geographical differences and the factors affecting dormancy induction as well as the post-dormancy phase. RBA investigations should take regional differences into consideration, since chilling accumulation differs from one area to another. Continuous global warming and climate change on a large scale will reduce chill accumulation and areas that currently have sufficient winter chill may become marginal. This increases the need for RBAs and for more research to find alternative methods of overcoming delayed foliation. Commercial production of deciduous fruit trees in warm areas requires exhaustive experimentation, both fundamental and applied, since inadequate chilling affects the dormancy cycle and negatively impacts production.

7. References

Adams, W.D. (2006). Recommended fruit and nut varieties. *Texas A&M AgriLife extension*. pp: 14.

Aihua, L., Shunyuan, J., Guang, Y., Ying, L., Na, G., Tong, C., Liping, K. and Luqi, H. (2018). Molecular mechanism of seed dormancy release induced by fluridone compared with cold stratification in Notopterygium incisum. *BMC Plant Biology*. **18**,116.

Allona, I., Ramos, A., Ibáñez, C., Contreras, A., Casado, R. and Aragoncillo, C. (2008). Molecular control of winter dormancy establishment in trees. *Spanish Journal of Agricultural Research*. **6**, 201– 210.

Amberger, A. (2013). Cyanamide in plant metabolism. *International Journal Plant Physiology and Biochemistry*. **5** (1), 1–10.

Anderson, J.V., Chao, W.S. and Horvath, D.P. (2001). A current review on the regulation of dormancy in vegetative buds. *Weed Science*. **49**, 581–589.

Andreini, L., Viti, R., Bartolini, S., Ruiz, D., Egea, J. and Campoy, J.A. (2012). The relationship between xylem differentiation and dormancy evolution in apricot flower buds (*Prunus armeniaca* L.): The influence of environmental conditions in two Mediterranean areas. *Trees*. **26**, 919–928.

Anzanello, R., Fialho, F.B., dos Santos, H.P., Bergamaschi, H. and Marodin, G.A.B. (2014). Bud dormancy in apple trees after thermal fluctuations. *Pesquisa Agropecuária Brasileira*. **49**, 457–464.

Apel, K. and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annual Review of Plant Biology*. **55**, 373–399.

ARC-Infruitec, 1997. *South African deciduous fruit cultivars catalogue*. 2nd ed. ARC Infruitec-Nietvoorbij, Stellenbosch, SA. (as per Tharaga, 2014).

Arias, O. and J. Crabbé. (1975). Les gradients morphogénétiques du rameau d'un an des végétaux ligneux en repos apparent. Données complémentaires fournies par l'étude de *Prunus avium* L. *Physiologie Végétale*. **13**, 69–81.

Arora, R, Wisniewski, M.E. and Scorza, R. (1992). Cold acclimation in genetically related (Sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch). Seasonal changes in cold hardiness and polypeptides of bark and xylem tissues. *Plant Physiology*. **99**, 1562–1568.

Arora, R., Rowland, L.J. and Tanino, K. (2003). Induction and release of bud dormancy in woody perennials: a science comes of age. *HortScience*. **38**, 911–921.

Athenstaedt, K. and Daum, G. (2006). The life cycle of neutral lipids : Synthesis, storage and degradation. *Cellular and Molecular Life Science*. **63**, 1355–1369.

Atkinson, C.J., Brennan, R.M. and Jones, H.G. (2013). Declining chilling and its impact on temperate perennial crops. *Environmental and Experimental Botany*. **91**, 48–62.

Avenant, E. and Avenant, J.H. (2014). Chill unit accumulation and necessity of rest breaking agents in South African table grape production regions. 37th World Congress of Vine and Wine and 12th General Assembly of the OIV, BIO Web of Conferences. **3**(01017), 1–4.

Baker, J.M. (1970). The Effects of oils on plants. *Environmental Pollution*. **I**, 27–44.

Balandier, P., Bonhomme, M., Rageau, R., Capitan, F. and Parisot, E. (1993). Leaf bud endodormancy release in peach trees: evaluation of temperature models in temperate and tropical climates. *Agricultural and Forest Meteorology.* **67**, 95–113.

Bangerth, F., Li, C-J. and Gruber, J. (2000). Mutual interaction of auxin and cytokinins in regulating correlative dominance. *Plant Growth Regulation.* **32**, 205–217.

Bartolini, S. and Giorgelli, F. (1994). Observations on development of vascular connections in two apricot cultivars. *Advances in Horticultural Science*. **8**, 97–100.

Baskin, J. and Baskin, C. (2004). A classification system for seed dormancy. *Seed Science Research*. **14**, 1–16.

Beauvieux, R., Wenden, B. and Dirlewanger, E. (2018). Bud dormancy in perennial fruit tree species: A pivotal role for oxidative cues. *Frontiers in Plant Science*. **9**, 657.

Ben Mohamed, H., Vadel, A.M., Geuns, J.M.C. and Khemira, H. (2012). Effects of hydrogen cyanamide on antioxidant enzymes' activity, proline and polyamine contents during bud dormancy release in 'Superior Seedless' grapevine buds. *Acta Plant Physiology*. ***34,*** 429–437.

Bergh, O. (1985a). Morphogenesis of *Malus domestica* cv. 'Starking' flower buds. *South African Journal of Plant and Soil.* **2,** 187–190.

Bergh, O. (1985b). Effect of the previous crop on cortical cell number of *Malus domestica* cv. 'Starking Delicious' apple flower primordia, flowers and fruit. *South African Journal of Plant and Soil.* **2**, 191–196.

Blommaert, K. L.J. (1963). Winter rest of deciduous fruit trees in relation to the problem of delayed foliation.

Stellenbosch University https://scholar.sun.ac.za

South African Journal of Science. **59** (7), 316–319.

Borchert, R. (1991). Growth periodicity and dormancy, pp 221-245. In: A.S. Raghavendra (Ed). Physiology of trees. John Wiley & Sons, NY.

Bonhomme, M., Rageau, R., Lacointe, A. and Gendraud, M. (2005). Influences of cold deprivation during dormancy on carbohydrate contents of vegetative and floral primordia and nearby structures of peach buds (*Prunus persica* L. Batch). *Scientia Horticulturae*. **105**, 223–240.

Borkowska, B. (1981). Dormancy and development of apple axillary buds investigated *in vitro*. *Acta Horticulturae*. **120**, 161–166.

Boyer, J. and Liu, R.H. (2004). Apple phytochemicals and their health benefits. *Nutrition Journal*. **3**, 5.

Bregoli, A.M., Nardozza, S., Andreotti, C, Magnani, R., Corelli-Grappadelli, L., Masia, A. and Costa G. (2006). Relationship between biometric and physiological parameters, application time and flower bud abscission induced by a dormancy breaking agent in Peach. *Acta Horticulturae*, **713**, 191–196.

Campoy, J. A., Ruiz, D. and Egea J. (2011). Dormancy in temperate fruit trees in a global warming context: a review, *Scientia Horticulturae.* **130**, 357–372.

Carvajal-Millán, E., Gardea, A.A., Rascón-Chu, A., Orozco, J.A., Ponce de León, N., Márquez-Escalante, J.A., Romo Chacón, A. and Guerrero Centro, V.M. (2007). Respiratory response of apple buds treated with budbreaking agents. *Thermochimica Acta*. **457**, 109–112.

Chandler, W.H. (1942). Deciduous orchards. Philadelphia, USA: Lea and Febiger. pp: 438.

Chao, W.S., Foley, M.E., Horvath, D.P. and Anderson, J.V. (2007). Signals regulating dormancy in vegetative buds. *International Journal of Plant Developmental Biology*. **1**, 49–56.

Chapman, K.D., Dyer, J.M. and Mullen, R.T. (2012). Biogenesis and functions of lipid droplets in plants. Thematic review series: Lipid droplet synthesis and metabolism: from yeast to man. *Journal of lipid research*. **53**, 215–226.

Chen, T.H.H., Howe, G.T. and Bradshaw Jr, H.D. (2002). Molecular genetic analysis of dormancy-related traits in poplars. *Weed Science*. **50**, 232–240.

Cheng, X., Ruyter-Spira, C. and Bouwmeester, H. (2013). The interaction between strigolactones and other plant hormones in the regulation of plant development. *Frontiers in Plant Science*. **4**, 199.

Clopton, S. and Saltman, P. (1995). Low-level of oxidative stress cause cell cycle specific arrest in cultured cells. *Biochemical Biophysics Research Communications.* **120**, 189–196.

Cohn, M. and Hilhorst, H. (2000). Alcohols that break seed dormancy: The anaesthetic hypothesis, dead or alive. Plant Dormancy (Eds Crabbé J., and Viemont J-D.), CAB International Wallingford. pp: 259–273

Cook, N.C., Rabe, E., Keulemans, J. and Jacobs, G. (1998). The expression of acrotony in deciduous fruit trees: A study of the apple rootstock M.9. *Journal of the American Society for Horticultural Science*. **123**, 30–34.

Cook, N.C. and Jacobs, G. (1999). Sub-optimal winter chilling impedes development of acrotony in apple shoots. *HortScience*. **34**, 1213–1216.

Cook, N.C., Bellen, A., Cronjé, P.J.R., de Wit, I., Keulemans, W., van den Putte, A. and Steyn, W. (2005). Freezing temperature treatment induces bud dormancy in 'Granny Smith' apple shoots. *Scientia Horticulturae*. **106**, 170–176.

Cook, N.C., Calitz, F.J., Allderman, L.A., and Louw, E.D. (2017). Diverse patterns in dormancy progression of apple buds under variable winter conditions. *Scientia Horticulturae.* **226**, 307–315.

Cook, N.C. and Jacobs, G. (2000). Progression of apple (*Malus* x *domestica* Borkh.) bud dormancy in two mild winter climates. *Journal of Horticultural Science*. **75,** 233–236.

Cook, S. J., Balmanno, K., Garner, A., Millar, T., Taverner, C. and Todd, D. (2000). Regulation of cell cycle re-entry by growth survival and stress signaling pathways. *Biochemical Society Transactions*. **28**, 233–240.

Cook, N.C. (2010). Apple production under conditions of sub-optimal winter chilling in South Africa. *Acta Horticulturae.* **872**, 199–204.

Cooke, J.E.K., Eriksson, M.E. and Junttila, O. (2012). The dynamic nature of bud dormancy in trees: Environmental control and molecular mechanisms. *Plant Cell Environment*. **35**, 1707–1728.

Costa, C., Stassen, P.J.C. and Mudzunga, J. (2004). Chemical rest breaking agents for the South African pome and stone fruit industry. *Acta Horticulturae*. **636**, 295–302.

Crabbé, J. and Barnola, P. (1996). In Plant dormancy. Physiology, biochemistry and molecular biology. Lang GA (Ed). *CAB International*, Wallingford, CT. USA. pp 83 –114.

Crawford, S., Shinohara, N., Sieberer, T., Williamson, L., George, G., Hepworth, J., *et al.* (2010). Strigolactones enhance competition between shoot branches by dampening auxin transport. Development. **137**, 2905 – 2913.

Creelman, R.A. and Mullet, J.E. (1995). Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences*. **92**, 4114 – 4119.

Creelman, R.A. and Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*. **48**, 355–381.

Cutting, J.G.M., Strydom, D.K., Jacobs, G., Bellstedt D.U., Van Der Merwe K.J. and Weiler E.W. (1991). Changes in xylem constituents in response to rest-breaking agents applied to apple before budbreak. *Journal of the American Society for Horticultural Science*. **116,** 680–683.

Darbyshire, R., Webb, L., Goodwin, I. and Barlow, S. (2011). Winter chilling trends for deciduous fruit trees in Australia. *Agricultural and Forest Meteorology*. **151,** 1074–1085.

Das, K. and Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science*. **2** (53), 1–13.

De Albuquerque I., Mello-Farias P.C., Yamamoto R.R., Chaves A.L.S., Malgarim M. B. and Herter F. G. (2017). Dormancy studies in apple trees cultivars grown under mild climate. *Journal of Experimental Agriculture International*. **18**, 1–6.

De Sousa, C.A.F. and Sodek, L. (2002). The metabolic response of plants to oxygen deficiency. *Brazilian Journal of Plant Physiology*. **14**, 83–94.

Dennis, F. (1994). Dormancy – What we know (and don't know). *HortScience*. **29** (11), 1249–1255.

Dennis, F. (2003). Problems in standardizing methods for evaluating the chilling requirements for the breaking of dormancy in buds of woody plants. *HortScience.* **28** (3), 347–350.

Dokoozlian, N.K. (1999). Chilling temperature and duration interact on the budbreak of 'perlette' grapevine cuttings. *HortScience.* **34**(6), 1054–1056.

Dozier, W.A., Powell, A.A., Caylor, A.W., McDaniel, N.R., Carden, E.L. and McGuire, J.A. (1990). Hydrogen cyanamide induces budbreak of peaches and nectarines following inadequate chilling. *HortScience*. **25**, 1573–1575.

Dun, E.A., de Saint Germain, A., Rameau, C., and Beveridge, C.A. (2012). Antagonistic action of strigolactone and cytokinin in bud outgrowth control. *Plant Physiology*. **158**, 487–498.

Dufourc, E.J. (2008). The role of phytosterols in plant adaptation to temperature. *Plant Signal Behavior*. **3**, 133–134.

Dulf, F.V., Oroian, L., Vodnar, D.C, Socaciu, C. and Pintea, A. (2013). Lipid classes and fatty acid regiodistribution in triacylglycerols of seed oils of two *Sambucus* species (*S. nigra* L. and *S. ebulus* L.). *Molecules*, **18**, 11768–11782.

Dzhangaliev, A.D. (2003). The wild apple tree of Kazakhstan. *Horticulture Review*. **29**, 65–304.

El Yaacoubi, A., Malagi, G., Oukabli, A., Citadin, I., Hafidi, M., Bonhomme, M. and Legave, J-M. (2016).

Differentiated dynamics of bud dormancy and growth in temperate fruit trees relating to bud phenology adaptation, case of apple and almond trees. *International Journal of Biometeorology*. **60**, 1695–1710.

El-Yazal, M.A. and Rady, M.M. (2012). Changes in nitrogen and polyamines during breaking bud dormancy in 'Anna' apple trees with foliar application of some compounds. *HortScience*. **136**, 75–80

Erez, A. (1987). Chemical control of budbreak. *HortScience.* **22,** 1240–1243.

Erez, A. (1995). Means to compensate for insufficient chilling to improve bloom and leafing. *Acta Horticulturae.* **395**, 81–95.

Erez, A., Couvillon, G.A. and Hendershott, C.H. (1979a). Quantitative chilling enhancement and negation in peach buds by high temperatures in a daily cycle. *Journal of the American Society for Horticultural Science*. **104**: 536–540.

Erez, A., Couvillon, G.A. and Hendershott, C.H. (1979b). The effect of cycle length on chilling negation by high temperatures in dormant peach leaf buds. *Journal of the American Society for Horticultural Science*. **104**, 573–576.

Erez, A., Fishman, S., Gat, Z. and Couvillon, G.A. (1988). Evaluation of winter climate for breaking bud rest using the dynamic model. Acta *Horticulturae.* **232**, 76–89.

Erez, A., Fishman, S., Linsley-Noakes, G.C. and Allan, P. (1990). The dynamic model for rest completion in peach buds. *Acta Horticulturae*. **279**, 165–174.

Faust, M., Erez, A., Rowland, L.J., Wang, S.Y. and Norman, H.A. (1997). Bud dormancy in perennial fruit trees: Physiological basis for dormancy induction, maintenance and release. *HortScience*. **32**, 623–629.

Fennell, A. and Line, M.J. (2001). Identifying differential tissue response in grape (*Vitis riparia*) during induction of endodormancy using nuclear magnetic resonance imaging. *Journal of the American Society for Horticultural Science*. **126**, 681–688.

Fennell, A., (1999). Systems and approaches to studying dormancy: Introduction to the workshop. *HortScience*. **34**, 1172–1173.

Fernie, A.R., Carrari F. and Sweetlove, L.J. (2004). Respiratory metabolism: Glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology*. **7**, 254–261.

Finetto, G.A. (1997). An investigation of the chilling requirement of some apple cultivars in the Po Valley. *Acta Horticulturae*. **441**, 49–57.

Finetto, G.A. (2014). An Investigation of chilling requirement of some scab resistant apple cultivars in Po Valley. *Acta Horticulturae*. **1059,** 115–122

Fishman, S., Erez, A., Couvillon, G.A. (1987). The temperature dependence of dormancy breaking in plants: Mathematical analysis of a two-step model involving cooperative transition. *Journal of Theoretical Biology*. **124**, 473– 483.

Fu , Y.S.H., Campioli, M., Vitasse, Y., De Boeck, H.J., Van den Berge, J., AbdElgawad, H., Asard, H., Piao, S., Deckmyn, G. and Janssens, I.A. (2014). Variation in leaf flushing date influences autumnal senescence and next year's flushing date in two temperate tree species. *PNAS*. **111**, 7355–7360.

Fuchigami, L.H. and Nee, C.C. (1987). Degree growth stage model and rest-breaking mechanisms in temperate woody perennials. *HortScience.* **22**, 836–845.

Gai, S., Zhang, Y., Liu C., Zhang, Y. and Zheng G. (2013). Transcript profiling of *Paoenia ostii* during artificial chilling induced dormancy release identifies activation of GA pathway and carbohydrate metabolism. *PloS One.* **8** (2), 1–11.

GAIN (Global Agricultural Information Network) report, (2016). South Africa - Republic of fresh deciduous fruit annual update on the South African deciduous fruit supply and demand. USDA Foreign Agricultural Service (Accessed 11th Feb. 2019).

GAIN (Global Agricultural Information Network) report, (2017). South Africa - Republic of fresh deciduous fruit semi-annual update on the South African deciduous fruit supply and demand. pp: 1–18.

Gendraud, M. (1975). Contribution á l'étude du métabolosme des nucléotides di- et tri-phosphates de tubercules de Topinambour cultivés in vitro en rapport avec leurs potentialités morphogénétiques. *Plant Science Lett.* **4**, 53–59.

Gendraud, M. and Lafleuriel, J. (1983). Caractéristiques de l'absorption du saccharose et du tétraphénylphosphonium par les parenchymes de tubercules de Topinambour, dormants et non dormants, cultivés in vitro. *Physiologie Végétale*. **21**, 1125–1133.

Ghariani, K. (1993). Chilling requirement affects plant response to hydrogen cyanamide in apple cultivars. Oregon State University. MSc Thesis. pp: 86.

Ghrab, M. and Ben Mimoun, M. (2014). Effective hydrogen cyanamide (Dormex®) application for budbreak, flowering and nut yield of pistachio trees cv. Mateur in warm growing areas. *Experimental Agriculture.* **50**, 398–406.

Gosling, P.G. and Ross, J.D. (1980). Pentose phosphate metabolism during dormancy breakage in *Corylus avellana* L. *Planta*. **148**, 362–366.

Guak, S. and Fuchigami, L.H. (2001). Effects of applied ABA on growth cessation, bud dormancy, cold acclimation, leaf senescence and N mobilization in apple nursery plants. *Journal of Horticultural Science and Biotechnology*. **76** (4), 459–464.

Grunwald, C. (1970). Sterol distribution in intracellular organelles isolated from tobacco leaves. *Plant Physiology*. **45**, 663–666.

Grunwald, C. (1971). Effects of free sterols, steryl ester and steryl glycoside on membrane permeability. *Plant Physiology*. **48**, 653–655.

Güthner, T. and Mertschenk, B. (2012). Cyanamides, Ullmann's encyclopedia of industrial chemistry, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. **10**, 645 – 664.

Häkkinen, R., Linkosalo, T. and Hari. P. (1998). Effects of dormancy and environmental factors on timing of bud burst in *Betula pendula*. *Tree Physiology*. **18**, 707–712.

Halaly, T., Pang, X., Batikoff, T., Crane, O., Keren, A., Venkateswari, J., Ogrodovitch, A., Sadka, A., Lavee, S. and Or, E. (2008). Similar mechanisms might be triggered by alternative external stimuli that induce dormancy release in grape buds. *Planta.* **228**, 79–88.

Halgryn, P.J., Theron K.I. and Cook, N.C. (2001). Genotypic response to chilling period of apple buds from two Western Cape localities. *South African Journal of Plant and Soil*. **18**, 21–27.

Hauagge, R. and Cummins, J.N. (1991b). Phenotypic variation of length of bud dormancy in apple cultivars and related *Malus* species. *Journal of the American Society for Horticultural Science*. **116**, 100–106.

Hauagge, R., Cummins, J.N. (1991a). Relationships among indices for the end of bud dormancy in apple cultivars and related *Malus* species under cold winter conditions. *Journal of the American Society for Horticultural Science*. **116**, 95–99.

Hawerroth, F.J., Herter, F.G., Petri, J.L., Marafon, A.C. and Leonetti, J.F. (2013). Evaluation of winter temperatures on apple budbreak using grafted twigs. *Revista Brasileira de Fruticultura, Jaboticabal - SP*. **35**, 713–721.

Heide, O.M. (2003). High autumn temperature delays spring budburst in boreal trees, counterbalancing the effect of climatic warning. *Tree Physiology*. **23**, 931–936.

Heide, O.M. (2011). Temperature rather than photoperiod controls growth cessation and dormancy in *Sorbus* species. *Journal of Experimental Botany*. **62**, 5397–5404.

Heide, O.M. and Prestrud, A.K. (2005). Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. *Tree Physiology.* **25**, 109–114.

Herter, F.G., Finardi, N.L. and Mauget, J.C. (1988). Dormancy development in apple trees cvs. 'Gala', 'Golden' and 'Fuji', in Pelotas, RS. *Acta Horticulturae*. **232**, 109–115.

Holland, D., Bar-Yaakov, I., Trainin, T. and Kamel Hatib, K. (2006). Old deciduous fruit trees of the Rosaceae in Israel and their utilization in modern agriculture and breeding. *Israel Journal of Plant Sciences*. **54**, 169–177.

HORTGRO. (2018). Key deciduous fruit statistics 2018. Deciduous Fruit Producers Trust, Paarl, South Africa. pp: 95.

Horvath, D. (2009). Common mechanisms regulate flowering and dormancy. *Plant Science.* **177**, 523–531

Horvath, D.P., Anderson, J.V., Chao, W.S. and Foley, M.E. (2003). Knowing when to grow: Signals regulating bud dormancy, *Trends in Plant Science.* **8**, 534–540.

Hummer, K. E. and Janick, J. (2009). Rosaceae: Taxonomy, economic importance, genomics. In *Genetics and genomics of Rosaceae. Springer, New York, NY*. pp: 1–17.

Ismail, S.Z., Khandaker, M.M., Mat, N. and Boyce, A.N. (2015) Effects of hydrogen peroxide on growth, development and quality of fruits: A review. *Journal of Agronomy.* **14**, 331-336.

Ito, A., Yaegaki, H., Hayama, H., Kusaba, S., Yamaguchi, I. and Yoshioka, H. (1999). Bending shoots stimulates flowering and influences hormone levels in lateral buds of Japanese pear. *Hortscience*. **34**, 1224–1228.

Ito, A., Sugiura, T., Sakamoto, D. and Moriguchi, T. (2013). Effects of dormancy progression and low-temperature response on changes in the sorbitol concentration in xylem sap of Japanese pear during winter season. *Tree Physiology*. **00**, 1–11

Jackson, J.E. and Bepete, M. (1995). The effect of hydrogen cyanamide (Dormex) on flowering and cropping of different apple cultivars under tropical conditions of sub-optimal winter chilling. *Scientia Horticulturae.* **60**, 293–304.

Jacobs, J.N., Jacobs, G.and Cook, N.C. (2002). Chilling period influences the progression of bud dormancy more than temperature in apple and pear shoots. *The Journal of Horticultural Science and Biotechnology*. **77**, 333–339.

Kozai, N., Beppu, K., Mochioka, R., Boonprakob, U., Subhadrabandhu, S. and Ikuo Kataoka, I. (2004). Adverse effects of high temperature on the development of reproductive organs in 'Hakuho' peach trees. *The Journal of Horticultural Science and Biotechnology*. **79**, 533–537.

Kuroda, H., Sagisaka, S. and Chiba, K. (1990). Seasonal changes in peroxide-scavenging systems of apple trees in relation to cold hardiness, *Journal of Japanese Society for Horticultural Science*. **59**, 399–408.

Kumar, G., Gupta, K., Pathania, S., Swarnkar, M.K., Rattan, U.K., Singh, G., Sharma, R.K. and Singh (2017). Chilling affects phytohormone and post-embryonic development pathways during budbreak and fruit set in Apple (*Malus x domestica* Borkh.). *Scientific Reports*. **7**, 42593.

Labuschagné, I.F., Louw, J.H., Schmidt, K. and Sadie, A. (2002a). Genotypic variation in prolonged dormancy symptoms in apple progenies. *HortScience*. **37**, 157–163.

Labuschagné, I.F., Louw, J.H., Schmidt, K. and Sadie, A. (2002b). Genetic variation in chilling requirement in apple progeny. *Journal of the American Society for Horticultural Science*. **127**, 663–672.

Lalanne-Tisné, G., Quero-García, J., Lafargue, M., Joly, J., Fouilhaux, L., Dirlewanger, E., Costes, E. and Legave, J.M. (2017). Comparison of phenotypic methodologies to characterize chilling and heat requirements of peach and apple cultivars. *Acta Horticulturae*. **1172**, 349–354.

Lang, G.A. (1987). Dormancy: a new universal terminology. *HortScience*. **22**, 817–820.

Lang, G.A., Early, J.D., Martin, G.C. and Darnell, R.L. (1987). Endo-, para-and eco-dormancy physiological terminology and classification for dormancy research. *HortScience*. **22**, 371–377.

Lavarenne, S., Champciaux, M., Barnola, P. and Gendraud, M. (1982). Métabolisme des nucléotides et

dormance des bourgeons chez le frêne. *Physiologie Végétal.* **20,** 371–376.

Leite, G.B., Bonhomme, M., Lacointe, A., Rageau, R., Sakr, S., Guilliot A, Maurel, K., Pétel. G. and Rodrigues, C.A. (2004). Influence of lack of chilling on budbreak patterns and evolution of sugar contents in buds and stem tissues along the one-year old shoot of the peach trees. *Acta Horticulturae.* **662**, 61–71.

Li, J., Xu, Y., Niu, Q., He, L., Teng, Y. and Bai, S. (2018). Abscisic Acid (ABA) promotes the induction and maintenance of Pear (Pyrus pyrifolia white pear group) flower bud endodormancy. *International Journal Molecules Sciences*. **19** (310), pp.15.

Li Y., Fang, W-C., Zhu, G-R., Cao, K., Chen, C-W., Wang, X-W. and Wang, L-R. (2016). Accumulated chilling hours during endodormancy impact blooming and fruit shape development in peach (*Prunus persica* L.). *Journal of Integrative Agriculture*. **15**, 1267–1274.

Li, D.M., Li, L., Tan, Y., Chen, X.D., Zhang, H.S., Gao, D.S. and Li, J. (2011). Effect of photoperiod on key enzyme activities of respiration in nectarine buds during dormancy induction. *Agricultural Sciences in China*. **10**, 1026–1031.

Lindsey, K., Pullen, M.L. and Topping, J.F. (2003). Importance of plant sterols in pattern formation and hormone signaling. *Trends in Plant Science*. **8**(11), 521–525.

Linsley-Noakes, G.C., Allan, P. and Matthee, G.W. (1994). Modification of rest completion models for improved accuracy in South African stone fruit. *Journal of the Southern African Society for Horticultural Sciences*. **4**, 13–15.

Linsley-Noakes, G.C., Louw, M. and Allan, P. (1995). Estimating daily positive Utah chill units using maximum and minimum temperatures. *Journal of the Southern African Society for Horticultural Sciences*. **5**, 19–24.

Liu, D., Faust, M., Millard, M.M., Line, M.J. and Stutte, G.W. (1993). States of water in summer-dormant apple buds determined by proton magnetic resonance imaging. *Journal of the American Society for Horticultural Science*. **118**, 632–637.

Los, D.A. and Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et biophysica acta*. **1666**, 142–157.

Luedeling, E. (2012). Climate change impacts on winter chill for temperate fruit and nut production: A review. *Scientia Horticulturae*. **144**, 218–229.

Luedeling, E., Girvetz, E.H., Semenov, M.A. and Brown, P.H. (2011). Climate change affects winter chill for temperate fruit and nut trees. *PLoS One* **6**, e20155.

Luedeling, E., Zhang, M. and Girvetz, E.H. (2009). Climate changes lead to declining winter chill for fruit and nut trees in California during 1950-2099. *PLoS One.* **4**, 1–9.

Lyrene, P.M. (2005). Breeding low-chill blueberries and peaches for subtropical areas. *Hortscience.* **40**(7).

Maguylo, K., Cook, N.C. and Theron, K.I. (2014). Primigenic and positional dominance among reproductive buds in shoots of two apple (*Malus x domestica* (Borkh.)) cultivars in a warmer and cooler site. *Trees*. **28**, 625–634.

McPherson, H.G., Richardson, A.C., Snelgar, W.P. and Currie, M.B. (2001). Effects of hydrogen cyanamide on budbreak and flowering in kiwifruit (*Actinidia deliciosa* 'Hayward'). *New Zealand Journal of Crop and Horticultural Science*. **29**, 277–285.

McPherson, H.G., Snelgar, W.P., Manson, P.G. and Snowball, A.M. (1997). Bud respiration and dormancy of kiwifruit (Actinidia deliciosa). *Annals of Botany*. **80**, 411–418.

Meï, C., Michaud, M., Cussac, M., Albrieux, C., Gros, V., Maréchal, E., Block, M.A., Jouhet, J. and Rébeillé F. (2015). Levels of polyunsaturated fatty acids correlate with growth rate in plant cell cultures. *Scientific Reports*. **5**, 15207.

Melke, A. (2015). The physiology of chilling temperature requirements for dormancy release and budbreak in temperate fruit trees grown at mild winter tropical climate. *Journal of Plant Studies*. **4**, 110–156.

Midgley, S.J.E. (2016). Commodity value chain analysis for apples. World Wide Fund-SA, Newlands, South Africa, pp: 11.

Midgley, S.J.E. and Lötze, E. (2011). Climate change in the Western Cape of South Africa: Trends, projections and implications for chill unit accumulation. *Acta Horticulturae*. **903**, 1127–1134.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*. **7**, 405–410.

Mohamed, A.K.A. (2008). The effect of chilling, defoliation and hydrogen cyanamide on dormancy release, budbreak and fruiting of 'Anna' apple cultivar. *Scientia Horticulturae*. **118**, 25–32.

Naor, A., Flaishman, M., Stern, R., Moshe, A. and Erez, A. (2003). Temperature effects on dormancy completion of vegetative buds in apple. *Journal of the American Society for Horticultural Science*. **128**, 636–641.

Nir, G. and S. Lavee. (1993). Metabolic changes during cyanamide induced dormancy release in grapevines. *Acta Horticulturae.* **329**, 271–274.

Nir, G., Shulman, Y., Fanberstein, L. and Lavee, S. (1986). Changes in the activity of catalase in relation to the dormancy of grapevine, *Vitis vinifera* L. buds. *Plant Physiology*. **81**, 1140–1142.

Noctor, G, Lelarge-Trouverie, C. and Mhamdi, A. (2015). The metabolomics of oxidative stress. *Phytochemistry*. **112**, 33–53.

Noctor, G. and Foyer, C.H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual Review Plant Physiology Plant Molecular Biology*. **49**, 249–279.

North, M.S. (2003). Alternative rest-breaking agents evaluated for 'Golden Delicious' apple trees. *South African Journal of Plant and Soil*. **20**, 59–63.

Notodimedjo, S., Sastrosumarto, S., Danoesastro, H. and Edwards, G.R. (1981). Shoot growth, flower initiation and dormancy of apple in the tropics. *Acta Horticulturae*. **120**, 179-186.

Ogundeji, A.A. and Jordaan, H.A. (2017). Simulation study on the effect of climate change on crop water use and chill unit accumulation. *South African Journal of Science*. **113**, 1–7.

Ohlrogge, J. and Browse, J. (1995). Lipid biosynthesis. *Plant Cell***.** **7**, 957–970.

Or, E., Vilozny, I., Eyal, Y. and Ogrodovitch, A. (2000). The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNFlike protein- kinase GDBRPK. *Plant Molecular Biology*. **43**, 483–494.

Or, E., Vilozny, I., Fennell, A., Eyal, Y. and Ogrodovitch, A. (2002). Dormancy in grape buds: Isolation and characterization of catalase cDNA and analysis of its expression following chemical induction of bud dormancy release. *Plant Science*. **162**, 121–130.

Orffer, C.J. and Goussard, P.G. (1980). Effect of hot water treatments on bud burst and rooting of grapevine cuttings. *Vitis.* **19**, 1–3.

Panneerselvam, R., Abdul Jaleel, C., Somasundaram, R., Sridharan, R. and Gomathinayagam, M. (2007). Carbohydrate metabolism in *Dioscorea esculenta* (Lour.) Burk. tubers and *Curcuma longa* L. rhizomes during two phases of dormancy. *Colloids and Surfaces B: Biointerfaces*. **59**, 59–66.

Panyushkina, I.P., Mukhamadiev, N.S., Lynch, A.M., Ashikbaev, N.A., Arizpe, A.H., O'Connor, C.D., Abjanbaev, D., Mengdbayeva, G.Z. and Sagitov A.O. (2017). Wild apple growth and climate change in Southeast Kazakhstan. *Forests*. **8** (406), pp.15

Parada, F., Noriega, X., Dantas, D., Bressan-Smith, R. and Pérez, F.J. (2016). Differences in respiration between dormant and non-dormant buds suggest the involvement of ABA in the development of endodormancy in grapevines. *Journal of Plant Physiology*. **201**, 71–78.

Pasa, S.P., Felippeto, J., Nava, G., da Silva, C.P., Brighenti, A.F. and Ciotta, M.N. (2016). Performance of 'Fuji Suprema' apple trees treated with budbreak promoters, in São Joaquim-SC. *Revista Brasileara fruiticultura Jaboticabal*. **40** (1), 1–10.

Pérez, F.J. and Lira, W. (2005). Possible role of catalase in post-dormancy budbreak in grapevines. *Journal*

of Plant Physiology. **162**, 301–308.

Pérez, F.J., Rubio, S. and Ormeño-Nuñez, J. (2007). Is erratic budbreak in grapevines grown in warm winters areas related to disturbances in mitochondrial respiratory capacity and oxidative metabolism? *Functional Plant Biology*. **34**, 624–632.

Pérez, F.J., Vergara, R. and Or, E. (2009). On the mechanism of dormancy release in grapevine buds: A comparative study between hydrogen cyanamide and sodium azide. *Plant Growth Regulation*. **59**, 145–152.

Pérez, F.J., Vergara, R. and Rubio, S. (2008). H_2O_2 is involved in the dormancy-breaking effect of hydrogen cyanamide in grapevine buds. *Plant Growth Regulation*. **55**, 149–155.

Petri, J.L. and Leite, G.B. (2004). Consequences of insufficient winter chilling on apple tree budbreak. *Acta Horticulturae*. **662**, 53–60.

Petri, J.L. and Stuker, H., (1995). Effect of mineral oil and hydrogen cyanamide concentrations on apple dormancy, cv. Gala. *Acta Horticulturae.* **395**, 161–167.

Piispanen, R. and Saranpää, P. (2004). Seasonal and within-stem variations of neutral lipids in silver birch (*Betula pendula*) wood. *Tree Physiology*. **24**, 991–999.

Pomeroy, M.K. and Raison, J.K. (1981). Maintenance of membrane fluidity during development of freezing tolerance of winter wheat seedlings. *Plant Physiology*. **68**, 382–385.

Porto, D.D., Bruneau, M., Perini, P., Anzanello, R., Renou, J-P., dos Santos, H.P., Fialho, F.B. and Revers, L.F. (2015). Transcription profiling of the chilling requirement for budbreak in apples: A putative role for FLC-like genes. *Journal of Experimental Botany*. **66**, 2659–2672.

Powell, L.E. (1986). The chilling requirement of apple and its role in regulating time of flowering in spring in cold winter climates. *Acta Horticulturae*. **179**, 129–133.

Preston, J.C. and Sandve, S.R. (2013). Adaptation to seasonality and the winter freeze. *Frontiers in Plant Science*. **4** (167), 1–18.

Pucciariello, C. and Perata, P. (2012). How plants sense low oxygen. *Plant Signaling and Behavior*. **7**, 813– 816.

Quan, I.J., Zhang, B., Shi, W.W. and Li, H.Y. (2008). Hydrogen peroxide in plants: A versatile molecule of the reactive oxygen species network. *Journal of Integrated Plant Biology* **50**(1), 2-18.

Quinn, P.J. (1981). The fluidity of cell membranes and its regulation. *Progress in Biophysics and Molecular Biology*. **38**, 1 – 104.

Quinn P.J. and W.P. William (1978). Plant lipids and their role in membrane function. *Progress in Biophysics and Molecular Biology*. **34**, 109-173

Quinn, P.J., Joo, F. and Vigh, L. (1989). The role of unsaturated lipids in membrane structure and stability. *Progress in Biophysics and Molecular Biology*. **53**, 71–103.

Rahemi, M. and Asghari, H. (2004). Effect of hydrogen cyanamide (dormex), volk oil and potassium nitrate on budbreak, yield and nut characteristics of pistachio (*Pistacia vera* L.). *Journal of Horticultural Science & Biotechnology*. **79**, 823–827.

Rai, R., Joshi, S., Roy, S., Singh, O., Samir, M. and Chandra, A. (2015). Implications of changing climate on productivity of temperate fruit crops with special reference to apple. *Journal of Horticulture*. **2**(2), 1– 6.

Ribas-Carbo, M., Taylor, N.L., Giles, L., Busquets, S., Finnegan, P.M., Day D.A., Lambers, H., Medrano, H., Berry, J.A. and Flexas, J. (2005). Effects of water stress on respiration in soybean leaves. *Plant Physiology*. **139,** 466–473.

Richardson, E.A., Seeley, S.D. and Walker, D.R. (1974). A model for estimating the release of rest for 'Redhaven' and 'Elberta' peach trees. *HortScience.* **9**, 331–332.

Rinne, P.L., Welling, A., Vahala, J., Ripel, L., Ruonala, R., Kangasjärvi, J., *et al.* (2011). Chilling of dormant buds hyperinduces *FLOWERING LOCUS T* and recruits GA-inducible 1, 3-β-glucanases to reopen

signal conduits and release dormancy in Populus. *Plant Cell*. **23**, 130–146.

Rohde, A. and Bhalerao, R.P. (2007). Plant dormancy in the perennial context. *Trends Plant Science.* **12**, 217–223.

Rubio, S., Donoso, A. and Pérez, F.J. (2014). The dormancy-breaking stimuli “chilling, hypoxia and cyanamide exposure” up-regulate the expression of α-amylase genes in grapevine buds. *Journal of Plant Physiology*. **171**, 373–381.

Sagisaka, S. (1972). Decrease of glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in xylem of *Populus gelrica* on budding. *Plant Physiology*. **50**, 750–755.

Sagisaka, S. (1974). Transition of metabolism in living poplar bark from growing to wintering stages and vice versa. *Plant Physiology*. **54**, 544–549.

Sagredo, X.K. (2008). Effect of rest-breaking and fruit thinning treatments on reproductive development in apple. Stellenbosch University PhD Dissertation. pp: 128.

Salisbury, F.B. and N.G. Marinos. (1985). The ecological role of plant growth substances. *Encyclopedia Plant Physiology*. **11**, 707-766.

Salvemini, F., Franzé, A., Iervolino, A., Filosa, S., Salzano, S. and Ursini, M.V. (1999). Enhanced glutathione levels and oxidoresistance mediated by increased glucose-6-phosphate dehydrogenase expression. *Journal of Biological Chemistry*. **274**, 2750–2757.

Scalabrelli, G. and Couvillon, G.A. (1986). The effect of temperature and bud type on rest completion and the GDH requirements for budbreak in ‘Red Haven’ peach. *Journal of the American Society for Horticultural Science*. **111**, 537–540.

Scandalios, J.G. (2005). Oxidative stress: Molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical and Biological Research*. **38**, 995–1014.

Schaller, H. (2003). The role of sterols in plant growth and development. *Progress in Lipid Research*. **42**, 163–175.

Schmid, K.M. and Ohlrogge, J.B. (2002). Lipid metabolism in plants. *Biochemistry of Lipids, Lipoprooteins and Membranes (4th Edn.)*. D.E. Vance and J.E. Vance (Edn.). pp: 34.

Schmitz, J.D., Herter, F.G., Regnard, J.L., Leite, G.B., Bonhomme, M., Cochard, H. and Lauri, P.É. (2015). Is acrotonic budburst pattern in spring a typical behavior of the low-chilling apple cultivar ‘Eva’ in mild winter conditions? An approach combining ex planta single-node cutting test and in planta bud water content during dormancy. *Scientia Horticulturae*. **188**, 84–88.

Seeley, S, (1990). Hormonal transduction of environmental stresses. *HortScience*. **25** (11), 1369–1376.

Seeley, S.D., Rojas-Martinez, R. and Frisby, J. (1998). Temperature effects during endodormancy induction on subsequent anthesis and growth of peach trees. *HortScience*. **33**, 452.

Shaltout, A.D. and Unrath, C.R. (1983). Rest completion prediction model for ‘starkrimson delicious’ apples. *Journal of the American Society for Horticultural Science.* **108**, 957–961.

Sheard, A.G. (2001). Measuring winter chilling in the South West region of KwaZulu-Natal during 2001 and its implications for deciduous fruit production: KZN Agri-Report No. N/A/2002/02. pp: 16.

Sheard, A.G. (2008). Factors leading to poor fruit set and yield of sweet cherries in South Africa. MSc Thesis, University of Stellenbosch. pp: 127.

Shi, K., Fu, L.J., Zhang, S., Li, X., Liao, Y.W.K; Xia, J.X., Zhou, Y.Z., Wang, R.Q., Chen, Z.X. and Yu, J.Q. (2013). Flexible change and cooperation between mitochondrial electron transport and cytosolic glycolysis as the basis for chilling tolerance in tomato plants. *Planta*. **237**, 589–601.

Shim, D., Ko, J-H, Kim, W-C., Wang, Q., Keathley, D.E. and Han, K-H. (2014). A molecular framework for seasonal growth-dormancy regulation in perennial plants. Nanjing Agricultural University, *Horticulture Research*. pp: 59**.**

Shulman, Y., Nir, G. and Lavee, S. (1986). Oxidative processes in bud dormancy and use of hydrogen cyanamide in breaking dormancy. *Acta Horticulturae*. **179**, 141–148.

Shulman, Y., Nir, G. and Lavee, S. (1983). The effect of cyanamide on the release from dormancy of grapevine buds. *Scientia Horticulturae*. **19**, 97–104.

Siller-Cepeda, J.H., Fuchigami, L.H. and Chen, T.H.H. (1992). Glutathione content in peach buds in relation to development and release of rest. *Plant Cell Physiology*. **33**, 867–872.

Singha, S. and L.E. Powell. (1978**)**. Response of apple buds culture *in vitro* to abscisic acid. *Journal of the American Society for Horticultural Science*. **103**, 620–622.

Smirnoff, N. and Arnaud, D. (2019). Hydrogen peroxide metabolism and functions in plants. *New Phytologist*. **221**, 1197-1214.

Snir, I. (1988). Effect of hydrogen cyanamide on budbreak in red raspberry. *Scientia Horticulture*. **34**, 75– 83

Sudawan, B., Chang, C.S., Chao, H.F., Ku, M.S.B. and Yen, Y.F. (2016). Hydrogen cyanamide breaks grapevine bud dormancy in the summer through transient activation of gene expression and accumulation of reactive oxygen and nitrogen species. *BMC Plant Biology*. **16** (202), 1–18.

Sugiura, T., Yoshida, M., Magoshi, J. and Ono, S. (1995). Changes in water of peach flower buds during endodormancy and ecodormancy measured by differential scanning calorimetry and nuclear magnetic resonance spectroscopy. *Journal of the American Society for Horticultural Science*. **120**, 134–138.

Swamy, P.M. and Sandhyarani, C.K. (1985). Contribution of the pentose phosphate pathway and glycolytic pathway to dormancy breakage and germination of peanut (*Arachis hypogaea* L.) seeds. *Journal of Experimental Botany*. **37**, 80–88.

Sweetlove, L.J., Beard, K.F.M., Nunes-Nesi, A., Fernie, A.R. and Ratcliffe, R.G. (2010). Not just a circle: flux modes in the plant TCA cycle. *Trends in Plant Science*. **15**, 462–470.

Taiz, L. and Zeiger, E. (2010). Plant Physiology. 5nd ed. Sinauer Associates Inc., Sunderland, M.A. pp: 782

Tabuenca, M.C. (1964). Necesidades de frio invernal de variedades de albaricoquero, melocotonero y peral. *Aula Dei*. **7**, 113–132.

Tan, Y., Gao, D.S., Li, L., Chen, X.D. and Xu, A.H. (2010). Respiratory response of dormant nectarine floral buds on chilling deficiency. *Agricultural Sciences in China*. **9,** 1448–1454.

Tan, Y., Li, L., Leng, C., Li, D., Chen, X. and Gao, D. (2013). Respiratory response of dormant nectarine vegetative buds to high temperature stress. *Journal of Integrative Agriculture*. **12**, 80–86.

Tanino, K.K. (2004). Hormones and endodormancy induction in woody plants. *Journal Crop Improvement.* **10**, 157–199.

Tharaga, P.C. (2014). Impacts of climate change on accumulated chill units at selected fruit production sites in South Africa. MSc Thesis, Bloemfontein University.

Theocharis, A., Clément, C. and Barka, E.A. (2012). Physiological and molecular changes in plants grown at low temperatures. *Planta*, **235,** 1091–1105.

Tian, W.N., Braunstein, L.D., Pang, J., Stuhmeier, K.M., Xi, Q., Tian, X. and Stanton, R.C. (1998). Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *Journal of Biological Chemistry*. **273**, 609–617.

Tiwari, B.S., Belenghi, B. and Levine, A. (2002). Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition and programmed cell death. *Plant Physiology.* **128**, 1271–1281.

Toro, G. Pinto, M. (2015). Plant respiration under low oxygen. *Chilean Journal Agricultural Research.* **75**, 1

Trejo-Martínez, M.A., Orozco, A., Almaguer-Vargas, G., Carvajal-Millán, E. and Gardea, A.A. (2009). Metabolic activity of low chilling grapevine buds forced to budbreak. *Thermochimica Acta*. **481**, 28-31.

Tamura, F., Tanabe, K., Itai, A., Tanaka, H. (1998). Protein changes in the flower buds of Japanese pear during breaking of dormancy by chilling or high-temperature treatment. *Journal of the American Society for Horticultural Science*. **123**, 532–536.

Upchurch, R.G. (2008). Fatty acid unsaturation, mobilization and regulation in the response of plants to

stress. *Biotechnology Letters*. **30**, 967–977.

Van der Schoot, C. and Rinne, P.L.H. (2011). Dormancy cycling at the shoot apical meristem: Transitioning between self-organization and self-arrest. *Plant Science*. **180**, 120–131.

Vanlerberghe, G.C. (2013). Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *International Journal of Molecular Science*. **14**, 6805–6847.

Viti, R., Bartolini, S. and Andre L. (2013). Apricot flower bud dormancy: Main morphological, anatomical & physiological features related to winter climate influence. Advances *Horticulturae science*. **27**, 5-17.

Vergara, R., Rubio, S. and Pérez, F.J. (2012). Hypoxia and hydrogen cyanamide induce budbreak and up-regulate hypoxic responsive genes (HRG) & VvFT in grapevine-buds. *Plant Molecular Biology*. **79**, 171–178.

Walton, E.F., Clark, C.J. and Boldingh, H.L. (1991). Effect of hydrogen cyanamide on amino acid profiles in kiwifruit buds during budbreak. *Plant Physiology*. **97**, 1256–1259.

Wang, C.Y. (2010). Alleviation of chilling injury in tropical and subtropical fruits. *Acta Horticulturae*. **864**, 267–273.

Wang, S.Y. and Faust, M. (1988). Changes of fatty acids and sterols in apple buds during budbreak induced by a plant bioregulator, thidiazuron. *Physiologia plantarum*. **72**, 115–120.

Wang, S.Y. and Faust, M. (1990a). Seasonal changes of membrane lipids in apple shoots. *Journal of the American Society for Horticultural Science*. **115**, 462–467.

Wang, S.Y. and Faust, M. (1990b). Changes in membrane lipids in apple buds during dormancy and budbreak. *Journal of the American Society for Horticultural Science*. **115**, 803–808.

Wang, S.Y. and Faust, M. (1992). Ascorbic acid oxidase activity in apple buds: Relation to Thidiazuron-induced lateral budbreak. *HortScience.* **27**, 1102–1105.

Wang, S.Y. and Faust, M. (1993). Biochemical events associated with resumption of growth in temperate zone fruit trees. *Acta Horticulturae*. **329**, 257–264.

Wang, S.Y., Faust, M. and Line M.J. (1994). Apical dominance in apple (*Malus domestica* Borkh): The possible role of indole-3-acetic acid (IAA). *Journal of the American Society for Horticultural Science*. **119**, 1215–1221.

Wang, S.Y., Jiao H.J. and Faust. M. (1991). Changes in superoxide dismutase activity during thidiazuron-induced budbreak of apple. *HortScience.* **26**, 1202–1204.

Wisniewski, M., Norelli, J. and Artlip, T. (2015). Overexpression of a peach CBF gene in apple: a model for understanding the integration of growth, dormancy and cold hardiness in woody plants. *Frontiers in Plant Science*. **6(**85), 1–13.

Zanol, G.C., Sekozawa, Y., Sugaya, S. and Gemma, H. (2010). Effect of hydrogen cyanamide on dormancy release, budbreak and fruiting in Japanese pear. *Acta Horticulturae*. **884**, 363–370.

Zhang, J. (2007). Chemical control of budbreak: A review. *Australian Nutgrower Ed.* pp: 8.

Zheng, C., Halaly, T., Acheampong, A.K., Takebayashi, Y., Jikumaru, Y., Kamiya, Y. and Or, E. (2015). Abscisic acid (ABA) regulates grape bud dormancy and dormancy release stimuli may act through modification of ABA metabolism. *Journal of Experimental Botany*. **66**, 1527–1542.

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PAPER 1

The effect of insufficient winter chill on respiration in dormant ‘Cripps Pink’ apple buds.

Abstract

Chilling temperatures allow apple trees to overcome their dormant state and resume growth in a synchronised manner in the next season. In warmer climates, e.g. the Western Cape region of South Africa, the lack of appropriate low temperatures to satisfy the chilling requirement of the buds leads to inadequate release of endodormancy, resulting in decreased and protracted budbreak, as well as, uneven and delayed foliation and bloom. This study investigates the effects of insufficient winter chill on the respiration rate and pathways such as, the tricarboxylic acid cycle (TCA), pentose phosphate pathway (PPP), alternative pathway (ALT) and cytochrome C (CYT), by comparing ‘Cripps Pink’ apple shoots from two contrasting winter climates. The respiration rate of the pathways was determined by quantifying the oxygen uptake using a Clark-type oxygen electrode in the presence of inhibitors targeting a specific pathway. Means were compared using a two-way ANOVA. Buds were also exposed to controlled amounts of chill and the results compared to the field exposed buds. An early dormancy entry, high maximum depth and early release were observed in the buds collected from the Koue Bokkeveld area with sufficient cold similar to laboratory chilled buds. Buds from the cold area had a higher total respiration rate at the end of the trial compared to buds from the warm area. This increase in respiration in the cold area at the beginning of spring is due to an increase in the TCA and the CYT pathways. The contribution of the TCA and CYT pathways of the buds exposed to insufficient winter chill (warmer Elgin area) was significantly lower. These buds maintained a higher PPP and ALT contribution to possibly compensate for the lack in energy production.

Key words: *apple buds, dormancy, insufficient chill, respiration pathways*

1. Introduction

Temperate-zone deciduous fruit trees show active growth in the warm seasons followed by a dormant winter period that ends with budbreak, in early spring, marking the start of the next growing season. This ‘activity-dormancy’ cycle is said to be a developmental adaptation to survive unfavourable winter conditions and synchronise growth in the spring (Cooke *et al.*, 2012). The dormancy period is of particular importance and was first defined by Lang (1987) as “the temporary suspension of visible growth of any plant structure containing a meristem” and later redefined by Rohde and Balherao (2007) as “the inability to resume growth from meristems and other organs and cells under favourable conditions”. Both definitions indicate dormancy as a property of the meristem that controls growth and is considered a complex, dynamic and multifaceted phenomenon (van der Schoot, 2011) that is affected by environmental as well as endogenous factors (Faust *et al.*, 1997; Graeber *et al.*, 2012). Chill accumulation has been reported as one of the key environmental factors that controls the onset and termination of dormancy, especially in members of the Rosaceae family, such as apple, that are not sensitive to photoperiod (Arora *et al.*, 2003; Heide and Prestrud, 2005). Apple trees, in general, need to accumulate between 400-1800 chill hours (Chill Hours model) to exit dormancy and start synchronised budbreak in spring (Baldocchi *et al.*, 2005). It has been shown that insufficient winter chill prolongs apple bud dormancy release (Cook and Jacobs, 2000), delays shoot growth (Cook and Jacobs, 1999) and fruit development (Atkinson *et al.*, 2013). The asynchrony of flowering, that in particular leads to mixed fruit maturities at harvest, is a major challenge for commercial apple producers (Cook and Jacobs, 1999; Petri and Leite, 2004). Apple trees that receive insufficient winter chilling develop a basitonic growth tendency while impeding development of acrotony and apical control (Cook and Jacobs, 1999). Other symptoms include delay in fruit set and ripening, small fruit size, low yields and low fruit quality (Atkinson *et al.*, 2013).

With climate change exposing temperate zones to declining winter chill, it is likely that warmer temperatures will negatively influence the productivity and sustainability of deciduous fruit production in warm growing regions (Luedeling *et al.*, 2009; Luedeling, 2012). Furthermore, studies show that not only a decline in chill accumulation during winter might affect dormancy and fruit tree development, but also a rise in heat accumulation as a result of global warming (Luedeling *et al.*, 2009; Campoy *et al.*, 2011). Although such increased temperatures might be beneficial for budbreak during a spring following a warm winter, it still remains a concern that chill accumulation (and thus dormancy release) in many apple cultivars will be decreasing in warm/warming winter production areas such as the Western Cape region of South Africa, where

a rise in air temperature is predicted for future autumn and winter periods (Midgley and Lötze, 2011).

Cook and Jacobs (2000) indicated that a lack of winter chill changes the endodormancy progression of 'Golden Delicious' and 'Granny Smith' apples by protracting the dormancy induction period and failing to reach high levels of endodormancy. Cook *et al.* (2017) stated that most apple producing areas in South Africa are accumulating chill units (Utah model) below the average requirement resulting in high variability in dormancy progression curves. In most of these areas the use of a rest breaking agent is part of the commercial production practice to ensure sufficient yields and sustainable production.

Respiration can be used to quantify growth and dormancy release during winter conditions (Myking, 1998) as low temperatures interfere with the oxygen consumption, decrease the respiration rate and subsequently lead to dormancy release (Atkin and Tjoelker, 2003). Munro *et al.* (2004) reported that increasing cold slows the flow of reduced electrons shutting down mitochondrial electron transfer and inhibiting cytochrome C activity. The electrons disturbance consequently increases the production of reactive oxygen species (ROS), such as O_2^- and H_2O_2 (Møller, 2001; Taiz and Zeiger, 2010) and, reduce the tricarboxylic acid cycle (TCA) activity and energy production (Fernie *et al.*, 2004). Under such conditions, general oxidative stress occurs and adaptive strategies activate the alternative oxidase and cytoplasmic pathways (Pentose Phosphate Pathway (PPP) and glycolysis) in order to maintain mitochondrial respiration and cope with the ATP demand (Atkin *et al.*, 2002; Møller, 2001; Vanlerberghe, 2013). Other studies indicate that this temporary state of inhibition is terminated once the specific chilling requirement is met and the spring conditions are favourable for metabolic activity (Pérez and Lira 2005; Hauagge and Cummins, 1991; Cooke *et al.*, 2012). Such conditions increase the respiration rate, ATP production and result in general growth resumption (Ruiz *et al.*, 2007; Trejo-Martínez *et al.*, 2009). Although the close relationship between temperature, dormancy release and mitochondrial or cytoplasmic respiration pathways has been well documented (Pérez *et al.*, 2009; Tan *et al.*, 2010; Tan *et al.*, 2013; Borovik and Grabelnych, 2016), most of the studies are based on induced cold stimuli and little attention has been given to studying respiration throughout the dormancy period under field conditions. There is also limited information on total respiration rate and individual respiration pathways during dormancy progression in apple buds and none available for South African warm winter production areas.

The aim of this study was to investigate the effect of insufficient winter chill on the respiration dynamics of apple buds by comparing the total respiration and that of four individual respiration pathways viz. Tricarboxylic Acid Cycle (TCA), Pentose Phosphate Pathway (PPP), Cytochrome

C pathway (CYT) and the Alternative oxidase pathway (ALT) from two climatically contrasting production areas.

2. Materials and Methods

2.1. Plant material and study site

'Cripps Pink', a medium chill (450 - 800 positive chill units, ARC-Infruitec, 1997 as per Tharaga, 2014) apple cultivar of high commercial importance in South Africa, was used. Shoots were collected from Elgin (34.2°S, 19.0°E, 305 m.a.s.l.) and the Koue Bokkeveld (33.4° S, 19.5° E, 945 m.a.s.l.) in the Western Cape region of South Africa during the dormancy periods of 2015 and 2016. The Koue Bokkeveld is described as a cold winter area with ±1400 Utah CU (sufficient chill) compared to Elgin, a warm winter area, with ±700 Utah CU (insufficient chill) (Midgley and Lötze, 2011). Three commercial orchards from each area were selected and temperature loggers (Tiny Tag, Gemini Data Loggers, UK) were placed in the orchards to record hourly temperature subsequently converted to Utah chill units (Richardson *et al.*, 1974). The orchards were between 11 and 19 years old with trees of similar size, training system and farming practices. All trees had M793 as a rootstock and similar soil types. Historically the tree phenology in the warmer area (Elgin) is on average 10-14 days later than the colder (Koue Bokkeveld) area.

2.2. Dormancy progression curves from field conditions

Thirty uniform, one-year-old shoots (40 cm) were collected randomly from each orchard every 14 days and every seven days during the last month of the dormancy period. Samples were collected over two consecutive seasons (2015 and 2016) starting end of April and ended at the time of application of rest breaking agent in late August. The shoots were defoliated (if necessary) and labelled in the field and brought to the laboratory where they were sorted into three replicates of ten shoots each.

The proximal 3 cm of each shoot was cut off to remove possible air embolisms, placed into a 5 L white plastic buckets (three bundles per bucket) containing 1L of water and 5% sodium hypochlorite (5 ml sodium/L H_2O household bleach). Forcing experiments were performed according to Cook and Jacobs (1999) and Dennis (2003), where by the shoots were placed in a growth chamber with a constant temperature (25 ^{0}C) and continuous illumination (215 µmol m^{-2} s^{-1}. PAR (photosynthetically active radiation), 93% "cool-white" fluorescent and 7% incandescent light source). The water and bleach mixture was changed every second or third day and the proximal 5 mm of each shoot was removed weekly. Terminal buds were monitored twice per week and the time it took for 50% of the terminal buds in each bundle to bloom was recorded.

Dormancy progression curves were constructed by plotting the number of days it took to 50% budbreak (BB) for every sampling point. When a shoot took more than 10 days to 50% BB, it was considered as endodormant as suggested by Cook and Jacobs (1999). The depth of dormancy was considered to increase with an increase in the number of days to 50% BB while 50% BB within less than 10 days was considered the complete release of endodormancy. The means of the replicates were compared via an ANOVA followed by Fischer's Least Significant Difference test if means were found to differ at a significance level of $p<0.05$. Data was analysed using XLStat Institute 2017 (version: 19.01.40777 (32 bit)).

2.3. Respiration measurements from field conditions

A random collection of one-year-old shoots (20-25 cm) were harvested from each orchard during the dormancy periods of 2015 and 2016. In 2015, sample collection was done every 14 days from 28 April to 2 August and thereafter on a weekly basis up until 1 September before the application of rest breaking agent (done after 1 September). In 2016, sample collection started on 28 April and shoots were collected every 21 days until 6 June, every 14 days from 6 June until 3 August and every 7 days from 3 August until 25 August before the application of rest breaking agent (done after 25 August). All shoots were brought to the laboratory and processed within 24 hours after collection.

The total respiration rate and the rate of the individual respiration pathways were measured using a method similar to Vassileva *et al.* (2009) and Tan *et al.* (2010 and 2013). Terminal buds were excised from the shoots and processed using three buds per replicate. The sample was weighed and each bud was cut lengthwise into four slices. Internal air was removed from the bud slices using a vacuum. Oxygen consumption was measured using an OxyGraph (Hansatech Instruments Ltd., England) fitted with a sample chamber containing a Clarke-type oxygen electrode. The sample was lowered into the liquid-filled chamber using a net and the oxygen consumption of the liquid was measured over a three-minute period and expressed as nmol g^{-1} fresh weight min^{-1}. For the quantification of the total respiration rate the chamber was filled with distilled water during the measurement. For determining the respiration rate of the different pathways, a buffer solution (10 mM HEPES, 10mM MES buffer pH 6.6 and 0.2 mM $CaCl_2$ (Sigma-Aldrich, South Africa)) containing pathway specific inhibitors was used. More specifically, malonic acid (Merck, South Africa), a competitive inhibitor of succinate dehydrogenase in the TCA cycle, Na_3PO_4 (Sigma-Aldrich, South Africa) a specific inhibitor of glucose-6-phosphate dehydrogenase in the PPP, KCN (Sigma-Aldrich, South Africa) a specific inhibitor of cytochrome C oxidase in the CYT pathway and salicylhydroxamic acid (SHAM) (Sigma-Aldrich, South Africa) a specific inhibitor of alternative oxidase in the ALT pathway was used.

After determination of the total respiration in distilled water, the rate of the TCA and PPP pathways were measured by replacing the distilled water with 3 M malonic acid to measure oxygen consumed by the PPP pathway. This was followed by replacing the chamber liquid with a combination of 3 M malonic acid and 0.6 M Na_3PO_4 to determine the residual respiration, which is the remaining respiration after the TCA and PPP are blocked. This was repeated using 2mM KCN and 30 mM SHAM to determine the oxygen consumption of the CYT and ALT pathways. The OxyGraph chamber was cleaned with distilled H_2O or buffer depending on the reaction and the electrode was polarised in-between assays. The OxyGraph Plus software (version 1.02) was used to monitor the reaction and capture the reaction rates. To prevent the interference of photosynthesis, all the experiments were conducted in the dark at 20 °C. Twelve replications were analysed during the 2015 winter season and eight replications during the 2016 season. The oxygen consumed by the TCA and PPP, CYT and ALT pathways was then calculated through subtraction according to Tan *et al.* (2010 and 2013) (Table 1). The residual respiration is interpreted as the oxygen consumption that persisted in the presence of both inhibitors sets, MA and Na_3PO_4 or KCN and SHAM. The means of the replicates were compared via an ANOVA followed by Fischer's Least Significant Difference if means were found to differ at a significance level of $p<0.05$. All data analyses were done using XLStat Institute as described above (section 2.2).

2.4. Respiration measurements from lab-chilled conditions

On 25 April 2016, shoots were randomly collected from an orchard in the Koue Bokkeveld area, defoliated and brought to the laboratory. The shoots (with 53 Utah CU already accumulated) were folded in moist paper and plastic before placing them in a cold room at 4˚C where they accumulated 24 Utah CU in 24 hours (Richardson *et al.*, 1974). Shoots were removed from the cold room every two weeks (from 324 until 1997 Utah CU). The dormancy progression and respiration rate of the buds were tested as indicated above (section 2.2). Three replications (ten shoots per replication) were used for dormancy progression and eight replications for the respiration rate assays. Respiratory parameters were determined according to Tan *et al.* (2010 and 2013) (Table 1).

3. Results

3.1. Temperature, chill accumulation and phenology

The climatic data confirmed differences between the two contrasting areas in both years. According to the Utah chill model the Koue Bokkeveld received 1537 and 1338 CU and Elgin 869

and 645 CU in 2015 and 2016, respectively (Fig.1A and B). Chill accumulation started earlier in the Koue Bokkeveld and by May in both years, it had already accumulated more chill compared to Elgin in the same period. The average temperatures for both years indicated that the lower lying Elgin region had warmer winter conditions compared to the Koue Bokkeveld. Average daily temperature declined throughout winter and was at its lowest in July in both areas in both years after which it increased again towards spring. Overall, 2016 was a warmer year in both areas. Phenological growth stages such as leaf drop and budswell occurred on average 12-14 days later in Elgin compared to the Koue Bokkeveld (arrows in Fig.1A and B). In both years budswell was associated with significant increases in bud weight (data not shown) and occurred between 22-24 Aug in the Koue Bokkeveld and 5-7 Sep in Elgin although it is generally accepted that growth stimulating reactions can occur in the bud tissue as soon as temperature conditions are favourable, thus long before the visible signs of budswell.

3.2. Dormancy progression curves from field conditions

The dormancy progression curves of the two contrasting areas are presented in Fig. 2. Analysis of variance indicated that there was a significant interaction between the areas and the day of the year ($p < 0.0001$), meaning that the two areas reacted differently to the environmental conditions presented during the trial. At the commencement of shoot collection in 2015 (end-April) the terminal buds from the Koue Bokkeveld were already at its highest level of dormancy. This level was maintained until mid-June after which it decreased significantly over a short period and by mid-August the growth inhibition was released completely (broken line in Fig. 2A). In contrast to this, the shoots from Elgin never entered a deep dormancy state and growth was never strongly inhibited during the winter period (solid line in Fig. 2A). A maximum dormancy level of only 21 days to 50% BB was reached in Elgin compared to a maximum of 42 days in the Koue Bokkeveld.

In 2016 the Koue Bokkevld buds were also already dormant by the end of April and reached a maximum level (42 days) by mid-May (Fig. 2B). This level was maintained until the end of June after which it started to decrease. In Elgin the buds increased their dormancy level in May and reached a maximum level in mid-June, 2 to 3 weeks later compared to the Koue Bokkeveld. The maximum level (34 days) in Elgin was significantly higher than the maximum level (21 days) reached in 2015. The Elgin buds started to exit dormancy at the same time as the buds from the Koue Bokkeveld and both areas had a dormancy level less than 10 days to 50% BB at the end of August.

In general, the dormancy progression for the colder production area (Koue Bokkeveld) was very similar over the two years and reached deeper dormancy levels early in the season compared to

the buds from the warmer winter area (Elgin). The dormancy curves of the warmer area differed significantly between the two seasons with 2015 showing a shallower endodormancy compared to 2016 although it being the warmer season.

3.3. Respiration measurements from field conditions

3.3.1. Total respiration

The total respiration rate of buds collected from the two areas throughout the two winter seasons varied between 23 and 41 nmol g^{-1} min^{-1}. In both years the rate declined with the progression of winter but an increase towards spring was only evident (in both areas) in 2015 (colder year) (Fig. 3). Statistically, there was a significant interaction between the areas and the day of the year ($p < 0.0001$) in both seasons (2015 and 2016) (Fig. 3).

In 2015, the total respiration rate decreased in both areas from ±39 nmol g^{-1} min^{-1} at the onset of the experiment until the beginning of July (Fig. 3A). By the end of July, the respiration of the buds from the Koue Bokkeveld continued to decrease and reached a minimum (23 nmol g^{-1} min^{-1}) while the buds from Elgin maintained their level of oxygen uptake after reaching a minimum of 25 nmol g^{-1} min^{-1}. In August, buds from both areas increased their respiration but the buds from the Koue Bokkeveld showed a faster recovery and overtook the Elgin buds in the second week of August. At the beginning of September, the buds from Elgin had a respiration rate 15% lower than that of the Koue Bokkeveld.

In 2016 the trial started with the highest respiration rate (>41 nmol g^{-1} min^{-1}) in both areas (Fig. 3B). In contrast to 2015, the respiration rates of buds in both areas remained relatively high and unchanged during May and only started to decrease towards the end of June. By the end of June, the levels had decreased but remained slightly higher (±37 nmol g^{-1} min^{-1}) than what was measured at the same period in 2015 (±28 nmol g^{-1} min^{-1}). In July 2016 the buds from the Koue Bokkeveld increased their respiration rate and again decreased towards August while the respiration from buds in Elgin remained consistently low. Toward September, both areas showed a slight increase in total respiration, but at the end of the trial they were not significantly different. Although the respiration rates in both areas declined during the winter of 2016, it never went lower than 35 nmol g^{-1} min^{-1}.

3.3.2. TCA, PPP pathways and residual respiration

TCA pathway and contribution to the total respiration

In both years, the respiration rate of the TCA pathway varied between 11 and 23 nmol g^{-1} min^{-1} and contributed to between 37% and 62% of the total respiration (Fig. 4 A-D). The ANOVA results from the TCA pathway showed a significant interaction (Areas*Days) for both 2015 ($p = 0.0010$) and 2016 ($p = 0.0060$) (Fig. 4A and B), indicating that buds from the contrasting areas react differently. Although the respiration patterns differed between the two years, the areas performed relatively similar until late August in both years.

In 2015, buds from both areas showed a significant decrease in the TCA respiration throughout the winter period, reaching a minimum in late July, compared to the relatively consistent slow rate of decline measured in both areas during 2016 (Fig. 4A and B). The two areas did, however, differ greatly during August in both years (2015 and 2016). In both years the Koue Bokkeveld buds showed a rapid increase in the TCA respiration towards budbreak compared to buds from the warmer region that remained relatively low. Thus, in summary, area differences in the TCA cycle were detected at the end of the winter period in both years with buds from Elgin performing at a lower rate. Elgin buds had a TCA respiration rate of 38% and 30% lower than that of Koue Bokkeveld buds at the end of the experiment in 2015 and 2016, respectively (Fig. 4A and B). This pattern remained similar when considering the percentage contribution that the TCA pathway made towards the total respiration (Fig. 4C and D). The decrease in both areas at the end of July 2015 was accentuated and the rapid increase in the buds from the cold winter area exceeded that of the warm area by 25% at the end of the trial (Fig. 4C). Although a relative constant contribution was observed in both areas until mid-July in 2016 (Fig. 4D), the Koue Bokkeveld then started to increase its contribution relative to Elgin that remained unchanged. The contribution in the Koue Bokkeveld buds was 26% higher compared to Elgin at the start of spring (Fig. 4D).

PPP pathway rate and contribution to the total respiration

During the two winter seasons, the respiration rate of the PPP pathway was between 4 and 11 nmol g^{-1} min^{-1} and contributed to between 11% and 30% of the total respiration (Fig. 5). In 2015 there was no significant interaction between the two areas over time, however, significant differences were detected for the main effects: day of the year ($p < 0.0001$) and areas ($p = 0.0010$) (Fig. 5A). Although the Elgin buds had a higher average PPP rate, both the Koue Bokkeveld and Elgin buds showed a decreasing PPP trend from the onset of the experiment until the end of May. This rate was maintained at a constant level until the end of July followed by a

sharp increase until the first week of August where after it remained constant till the end of the trial (Fig. 5A). In 2016, there was a significant interaction between the areas and the day of the year ($p = 0.0070$) (Fig. 5B). The buds from Elgin showed a steady rate of 8 nmol g^{-1} min^{-1} from the onset of the trial (end of April) until mid-July, followed by a decline. The PPP rate of the Koue Bokkeveld buds showed a decreasing pattern throughout the entire winter and dropped below that of the Elgin buds already at the end of April. Similar to the 2015 season, the buds from Elgin and the Koue Bokkeveld reached a minimum PPP rate at the beginning of August 2016. Buds, from both areas, then showed a slight increase in the PPP rate towards spring but the buds from the Koue Bokkeveld remained significantly lower than that from Elgin. The PPP rate in the Elgin buds was 42% higher compared to the Koue Bokkeveld buds at the end of the trial (Fig. 5B).

A relatively similar trend was observed for the percentage contribution of the PPP pathway to the total respiration (Fig. 5C and D) in both years. In 2015, there was no significant interaction detected, but significant differences were detected for both of the main effects days ($p < 0.0001$) and areas ($p = 0.0010$) (Fig. 5C). While the Elgin buds had a higher percentage compared to the Koue Bokkeveld, a declining trend in the PPP contribution was observed from the start of the experiment until the end of May in buds from both areas. Levels then remained constant until the end of July followed by a significant increase until the beginning of August only to decline again until the end of the experiment (Fig. 5C). In 2016, a significant interaction between 'Days' and 'Areas' where observed ($p = 0.0010$) (Fig.5 D). Similar to the PPP rate (Fig. 5B), the contribution of the PPP pathway, in buds from both areas, also declined from the beginning of the trial until August and then increased significantly towards spring. Again the level in the Elgin was significantly higher (43%) compared to the Koue Bokkeveld.

Residual rate and contribution to the total respiration rate

The residual respiration rate (Fig. 6) indicates the respiration that persists in the presence of the TCA (malonic acid) and PPP (Na_3PO_4) inhibitors (Table 1). The residual respiration was between 4 and 17 nmol g^{-1} min^{-1} and contributed between 16% and 39% of the total respiration during the two winter seasons (Fig. 6A-D). The residual respiration capacity of both areas was higher during 2016 compared to 2015. The analysis of variance showed significant main effects ($p = 0.0270$ for 'Areas' and $p < 0.0001$ for 'Days') during the 2015 season (Fig. 6A) and a significant interaction ($p < 0.0001$) effect for the 2016 winter seasons (Fig. 6B). In 2015 the residual respiration had a higher average rate in the Koue Bokkeveld buds compared to the Elgin buds although the buds from both locations showed a similar trend of initial decrease with a minimum level during July followed by a rapid increase before a second decrease towards spring (Fig. 6A). In 2016, however, the residual rate in buds from the two areas behaved differently. The Koue Bokkeveld

levels remained stable from the end of April until the end of July when it dropped and then remained relatively low until budbreak. The residual respiration from buds from Elgin showed a stable level from end of April 2016 until the end of June when it decreased briefly and recovered again to remain stable (and similar to the Koue Bokkeveld) until spring (Fig. 6B). The residual's contribution to the total respiration showed a significant interaction ($p < 0.0020$) in 2015 (Fig. 6C). In general, the residual's contribution in both areas remained constant from the end of April until the end of July and then increased significantly in both areas followed by a decrease in the Koue Bokkeveld buds while the Elgin buds remained constant towards spring. In 2016, buds from both areas were only affected by time ('Days') ($p < 0.0001$) (Fig. 6D) with the contribution showing a downwards trend throughout the season in both areas.

3.3.3. CYT, ALT pathways and residual respiration

CYT pathway rate and contribution to the total respiration rate

The range of the CYT respiration pathway was between 9 and 21 nmol g^{-1} min^{-1} for the two seasons and at any given time, contributed between 36% and 52% to the total respiration (Fig. 7A-D). The CYT respiration rate indicated a significant interaction effect between the areas (Koue Bokkeveld and Elgin) and the day of the year in both 2015 ($p = 0.0060$) and 2016 ($p = 0.0010$) (Fig.7 A and B). During 2015, the CYT rate showed a decreasing trend from the onset of the experiment (end of April) until the end of July in buds from both areas. From August until the start of budbreak the buds from the Koue Bokkeveld significantly increased while the Elgin buds remained constant and at the end of the trial the buds from the Koue Bokkeveld were 23% higher compared to Elgin. During the 2016 winter season, there was a similar initial decreasing trend in both areas that continued until late June but did not reach such low levels as seen in 2015. The CYT rate of the buds from Koue Bokkeveld then increased sharply while that of buds from Elgin remained constant. The CYT rate in buds from Koue Bokkeveld remained relatively higher than that of Elgin until end of the experiment (Fig.7 B). During the 2015 season, CYT contribution to the total respiration showed a significant main area effect ($p = 0.0110$) and the average level was higher for the Koue Bokkeveld buds compared to that from Elgin (Fig.7C). In 2016, a significant interaction effect between the areas and the day was detected on the percentage contribution of the CYT pathway ($p = 0.0010$) (Fig.7D). The pattern of the percentage contribution of the CYT pathway mimicked that of the CYT respiration rate for both areas but accentuated the difference between the two areas towards the spring.

ALT pathway rate and contribution to the total respiration

The ALT respiration pathway had a range between 3 and 9 nmol g^{-1} min^{-1} for the two seasons, which lead to a contribution range of between 8% and 24% of the total respiration (Fig 8A-D). There were significant main effects (Areas, $p < 0.0001$ and Days of the year, $p = 0.0040$) during the 2015 season (Fig. 8A) showing a decrease in both areas from the end of April until July, a considerable increase in August, with levels decreasing again towards the end of August and beginning of September. The Elgin buds had higher average level of ALT pathway rate compared to that of the Koue Bokkeveld buds in 2015. During the 2016 season, there was a significant interaction effect between the areas and the day of the year ($p = 0.0360$) (Fig. 8B). In this year, the rate of the ALT pathway showed a progressive decrease from the beginning of the experiment (end of April), with a faster rate notable for the Koue Bokkeveld buds and at the end of the trial the level was 30% lower than the buds from Elgin. Both buds from warm and cold areas reached a minimum ALT rate in mid-August where after there was a slight increase in both areas. The pattern for the percentage contribution of ALT pathway was similar to the results of ALT pathway rate. A significant main effect (areas) ($p < 0.0001$) was recorded during 2015 season and the contribution average of ALT was considerably higher in Elgin buds than that of Koue Bokkeveld (Fig. 8C). During the 2016 season, a significant interaction effect between the areas and day of the year was observed ($p = 0.0140$) (Fig. 8D). Similar to the ALT rate pattern, the contribution of ALT was reduced in buds from both areas but buds from the colder region decreased faster towards the start of spring while the buds from the warmer region maintained a higher level.

Residual respiration rate and contribution to the total respiration

The amount of oxygen uptake in the presence of both a CYT (SHAM) and ALT (KCN) inhibitor is shown in Fig. 9. The rate was generally in the range of 9 and 19 nmol g^{-1} min^{-1} in both years and contributed to between 32% and 48% of the total respiration. Analysis of variance showed a significant interaction in both the 2015 ($p = 0.0010$) and 2016 ($p = 0.0380$) winter seasons (Fig. 9A and B). During 2015, the residual rate decreased gradually in buds taken from both areas until the end of July. After July the residual rate increased significantly in the Koue Bokkeveld buds until budbreak, but remained constant in the buds from Elgin (Fig. 9A). In 2016, the residual rate in buds taken from both areas showed a decreasing trend from the onset of the trial (end of April) until July, followed by a relatively constant level until the end of the trial (end of August) (Fig. 9B). When considering the contribution of the residual to the total respiration, a significant interaction was observed in both the 2015 ($p = 0.0260$) and the 2016 ($p = 0.0010$) winter seasons, indicating that buds from the contrasting areas reacted differently in both years. Apart from some fluctuations, the contribution of the residual to the total respiration from both areas and across

both seasons showed similar trends. However, the contribution as the residual rate in 2015 was significantly higher in the Koue Bokkeveld buds compared to the Elgin buds during the last week of the trial (Fig. 9C). This was not observed in 2016 (Fig. 9D).

3.4. Dormancy progression curves and respiration of lab-chilled buds

3.4.1. Dormancy progression curve

The dormancy progression for the lab-chilled treatment revealed a significant decrease in the number of days to 50% budburst from 28 days at the onset of the experiment (end of April) (53 Utah CU) to 12 days at 377 Utah CU accumulation (Fig. 10). This low level of growth inhibition was maintained with a decreasing trend until 1944 CU were accumulated at the end of the trial and 50% BB occurred within 5 days.

3.4.2. Respiration from induced lab-chilled conditions

The total respiration rate recorded for the lab-chilled buds was between 40 and 45 nmol $g^{-1}min^{-1}$ and increased significantly from a level of 40 nmol g^{-1} min^{-1} at 53 accumulated CU to 44 nmol g^{-1} min^{-1} at 377 CU (Fig.11). As the chill units accumulated further, the respiration rate remained relatively constant until the end of the trial. The rate of the TCA pathway varied between 17 and 22 nmol g^{-1} min^{-1} (Fig. 12). The initial level of 17 nmol g^{-1} min^{-1} increased to 22 nmol g^{-1} min^{-1} after 377 CU were accumulated; thereafter it remained constant until the completion of the trial. The PPP pathway was between 4 and 8 nmol g^{-1} min^{-1} (Fig 12). The activity of the PPP was at a constant level between 53 and 701 CU after which it declined significantly and then remained constant and only decreased again to 4.3 nmol g^{-1} min^{-1} at 1997 CU accumulation (Fig. 12), the lowest point in the trial. The residual respiration (oxygen consumption persisting in the presence of TCA and PPP inhibitors) was between 14 and 19 nmol g^{-1} min^{-1} (Fig. 12). Its level remained constant from the beginning of the trial until 701 CU were accumulated; thereafter it increased until 1025 CU accumulated before it remained constant for the remainder of the trial (Fig. 12). The rate of the CYT pathway remained between 17 and 20 nmol g^{-1} min^{-1} throughout the trial with an increasing trend (Fig. 13). The ALT pathway was between 4 and 7nmol g^{-1} min^{-1} and showed a downtrend toward the end of the trial (Fig.13). The residual initially at 40 nmol g^{-1} min^{-1} decreased significantly to 32 nmol g^{-1} min^{-1} after 377 CU accumulation and then increased significantly to 39 nmol g^{-1} min^{-1} after 1025 CU were accumulated before it remained constant until the completion of the experiment (Fig. 13). The contribution of the different pathways to the total respiration rate behaved in a similar pattern as their respective rates.

4. Discussion

4.1. Temperature, chill accumulation and dormancy progression

Climatic conditions reported during the two experimental seasons were typical of what is expected in the Elgin and Koue Bokkeveld areas of the Western Cape, South Africa (Midgley and Lötze, 2011) with Elgin having a warmer winter compared to the Koue Bokkeveld. Similar data on climatic conditions for the two areas was presented by Cook and Jacobs (2000), Halgryn *et al.* (2001) and Midgley and Lötze (2011). Using the Utah model, the accumulation of chill units started earlier and increased faster in the Koue Bokkeveld compared to Elgin in both seasons. On a seasonal level, 2016 was a warmer year in both areas with a lower amount of chill accumulation (640 and 1300 CU) compared to 2015 (860 and 1500 CU) for Elgin and Koue Bokkeveld, respectively.

The dormancy progression curves of the apple buds collected from Elgin and the Koue Bokkeveld differed between the two areas and between the two seasons in correspondence with the differences in climatic conditions and resultant chill accumulation. In the colder 2015, the Koue Bokkeveld buds were already deeply dormant at the onset of the trial before any chill accumulation had been recorded, while buds from the warmer Elgin failed to reach such high dormancy levels and showed a relative flat dormancy level, i.e. below 20 days to budbreak, throughout the winter. In the warmer 2016 season when chill accumulation was lower in both areas, the dormancy progression curve for Elgin, although lower compared to the Koue Bokkeveld for 2016, reached a much higher maximum dormancy level compared to 2015. This was somewhat unexpected as previous research in South Africa has shown that dormancy levels tend to be shallower in warmer seasons (Cook *et al.*, 2017). The deeper dormancy, before accumulation of any Utah chill units, in the Koue Bokkeveld samples, was also detected in the lab-chilled buds collected in the same area at the end of April 2016. Overall the results obtained on the dormancy progression in this study, were comparable with results observed for 'Golden Delicious' and 'Granny Smith' in the same locations as recorded by Cook and Jacobs (2000) and by Cook *et al.* (2017). In cold winter areas, dormancy progression reflects the amount of accumulated chill units (Hauagge and Cummins, 1991) while abnormal dormancy progression has been attributed to inadequate chilling in apple (Cook and Jacobs, 2000) and in peach (Leite and Bonhomme, 2004).

Considering the dormancy progression and respiration data generated in both years of the study, two environmental and physiological stages/states/phases are recognisable, regardless of chill accumulation. Temperatures increased steadily from August in both areas in both years and the dormancy progression curves show that in both areas 50% of the terminal buds took less than ten days to start growing from mid-August onwards. It is also around this time that many of the

respiration graphs show a change in activity. From this we identified the dormant phase as the period from the onset of the experiment until mid-August followed by a growth resumption phase where conditions are favourable for growth. These two phases are separated by a vertical line at mid-August on all the graphs. Interestingly, the visual signs of growth (budswell) were recorded shortly after growth resumption between 22-24 August in the Koue Bokkeveld, but only 12-14 days later on 5-7 September in Elgin in both years.

The dormant phase can be seen as a period of continued decrease in temperature and an increase in chill accumulation in both the Elgin and Koue Bokkeveld areas. This decrease in temperature is known to reduce plant respiration (Atkin and Tjoelker, 2003). Growth resumption is associated with the recommencement of chemical reactions and enzyme activity, including respiration, as shown for grapevine buds by Gardea *et al.* (1994) and occurs before any visible signs of growth are noticeable. To simplify the explanation of these findings regarding the respiration dynamics we continue this discussion by referring to these two phases as the 'dormant' phase (from the onset of the experiment until 16 August for the Koue Bokkeveld and Elgin area) and 'growth resumption' phase (from 16 August until the end of the trial).

4.2. The 'dormant' phase

During the winter period (dormant phase), metabolic activity is almost absent in a deciduous tree and the main respiration pathways responsible for energy production (total respiration, TCA and CYT) decline or remaine constant as enzyme capacity is reduced at low temperatures (Atkin and Tjoelker, 2003). Since all temperate trees require chill accumulation during winter to resume growth in spring, knowledge of the dynamics of the different respiration pathways during the dormant phase can potentially reveal the physiological effect of chilling temperatures.

In general, the CYT pathway is known as the main and terminal pathway of oxidative phosphorylation in the inner mitochondrial membrane (Taiz and Zeiger, 2010; Toro and Pinto, 2015) as it maintains the electron transport between complexes III and IV which in turn drives the H^+ gradient across the membrane to produce ATP. Different stresses such as chill/ heat temperatures or rest breaking agents are known to negatively affect CYT activity (Ribas-Carbo *et al.*, 2000; Atkin *et al.*, 2002; Tan *et al.*, 2013; Borovik and Grabelnych, 2016) by reducing the O_2 consumption and/or inhibiting enzyme activities leading to oxidative stress (Taiz and Zeiger, 2010). This suggests that bud growth can be controlled by internal stress or environmental conditions that cripple the CYT pathway. In agreement with literature reporting a decrease in the respiration rate in response to colder temperatures (Kurimoto *et al.*, 2004; Atkin *et al.*, 2005), especially deciduous woody plants (Myking, 1998) such as apple buds (Young, 1990), we found

a decrease in CYT activity, total respiration and the TCA cycle during the dormant phase. The downwards trend in the TCA activity in the buds of both areas suggests that bud growth was limited by environmental conditions although some energy was still produced for maintenance reactions (Fernie *et al.*, 2004). Other studies show that lower activity of the TCA enzyme, isocitrate dehydrogenase, was detected in the dormant apple buds compared with non-dormant buds (Wang *et al.*, 1991). Similarly, TCA enzyme activity, as well as ATP synthase, were low in dormant buds of peonias (*Paoenia ostii)* (Gai *et al.*, 2013). Young (1990) reported that a decline in energy activation, due to decreased metabolic activity, coincided with increased chilling in apple buds. A decrease in the total respiration, CYT and TCA pathway rates due to a decrease in the temperature of the chamber fluid (40 °C, 30 °C, 20 °C, 10 °C and 5 °C) was also evident preliminary trials on apple buds using the Oxytherm (Hansatech Instruments, England) (data not shown). However, the respiration capacity was not compromised in the lab-chilled buds in the same way as the field chilled buds.

In general, plants respond to low temperature stress by increasing their PPP and ALT rates (Vanlerberghe and McIntosh, 1992; Tan *et al.*, 2010). The pesent results are not consistent with this; the activity of PPP and ALT was significantly lower at the end of the dormant phase compared to initial rates in both areas and both seasons. The PPP pathway protects against oxidative stress associated with cold and maintains the redox state by producing NADPH to generate and compensate for the lack of energy in the plant cells (Taiz and Zeiger, 2010; Vergara and Pérez, 2010), while the ALT pathway aims to maintain mitochondrial respiration (Juszczuk and Rychter, 2003; Vanlerberghe, 2013). A downwards trend in the PPP and the ALT pathways generally leads to a decrease in oxidative stress (Juszczuk and Rychter, 2003; Beauvieux *et al.*, 2018). In this study, however, although PPP and the ALT activities decreased in buds from both areas, they were consistently higher in buds from the warmer Elgin area compared to the colder Koue Bokkeveld, which may suggest differences in levels of stress in the two areas.

Only a few studies have reported on the seasonal change in the PPP and the ALT activity in temperate fruit trees and most of these disagree with the present results. Kuroda *et al.* (1990) indicated that cold hardiness in apple buds is closely associated with a rise in the activity of peroxide-scavenging systems, as well as PPP enzymes activity. Under seasonal experiments, Sagisaka (1972 and 1974) also found that PPP enzymes increased from winter to spring and then declined until early summer in sufficiently chilled Poplar (*Populus gelrica*) twigs. Similarly, a higher activity of these enzymes was detected during the dormant period when compared with the growing phase in apple buds (Wang *et al.*, 1991) and seeds (Swamy and Sandhyarani, 1986). Other studies also reported that an increase in the alternative oxidase concentration occurs when

plants are exposed to stress (Vanlerberghe and McIntosh, 1992; Ribas-Carbo *et al.*, 2000). On the contrary, however, Atkin *et al.* (2002) highlighted that ALT activity can also be low at lower temperatures (10 °C to 25 °C) in 'Stevens' soybean (*Glycine max)*, which is consistent with this study. Further studies are needed to confirm the pattern and roles of the PPP and the ALT pathways during the 'dormant' phase.

The present data did not show a clear connection between the TCA and the PPP or the CYT and the ALT pathways as suggested in literature. Usually, a higher PPP activity occurs when the TCA pathway declines and a higher level of ALT activity when the CYT pathway is limited (Tan *et al.*, 2010 and 2013). For instance, once the CYT pathway is inhibited, the electrons are temporarily taken up by the ALT pathway to prevent mitochondrial oxidation (Ribas-Carbo *et al.*, 2000; Taiz and Zeiger, 2010; Borovik and Grabelnych, 2016). In this study, the cold area showed a decrease in PPP and ALT activity during the dormant phase, possibly due to decreased enzymatic activity because of low environmental temperatures. The effect was less in the warmer area where the PPP and ALT activities were significantly higher during the latter part of the dormant phase.

4.3. The growth resumption phase

During the growth resumption phase, the significant increase in all of the main respiration pathways (total respiration, TCA and CYT) detected in the Koue Bokkeveld buds was in sharp contrast to the consistently lower levels found in the Elgin buds. Scalabrelli and Couvillon (1986) indicated that partially chilled buds need higher temperatures (>20 ˚C) to induce growth after the dormant phase compared to fully chilled buds that will sprout at relatively low temperatures (10˚C). This was evident in the current results as the Koue Bokkeveld buds, which accumulated sufficient chill, resumed growth 12-14 days before the buds from Elgin despite similar low dormancy levels in August. This difference in the timing of growth resumption between the two areas have also been reported by Cook and Jacobs (2000) and Cook *et al.* (2017) where buds from the Koue Bokkeveld started growing before those in Elgin. Furthermore, apple trees in Elgin show delayed foliation and protracted bloom in the absence of chemical rest breaking agents due to insufficient chill accumulation. This off-set in the phenology between the two regions could be the result of the different chill accumulation levels in the areas. In the Koue Bokkeveld the chill requirement of the buds were met and they were able to sprout under relatively cool spring conditions. In Elgin, by comparison, buds accumulated less chill, the chill requirement was not fully met, and these buds subsequently required warmer spring temperatures for growth resumption. As budbreak and growth demand energy (produced through respiration), it can only commence once the respiration pathways resume their activities after the dormant phase. Thus, it is proposed that insufficient winter chill could possibly delay budbreak due to the lack of available energy as a result of low

respiration rates post dormancy. Without sufficient chill, the respiration mechanics are not yet in place to provide the energy needed for growth.

Both CYT and TCA activity play an important role during the growth resumption phase. A rise in their activity would provide energy for uniform and early budbreak. Once the CYT pathway is activated, the H^+ proton gradient across the mitochondrial membrane is restored while the activity of the TCA pathway increases and produces NADH and $FADH_2$, which will result in energy production through oxidative phosphorylation (Fernie *et al.*, 2004; Taiz and Zeiger, 2010). In contrast, in the absence of increasing TCA and CYT activity in the Elgin buds during the growth resumption phase, energy levels would be restricted, which could delay the onset of budbreak, reduce the number of breaks and protract the flowering period; all typical symptoms of warm winter areas. ATP synthesis is known to be associated with a wider metabolic activity network such as oxidation, decarboxylation and dehydrogenisation (Fernie *et al.*, 2004; Sweetlove *et al.*, 2010; Araújo *et al.*, 2012) which leads to dormancy release and growth resumption. Usually, the bulk of the energy for growth is provided by the TCA pathway and if it is restricted during dormancy release, budbreak is effected (Tan *et al.*, 2010). A recent study indicated that most metabolites involved in the TCA cycle, such as citrate, 2-oxoglutarate, fumarate and malate, increased their levels in Japanese pear flower buds exposed to sufficient chilling (6 °C) in comparison to buds with insufficient chilling (6/ 18 °C (150 h/150 h)) (Horikoshi *et al.*, 2018). An increase in CYT activity and ATP synthesis was also largely associated with plant growth and maintenance respiration (Florez-Sarasa *et al.*, 2007). Similar to this study, others also reported an increase in respiration rate at the end of the dormant stage and towards budbreak in sufficiently chilled kiwifruit buds (McPherson *et al.*, 1997) and grapevine buds (Gardea *et al.*, 1994; Pérez *et al.*, 2007; Potjanapimon *et al.*, 2008). Findings from this study suggested that sufficient winter chill and warmer external temperatures favour an increase in the main respiration pathways.

The two alternative pathways (PPP and ALT) are also involved in the growth resumption phase. Under warmer winter conditions, an increase in PPP and ALT activity was observed in the buds from Elgin compared to those from the Koue Bokkeveld where the rates of the two pathways were lower. The high activity in PPP in the Elgin buds could be a strategy to produce ATP for the energy demanding process of budswell and budburst while the main energy producing pathways are reduced. Similarly, high activity of the ALT pathway decreases the oxidation within the mitochondrial membrane by reducing O_2 to H_2O and preserving the redox state (Taiz and Zeiger, 2010). Although the ALT pathway cannot transfer H^+ across membranes, it can allow for the production of low amounts of energy (Taiz and Zeiger, 2010; Toro and Pinto, 2015). Wang and Faust (1991) indicated that during growth resumption in TDZ-induced apple buds, the activity of

the two PPP enzymes (G6PDH and 6PGDH) decreased. Tan *et al.* (2010) suggested that under sufficient chilling of nectarine buds prior to dormancy release, TCA activity increases while PPP activity declines. Similarly, Millenaar *et al.* (1998) highlighted that the ALT pathway is activated when the CYT activity is limited and can stabilise the redox state of the mitochondrial ubiquinone pool by preventing an increase of radical oxygen species and products of fermentation. It seems that when the activity of TCA and CYT is not accelerated during dormancy release, the activity of PPP and ALT increases concomitantly to mitigate the low energy status at a time of high energy demand.

5. Conclusions

This study confirms that the respiration dynamics in apple buds vary from one area to another in relation to climatic differences. In an area with sufficient winter chill, 'Cripps Pink' buds showed a typical dormancy progression, depth and release and the respiration pathway activity supported the energy requirement for growth. The decrease in temperature during the dormant phase of the buds contributed to the decline in the respiration rate during winter. Sufficient chill accumulation in buds during the dormant phase, as well as warmer external temperatures during the growth resumption phase resulted in a high and increasing total respiration, TCA and CYT rates which contribute to the production of energy for the budswell and budbreak processes. Under insufficient chill accumulation the apple buds showed an atypical dormancy progression and respiration dynamic. During the dormant phase, the main respiration pathways failed to increase and during the growth resumption phase the PPP and ALT pathways increased, to possibly compensate for the lack in energy production (via TCA and CYT) whereas increasing the ALT pathway could also counter the high stress levels associated with incomplete dormancy.

The current results contribute to the investigation of dormant buds respiration under field conditions with insufficient winter cold. Since chilling temperatures are predicted to decline with global warming and hamper the cultivation of temperate tree fruits in areas with marginal winter condition, it is important to gain knowledge on the changes brought about by inadequate winter conditions. This study supports the use of RBA during growth resumption in warm winter areas as it can increase respiration (as tested in chapter 3), especially the TCA activity that is associated with ATP production and the initiation of other metabolic pathways. Further testing, both theoretical and experimental, is necessary to determine the role of ROS species under warm winter field conditions to confirm the link between respiration and oxidative stress management. Future research will be more meaningful if a study site from the northern hemisphere is included

to represent typical winter conditions. Results from such work will assist in the development of practical solutions to mitigate the threat of climate change to apple production.

6. References

Araújo, W.L., Nunes-Nesi, A., Nikoloski, Z. and Sweetlove, L.J. (2012). Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. *Plant Cell Environment*. **35**, 1–21.

ARC-Infruitec, 1997. *South African deciduous fruit cultivars catalogue*. 2nd ed. ARC Infruitec-Nietvoorbij, Stellenbosch, SA. (as per Tharaga, 2014).

Arora, R., Rowland, L.J. and Tanino, K. (2003). Induction and release of bud dormancy in woody perennials: a science comes of age. *HortScience*. **38**, 911–921.

Atkin, O.K., Zhang, Q. and Wiskich, J.T. (2002). Effect of temperature on rates of alternative and cytochrome pathway respiration and their relationship with the redox poise of the quinone pool. *Plant Physiology*. **128**, 212–222.

Atkin, O.K. and Tjoelker, M.G. (2003). Thermal acclimation and the dynamic response of plant respiration to temperature. *Trends in Plant Science*. **8**, 343–351.

Atkin, O.K., Bruhn, D., Hurry, V.M. and Tjoelker, M.G. (2005). The hot and the cold: unravelling the variable response of plant respiration to temperature. *Functional Plant Biology*. **32**, 87–105.

Atkinson, C.J., Brennan, R.M. and Jones, H.G. (2013). Declining chilling and its impact on temperate perennial crops. *Environmental and Experimental Botany*. **91**, 48–62.

Baldocchi, D., Wong, S. and Gutierrez, A. (2005). An assessment of impacts of future CO_2 and climate on agriculture. *A Report From: California climate change center*. pp: 33.

Beauvieux, R., Wenden, B. and Dirlewanger, E. (2018). Bud Dormancy in Perennial Fruit Tree Species: A Pivotal Role for Oxidative Cues. *Frontiers in Plant Science*. **9**, 657.

Borovik, O.A. and Grabelnych, O.I. (2016). Distribution of the respiratory pathways in the isolated mitochondria from etiolated leaves of winter wheat and rye after the action of low temperature. *Journal of Stress Physiology and Biochemistry*. **12**, 38–49.

Campoy, J. A., Ruiz, D. and Egea J. (2011). Dormancy in temperate fruit trees in a global warming context: a review. *Scientia Horticulturae*. **130**, 357–372.

Cook, N.C. and Jacobs, G. (1999). Suboptimal winter chilling impedes development of acrotony in apple shoots. *Hortscience*. **34**, 1213–1216.

Cook, N.C. and Jacobs, G. (2000). Progression of apple (*Malus x domestica* Borkh.) bud dormancy in two mild winter climates. *Journal of Horticultural Science and Biotechnology*. **75**, 233–236.

Cook, N.C., Calitz, F.J., Allderman, L.A., Steyn, W.J. and Louw, E.D. (2017). Diverse patterns in dormancy progression of apple buds under variable winter conditions. *Scientia Horticulturae.* **226**, 307–315.

Cooke, J.K., Eriksson, M.E. and Junttila, O. (2012). The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. *Plant Cell Environment*. **35**, 1707–1728.

Faust, M., Erez, A., Rowland, L.J., Wang, S.Y. and Norman, H.A. (1997). Bud dormancy in perennial fruit trees: Physiological basis for dormancy induction, maintenance and release. *HortScience*. **32**, 623–629.

Fernie, A.R., Carrari, F. and Sweetlove, L.J. (2004). Respiratory metabolism: Glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology*. **7**, 254–261.

Florez-Sarasa, I.D., Bouma, T.J., Medrano, H., Azcon-Bieto, J. and Ribas-Carbo, M. (2007). Contribution of the cytochrome and alternative pathways to growth respiration and maintenance respiration in

Arabidopsis thaliana. *Physiologia Plantarum*. **129**, 143–151.

Gai, S., Zhang, Y., Liu, C., Zhang, Y. and Zheng, G. (2013). Transcript profiling of *Paoenia ostii* during artificial chilling induced dormancy release identifies activation of GA pathway and carbohydrate metabolism. *PLoS ONE.* **8** (2), 1– 11.

Gardea, A.A., Moreno, Y.M., Azarenko, A.N., Lombard, P.B. and Daley, L.S. (1994). Changes in metabolic properties of grape buds during development. *Journal of the American Society for Horticultural Science*. **119**, 756–760.

Graeber, K., Nakabayashi, K., Miatton, E., Leubner-Metzger, G. and Soppe, W.J.J. (2012). Molecular mechanisms of seed dormancy. *Plant, Cell and Environment*. **35**, 1769–1786.

Halgryn, P. J., Theron, K. I. and Cook, N. C. (2001). Genotypic response to chilling period of apple buds from two Western Cape localities. *South African Journal of Plant and Soil*. **18**, 21–27.

Hauagge, R. and Cummins, J.N. (1991). Seasonal variation in intensity of bud dormancy in apple cultivars and related *Malus* species. *Journal of the American Society for Horticultural Science*. **116**, 107–115.

Heide, O. M. and Prestrud, A. K. (2005). Low temperature, but not photoperiod, control growth cessation and dormancy induction and release in apple and pear. *Tree Physiology*. **25**, 109–114.

Horikoshi, H.M., Sekozawa, Y., Kobayashi, M., Saito, K., Kusano, M. and Sugaya, S. (2018). Metabolomics analysis of 'Housui' Japanese pear flower buds during endodormancy reveals metabolic suppression by thermal fluctuation. *Plant Physiology and Biochemistry*. **126**, 134–141.

Juszczuk, I.M. and Rychter, A.M. (2003). Alternative oxidase in higher plants. *Acta Biochimica*. **50**, 1257–1271.

Kurimoto, K., Day, D.A., Lambers, H. and Noguchi, K. (2004). Effect of respiratory homeostasis on plant growth in cultivars of wheat and rice. *Plant Cell Environment*. **27**, 853–862.

Kuroda, H., Sagisaka, S. and Chiba, S. (1990). Seasonal changes in peroxide-scavenging systems of apple trees in relation to cold hardiness. *Journal of the Japanese Society for Horticultural Science*. **59**, 399–408.

Lang, G.A. (1987). Dormancy: a new universal terminology. *HortScience*. **22**, 817–820.

Leite, G.B., Bonhomme, M., Lacointe, A., Rageau, R., Sakr, S., Guilliot, A., Maurel, K., Petel, G. and Couto-Rodriguez, A., (2004). Influence of lack of chilling on budbreak patterns and evolution of sugar contents in buds and stem tissues along the one-year-old shoot of the peach trees. *Acta Horticulturae*. **662**, 61–71.

Luedeling, E., Zhang, M. and Girvetz, E.H. (2009). Climatic changes lead to declining winter chill for fruit and nut trees in California during 1950-2099. *PLoS One.* **4** (7), 1– 9.

Luedeling, E. (2012). Climate change impacts on winter chill for temperate fruit and nut production: *Scientia Horticulturae*. **144**, 218–229.

McPherson, H.G., Shelgar, W.P., Manson, P.G. and Snowball, A.M. (1997). Bud respiration and dormancy of kiwifruit (*Actinidia deliciosa*). *Annals of Botany*. **80**, 411–418.

Midgley, S.J.E. and Lötze, E. (2011). Climate change in the Western Cape of South Africa: Trends, projections and implications for chill unit accumulation. *Acta Horticulturae*. **903**, 1127–1134.

Millenaar, F.F., Benschop, J.J., Wagner, A.M. and Lambers, H. (1998). The role of the alternative oxidase in stabilizing the in vivo reduction state of the ubiquinone pool and the activation state of the alternative oxidase. *Plant Physiology*. **118**, 599–607.

Møller, I.M. (2001). Plant mitochondria and oxidative stress: Electron transport, NADPH turnover and metabolism of reactive oxygen species. *Annual Review Plant Physiology, Plant Molecular Biology*. **52**, 561–591.

Munro, K.D., Hodges, D.M., DeLong, J.M., Forney, C.F. and Kristie, D.N. (2004). Low temperature effects

on ubiquinone content, respiration rates and lipid peroxidation levels of etiolated seedlings of two differentially chilling-sensitive species. *Physiologia Plantarum*. **121**, 488–497.

Myking, T. (1998). Interrelations between respiration and dormancy in buds of three hardwood species with different chilling requirements for dormancy release. *Trees*. **12**, 224–229.

Pérez, F.J. and Lira, W. (2005). Possible role of catalase in post-dormancy bud break in grapevines. *Journal of Plant Physiology.* **162**, 301–308.

Pérez, F.J., Rubio, S. and Ormeño-Nuñez, J. (2007). Is erratic budbreak in grapevines grown in warm winters areas related to disturbances in mitochondrial respiratory capacity and oxidative metabolism? *Functional Plant Biology*. **34**, 624–632.

Pérez, F.J., Vergara, R. and Or, E. (2009). On the mechanism of dormancy release in grapevine buds: a comparative study between hydrogen cyanamide and sodium azide. *Plant Growth Regulation*. **59**, 145–152.

Petri, J.L. and Leite, G.B. (2004). Consequences of insufficient winter chilling on apple tree budbreak. *Acta Horticulturae*. **662**, 53–60.

Potjanapimon, C., Fujioka, N., Fukuda, F. and Kubota, N. (2008). Physiological aspects of bud associated with breaking dormancy in grapevines. *Scientific reports Fac. Agric. Okayama University*. **97**, 41–47.

Ribas-Carbo, M., Aroca, R., Gonzàlez-Meler, M.A., Irigoyen, J.J. and Sanchez-Diaz, M. (2000). The electron partitioning between the cytochrome and alternative respiratory pathways during chilling recovery in two cultivars of maize differing in chilling sensitivity. *Plant Physiology*. **122**, 199–204.

Richardson, E.A., Seeley, S.D. and Walker, D.R. (1974). A model for estimating the completion of rest for 'Red haven' and 'Elberta' peach trees. *HortScience.* **9**, 331–332.

Rohde, A. and Bhalerao, R.P. (2007). Plant dormancy in the perennial context. *Trends Plant Science.* **12**, 217–223.

Rubio, S., Donoso, A., Pérez F.J. (2014). The dormancy-breaking stimuli "chilling, hypoxia and cyanamide exposure" up-regulate the expression of α-amylase genes in grapevine buds. *Journal of Plant Physiology*. **171**, 373–381.

Ruiz, D., Campoy, J.A. and Egea, J. (2007). Chilling and heat requirements of apricot cultivars for flowering. *Environmental and Experimental Botany*. **61**, 254–263.

Sagisaka, S. (1972). Decrease of glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in xylem of *Populus gelrica* on budding. *Plant Physiology*. **50**, 750–755.

Sagisaka, S. (1974). Transition of metabolism in living poplar bark from growing to wintering stages and vice versa. *Plant Physiology*. **54**, 544–549.

Scalabrelli, G. and Couvillon, G.A. (1986). The effect of temperature and bud type on rest completion and the GDH requirements for bud break in "Red Haven" peach. *Journal of the American Society for Horticultural Science*. **111**, 537–540.

Swamy, P.M. and Sandhyarani, C.K. (1985). Contribution of the pentose phosphate pathway and glycolytic pathway to dormancy breakage and germination of peanut (*Arachis hypogaea* L.) seeds. *Journal of Experimental Botany.* **37**, 80–88.

Sweetlove, L.J., Beard, K.F.M., Nunes-Nesi, A., Fernie, A.R. and Ratcliffe, R.G. (2010). Not just a circle: flux modes in the plant TCA cycle. *Trends in Plant Science*. **15**, 462–470.

Taiz, L. and Zeiger, E. (2010). Plant Physiology. 5nd ed. Sinauer Associates Inc., Sunderland, M.A. pp: 782

Tan, Y., Gao, D. S., Li, L., Chen, X. D. and Xu, A. H. (2010). Respiratory response of dormant nectarine floral buds on chilling deficiency. *Agricultural Sciences in China*. **9**, 1448–1454.

Tan, Y., Li, L., Leng, C., Li, D., Chen, X. and Gao, D. (2013). Respiratory response of dormant nectarine vegetative buds to high temperature stress. *Journal of Integrative Agriculture*. **12**, 80–86.

Tharaga, P.C. (2014). Impacts of climate change on accumulated chill units at selected fruit production sites in South Africa. MSc Thesis, Bloemfontein University.

Toro, G. and Pinto, M. (2015). Plant respiration under low oxygen. *Chilean Journal of Agricultural Research*. 75, 57–70.

Trejo-Martínez, M.A., Orozco, A., Almaguer-Vargas, G., Carvajal-Millán, E. and Gardea, A.A. (2009). Metabolic activity of low chilling grapevine buds forced to budbreak. *Thermochimica Acta*. **481**, 28–31.

Van der Schoot, C. and Rinne, P.L.H. (2011). Dormancy cycling at the shoot apical meristem: transitioning between self-organization and self-arrest. *Plant Science*. **180**, 120–131.

Vanlerberghe, G.C. and Mcintosh, L. (1992). Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco. *Plant Physiology*. **100**, 115–119.

Vanlerberghe, G.C. (2013). Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *International Journal of Molecular Sciences*. **14**, 6805–6847.

Vassileva, V., Simova-Stoilova, L., Demirevska, K. and Feller, U. (2009). Variety-specific response of wheat (*Triticum aestivum*, L.) leaf mitochondria to drought stress. *Journal of Plant Research*. **122**, 445–454.

Vergara, R. and Pérez, F.J. (2010). Similarities between natural and chemically induced bud-endodormancy release in grapevine (*Vitis vinifera* L.). *Scientia Horticulturae*. **125**, 648–653.

Wang, S.Y., Jiao, H.J. and Faust, M. (1991). Changes in metabolic enzyme activities during thidiazuron-induced lateral budbreak of apple. *HortScience*. **26**, 171–173.

Young, E. (1990). Changes in respiration rate and energy of activation after chilling and forcing dormant apple trees. *Journal of the American Society for Horticultural Science*. **115**, 809–814.

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TABLES

Table 1. Summary of the calculation of the respiratory parameters (similar to Tan *et al.*, 2010 and 2013)

Abbreviation	Parameter	Measurement
R	Total O_2 uptake of buds	O_2 uptake in water/buffer
$Res_{tca\&ppp}$	Residual respiratory rate	O_2 uptake in the presence of MA and Na_3PO_4
R_{tca}	Rate of TCA	R minus respiratory rate in the presence of MA
R_{ppp}	Rate of PPP	R minus $Res_{tca\&ppp}$ and R_{tca}
Con_{tca}	% Contribution of TCA	Dividing R_{tca} with R x100
Con_{ppp}	% Contribution of PPP	Dividing R_{ppp} with R x100
$Res_{cyt\&alt}$	Residual respiratory rate	O_2 uptake in the presence of KCN and SHAM
R_{cyt}	Rate of CYT	R minus respiratory rate in the presence of KCN
R_{alt}	Rate of ALT	R minus $Res_{cyt\&alt}$ and R_{cyt}
Con_{cyt}	% Contribution of CYT	Dividing R_{cyt} with R x100
Con_{alt}	% Contribution of ALT	Dividing R_{alt} with R x100

FIGURES

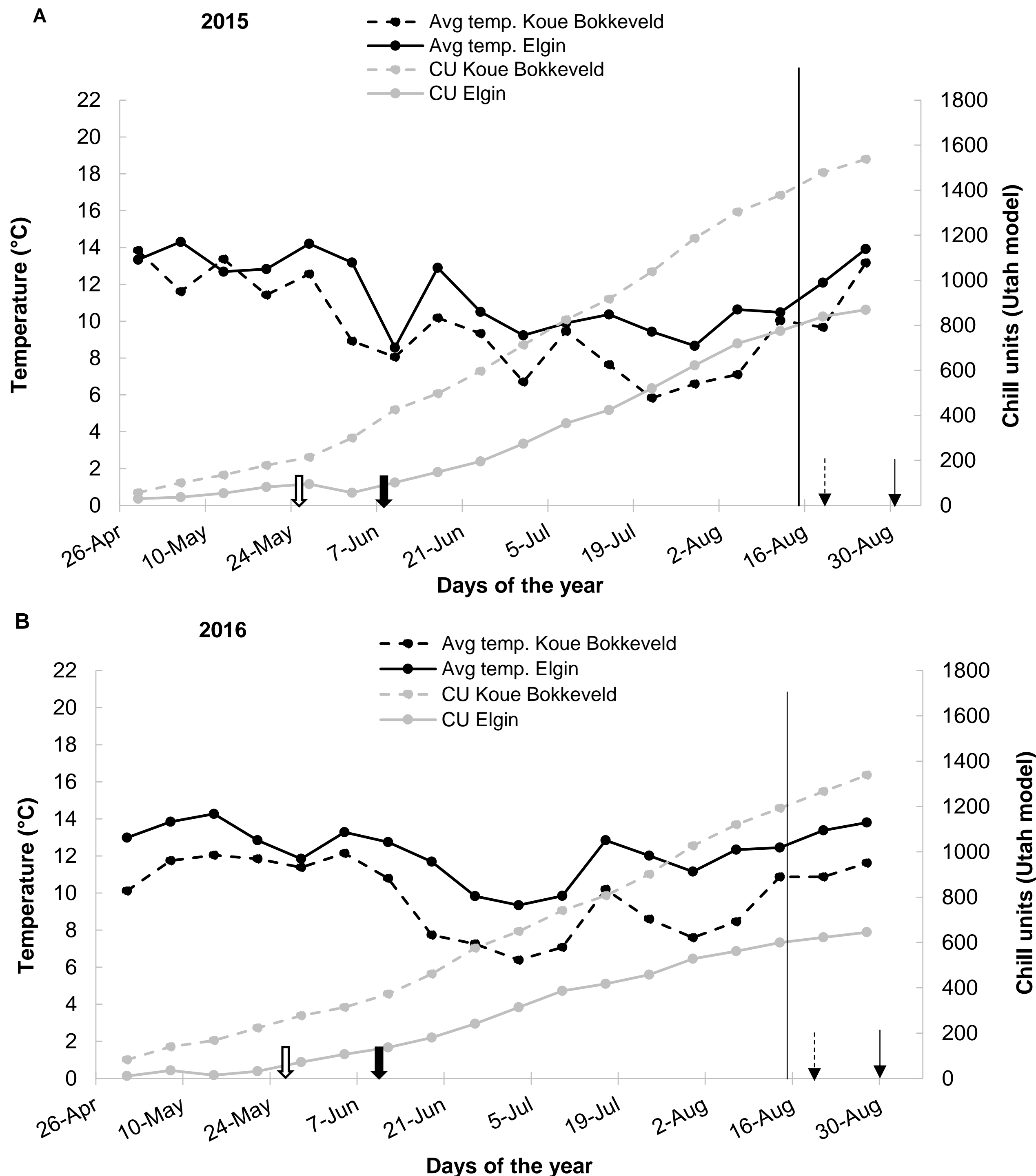


Fig. 1: Chill units (CU) (grey) and average daily temperature (black) for 2015 (A) and 2016 (B) in the Koue Bokkeveld (_ _ _) and Elgin (____). Block arrows indicate an estimative period of leaf drop in the Koue Bokkeveld (white) and in Elgin (black). Line arrows show budswell in the Koue Bokkeveld (dotted) and Elgin (solid). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.

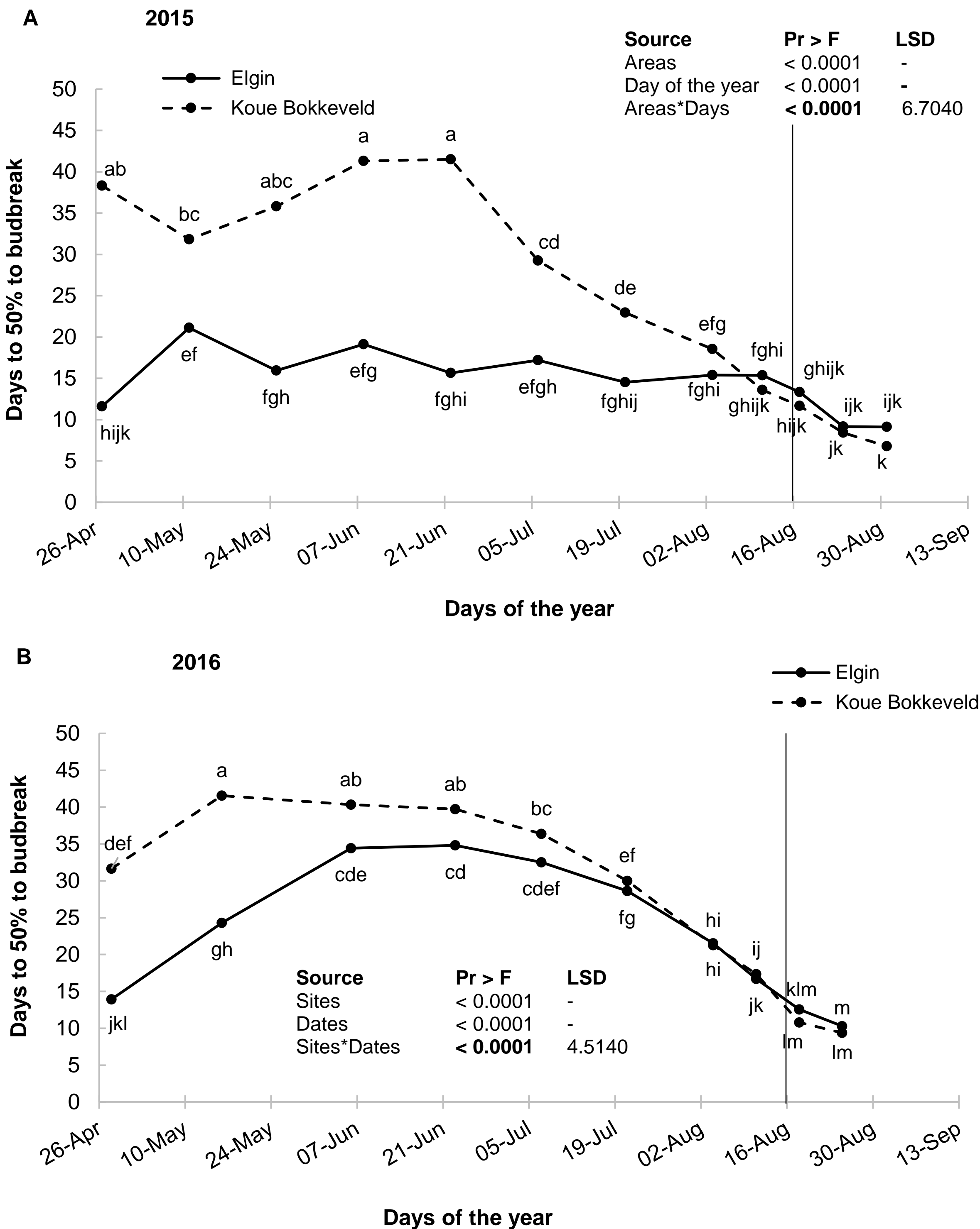


Fig. 2: Dormancy progression curves of 'Cripps Pink' buds sampled in the Koue Bokkeveld (_ _ _) and Elgin (____) during 2015 (A) and 2016 (B). Letters indicate significant differences at p=0.05. The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.

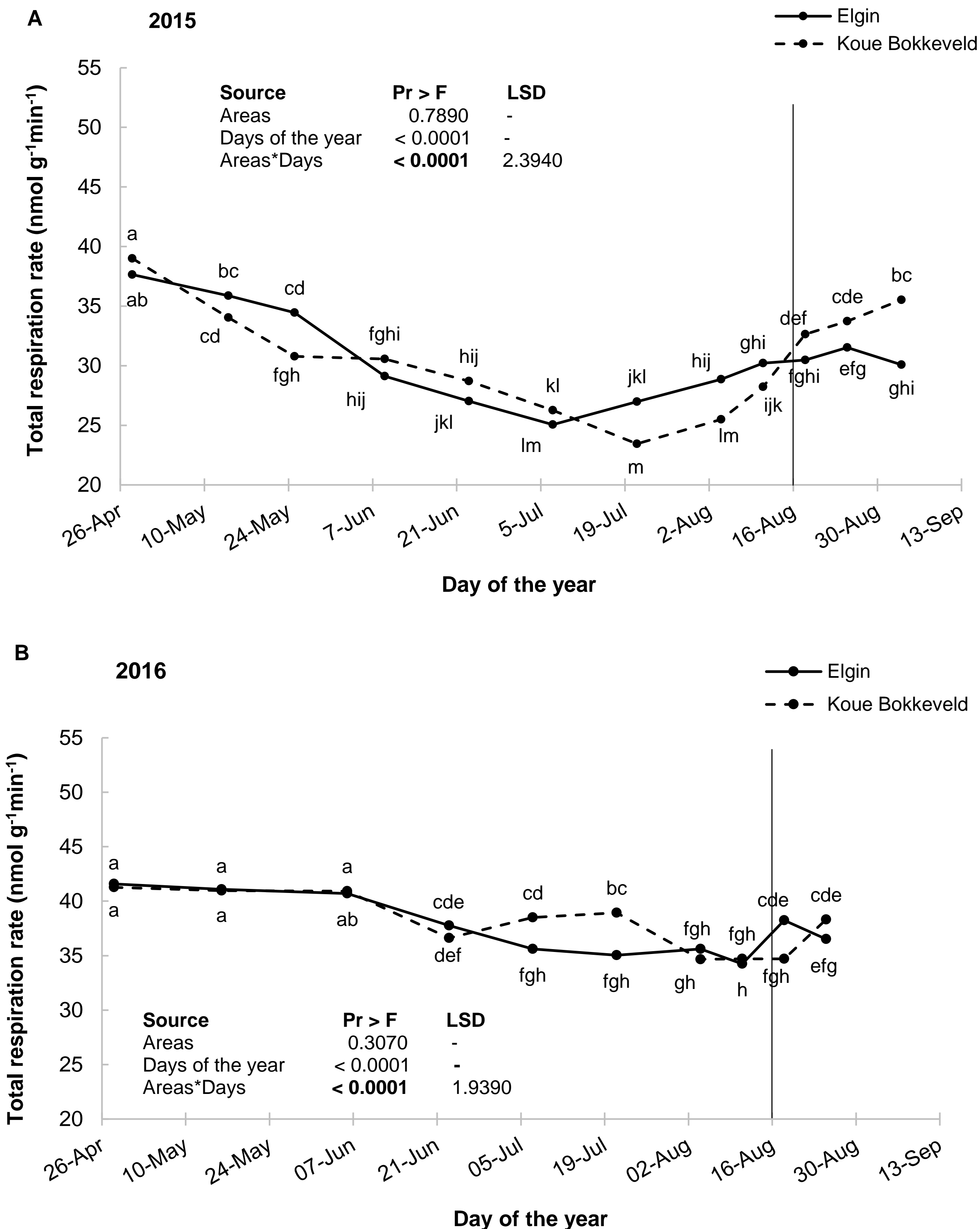
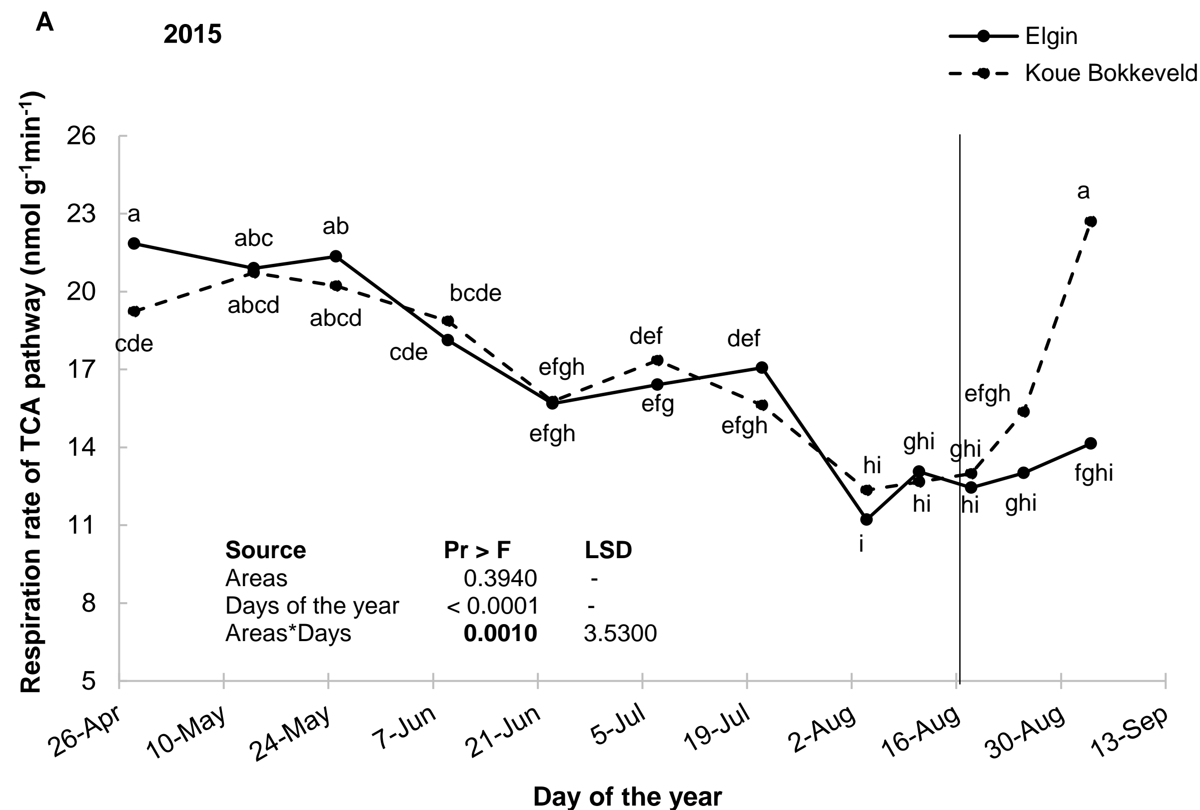
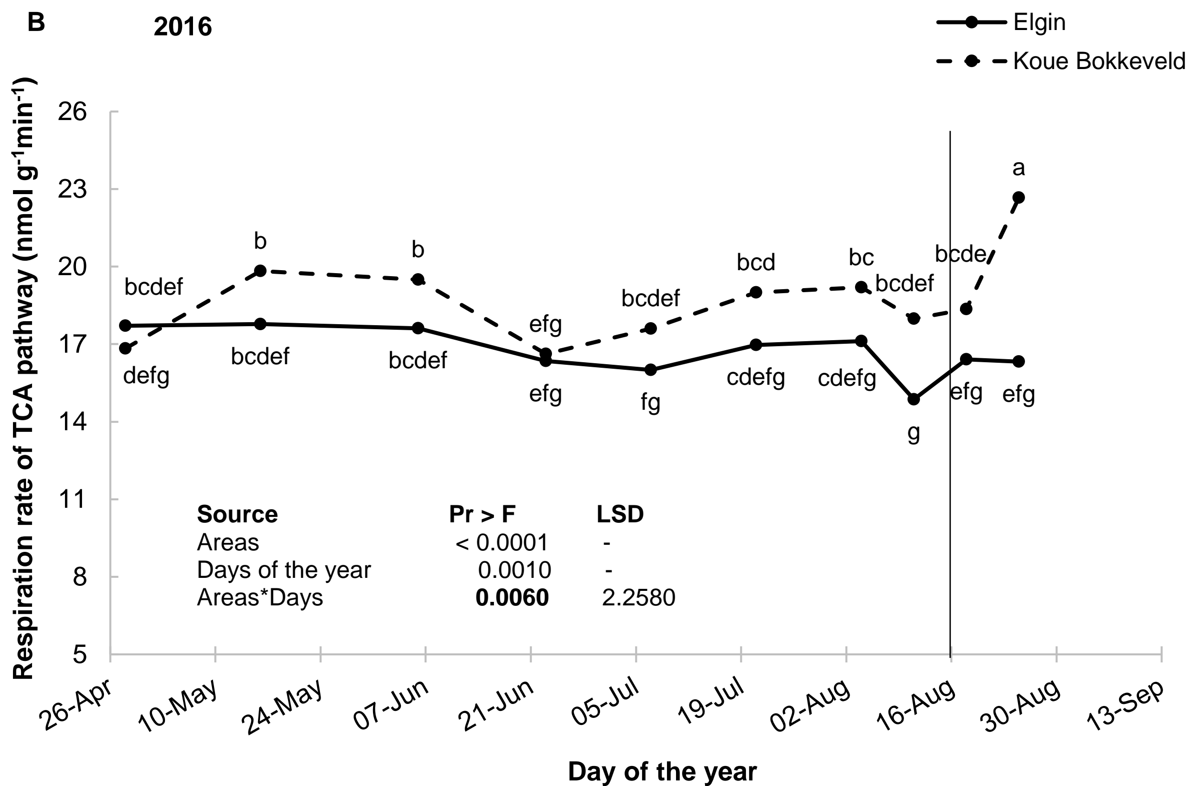


Fig. 3: Total respiration rate of 'Cripps Pink' apple buds from the Koue Bokkeveld (_ _ _) and Elgin (_____) for the 2015 (A) and 2016 (B) winter seasons. Letters indicate significant differences between the means (p=0.05). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.


A
2015
Elgin
Koue Bokkeveld
Respiration rate of TCA pathway (nmol g-1min-1)
26
23
20
17
14
11
8
5
a
abc
ab
cde
abcd
abcd
bcde
cde
efgh
efgh
def
efg
def
efgh
hi
i
ghi
hi
ghi
hi
efgh
ghi
a
fghi
Source Pr > F LSD
Areas 0.3940 -
Days of the year < 0.0001 -
Areas*Days 0.0010 3.5300
26-Apr
10-May
24-May
7-Jun
21-Jun
5-Jul
19-Jul
2-Aug
16-Aug
30-Aug
13-Sep
Day of the year


B
2016
Elgin
Koue Bokkeveld
Respiration rate of TCA pathway (nmol g-1min-1)
26
23
20
17
14
11
8
5
bcdef
defg
b
bcdef
b
bcdef
efg
efg
bcdef
fg
bcd
cdefg
bc
cdefg
bcdef
g
bcde
efg
a
efg
Source Pr > F LSD
Areas < 0.0001 -
Days of the year 0.0010 -
Areas*Days 0.0060 2.2580
26-Apr
10-May
24-May
07-Jun
21-Jun
05-Jul
19-Jul
02-Aug
16-Aug
30-Aug
13-Sep
Day of the year

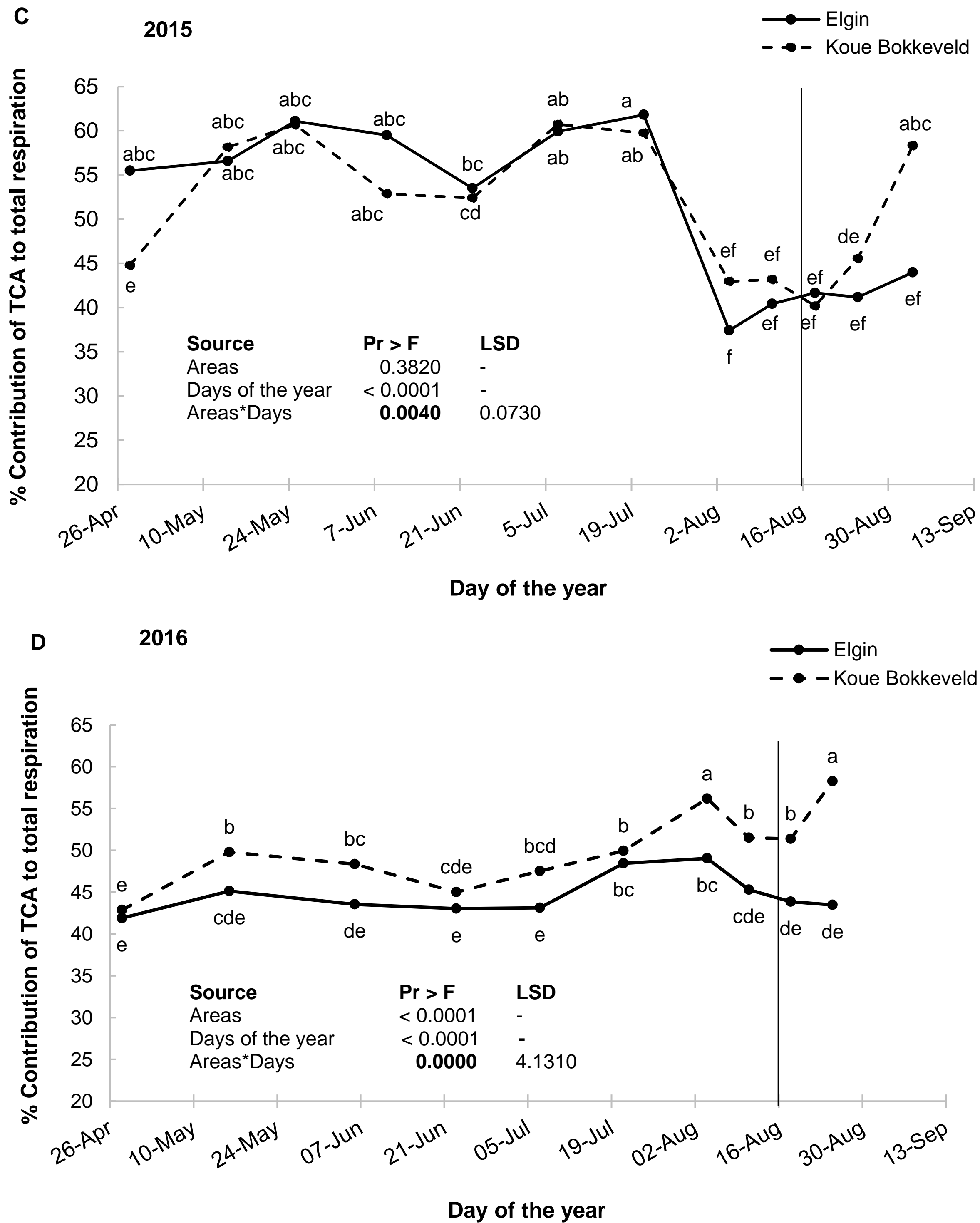
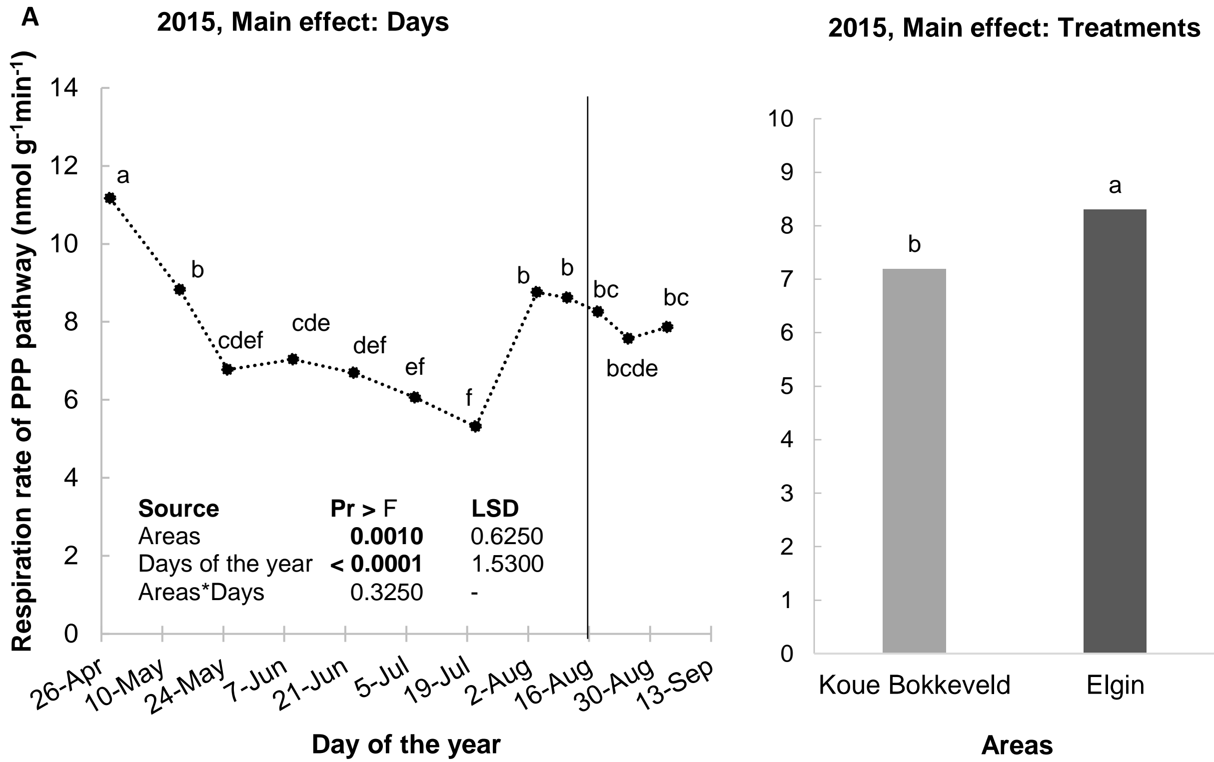
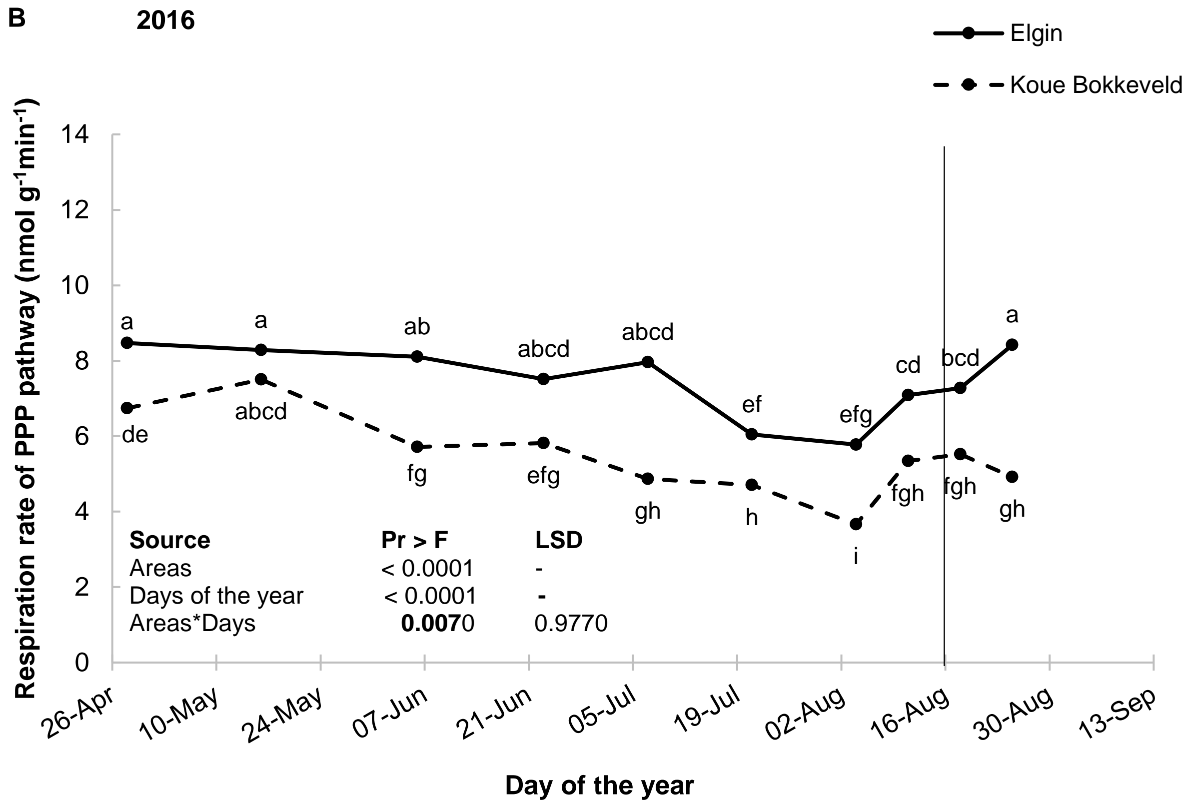


Fig. 4: TCA pathway rate (A and B) and % contribution to the total respiration (C and D) of 'Cripps Pink' apple buds sampled in the Koue Bokkeveld (_ _ _) and Elgin (____) during the 2015 (A and C) and 2016 (B and D) winter seasons. Letters indicate significant differences between the means (p=0.05). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.


A
2015, Main effect: Days
Respiration rate of PPP pathway (nmol g-1min-1)
a
b
cdef
cde
def
ef
f
b
b
bc
bcde
bc
Source Pr > F LSD
Areas 0.0010 0.6250
Days of the year < 0.0001 1.5300
Areas*Days 0.3250 -
26-Apr
10-May
24-May
7-Jun
21-Jun
5-Jul
19-Jul
2-Aug
16-Aug
30-Aug
13-Sep
Day of the year
2015, Main effect: Treatments
b
a
Koue Bokkeveld
Elgin
Areas


B
2016
Elgin
Koue Bokkeveld
Respiration rate of PPP pathway (nmol g-1min-1)
a
a
ab
abcd
abcd
ef
efg
cd
bcd
a
de
abcd
fg
efg
gh
h
i
fgh
fgh
gh
Source Pr > F LSD
Areas < 0.0001 -
Days of the year < 0.0001 -
Areas*Days 0.0070 0.9770
26-Apr
10-May
24-May
07-Jun
21-Jun
05-Jul
19-Jul
02-Aug
16-Aug
30-Aug
13-Sep
Day of the year

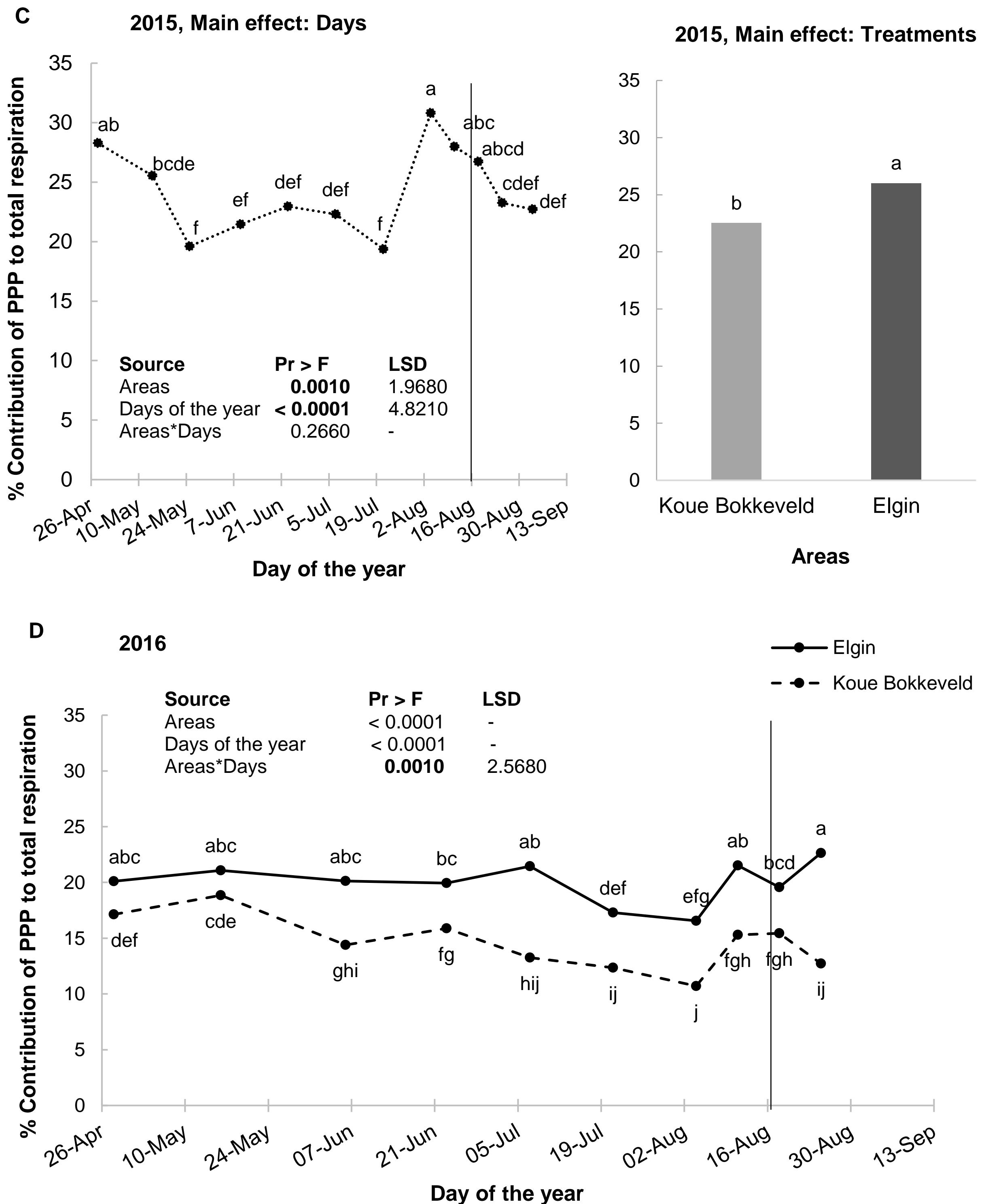
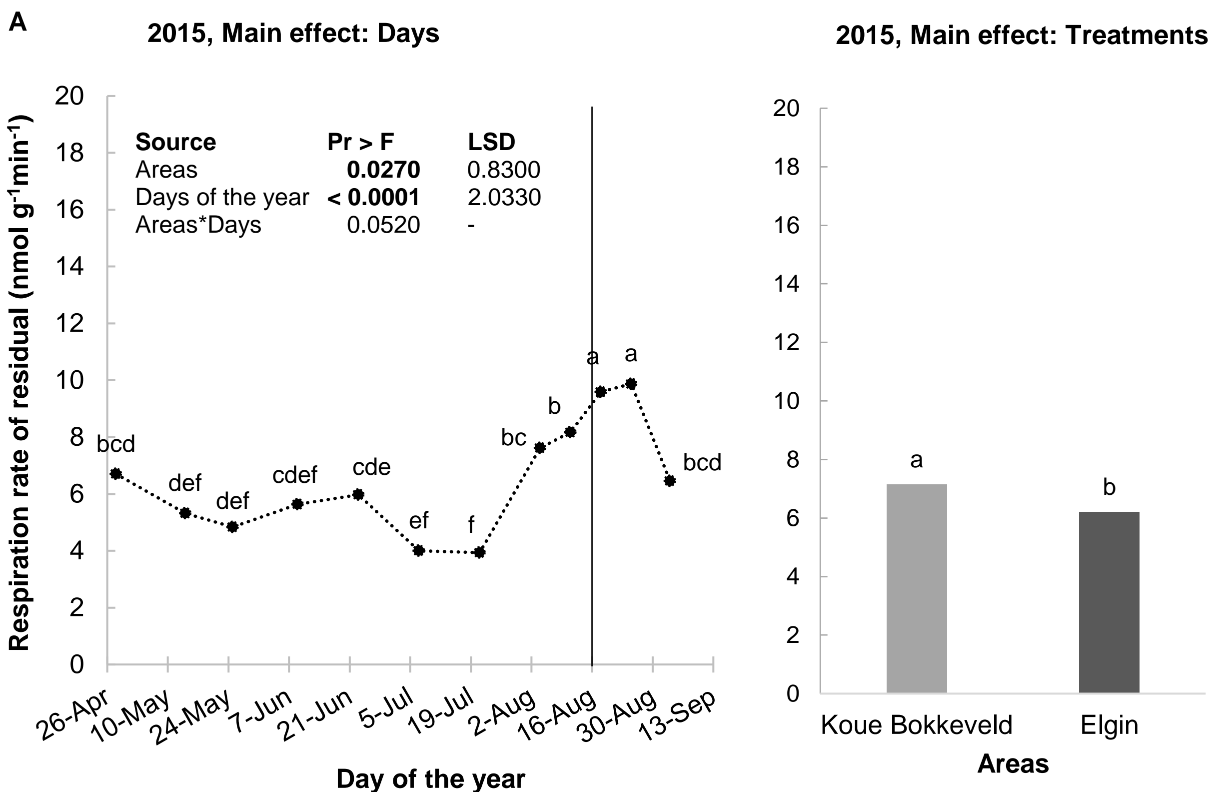
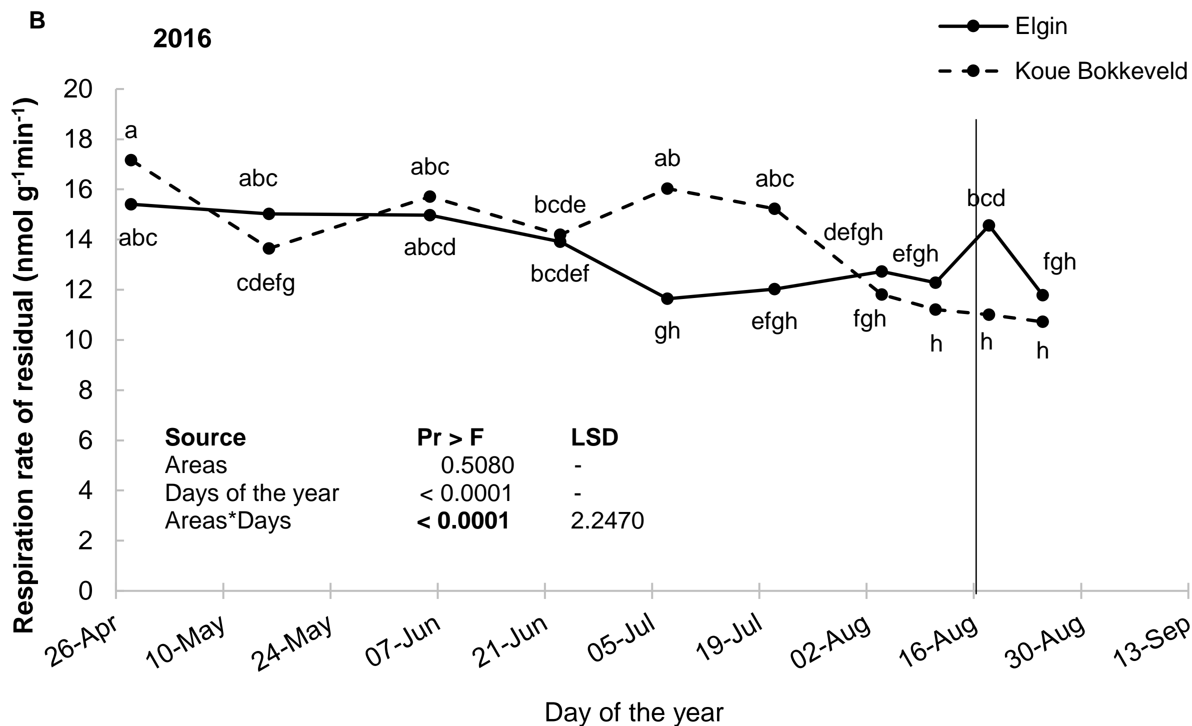
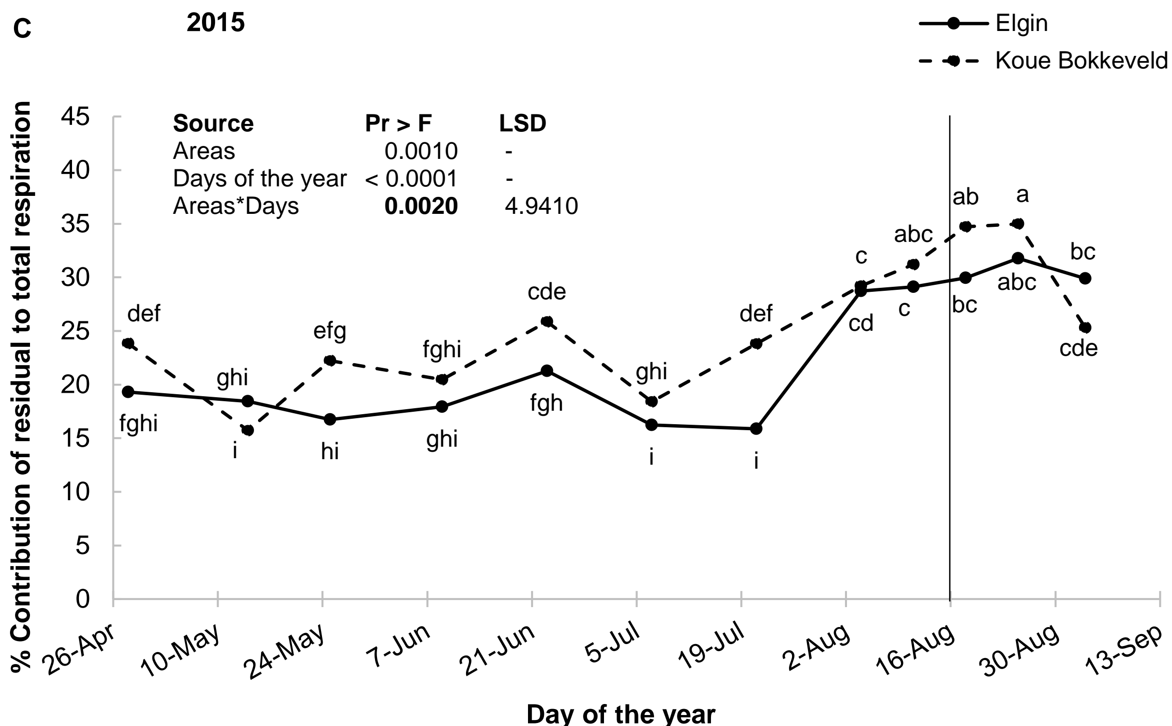


Fig. 5: PPP pathway rate (A and B) and PPP contribution to the total respiration (C and D) of 'Cripps Pink' apple buds from Koue Bokkeveld (_ _ _) and Elgin (____) during the 2015 (A and C) and 2016 (B and D) winter seasons. Letters indicate significant differences between the means (p=0.05). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.


A
2015, Main effect: Days
Source Pr > F LSD
Areas 0.0270 0.8300
Days of the year < 0.0001 2.0330
Areas*Days 0.0520 -
Respiration rate of residual (nmol g-1min-1)
Day of the year
bcd
def
def
cdef
cde
ef
f
bc
b
a
a
bcd
26-Apr
10-May
24-May
7-Jun
21-Jun
5-Jul
19-Jul
2-Aug
16-Aug
30-Aug
13-Sep
2015, Main effect: Treatments
a
b
Koue Bokkeveld
Elgin
Areas


B
2016
Elgin
Koue Bokkeveld
Respiration rate of residual (nmol g-1min-1)
Source Pr > F LSD
Areas 0.5080 -
Days of the year < 0.0001 -
Areas*Days < 0.0001 2.2470
a
abc
abc
cdefg
abc
abcd
bcde
bcdef
ab
gh
abc
efgh
defgh
fgh
efgh
h
bcd
h
fgh
h
26-Apr
10-May
24-May
07-Jun
21-Jun
05-Jul
19-Jul
02-Aug
16-Aug
30-Aug
13-Sep
Day of the year



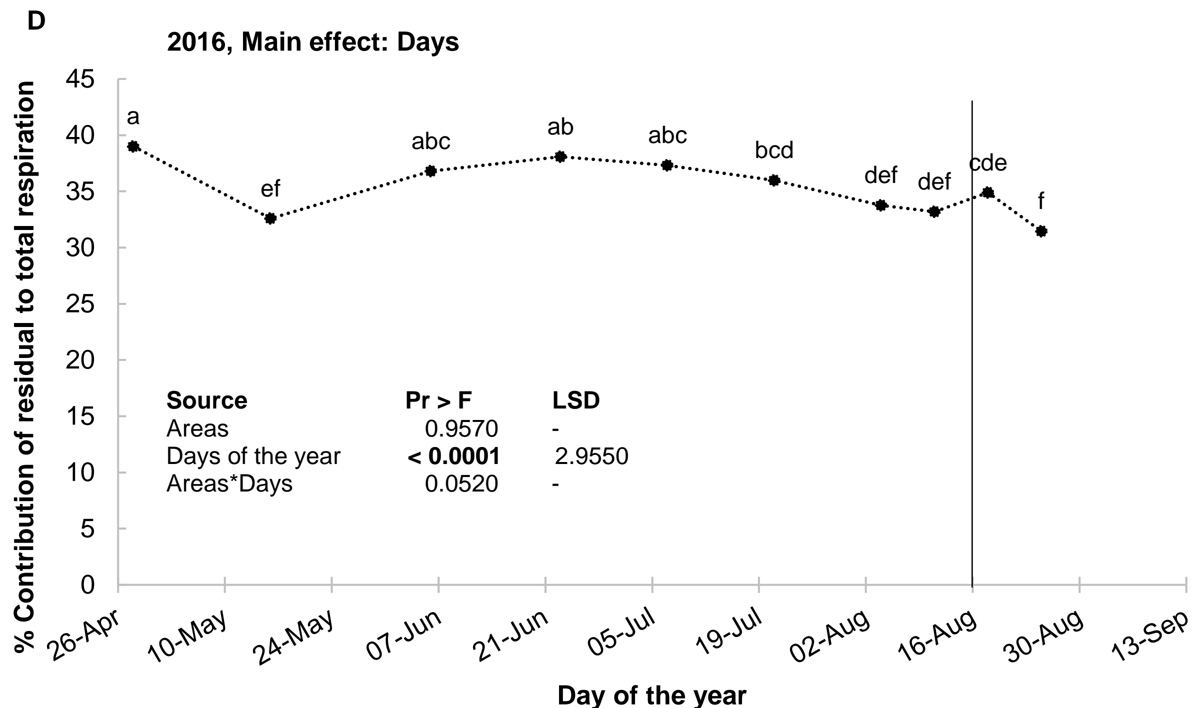
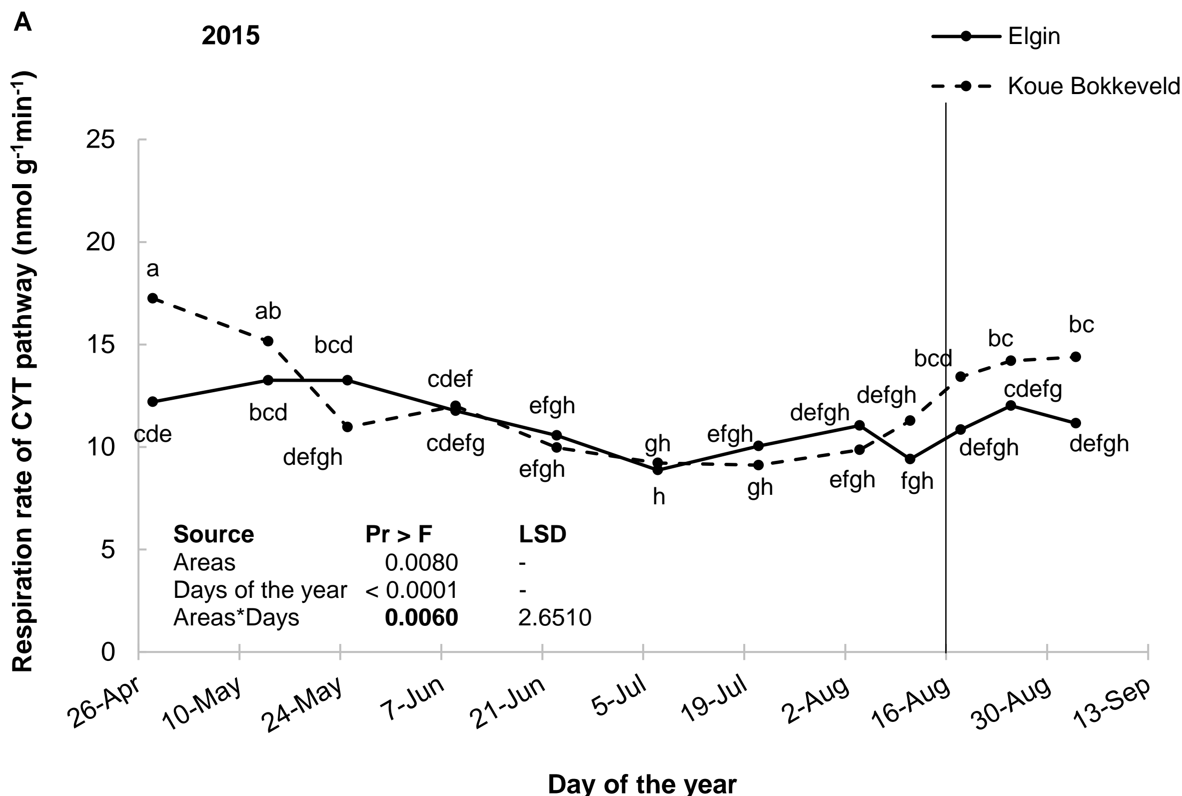
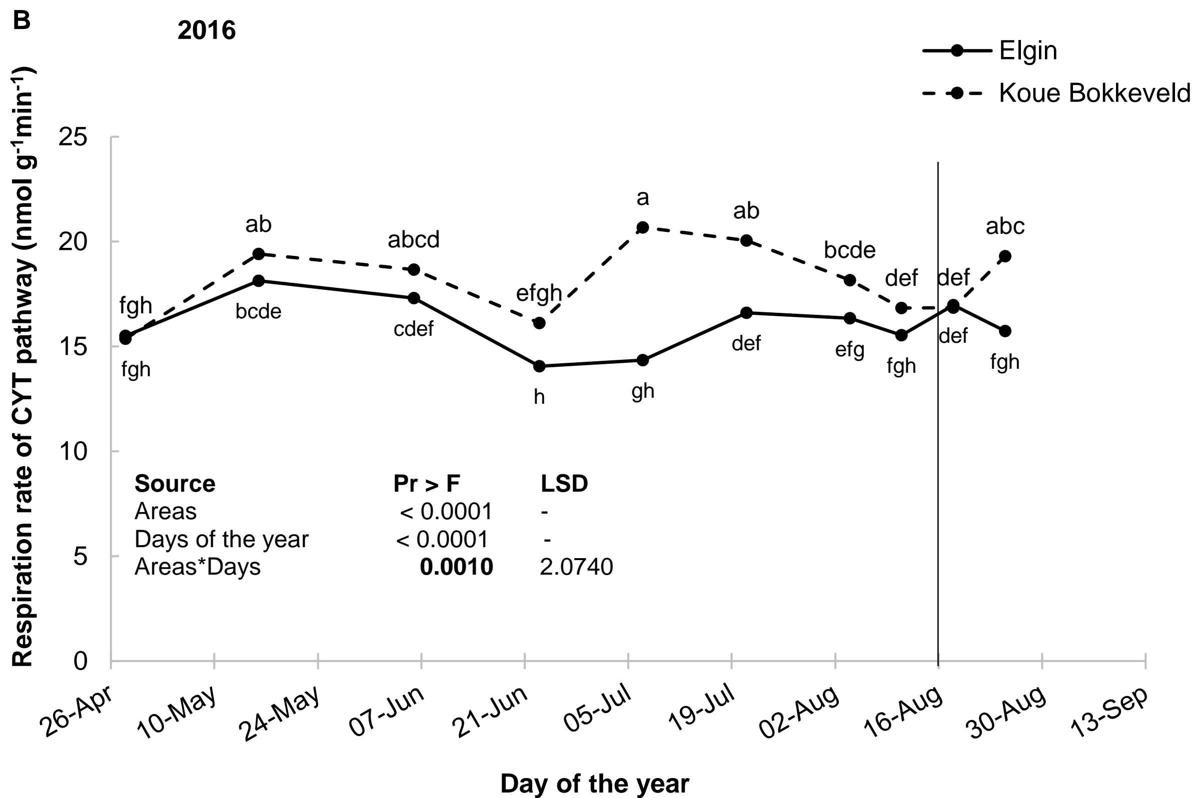
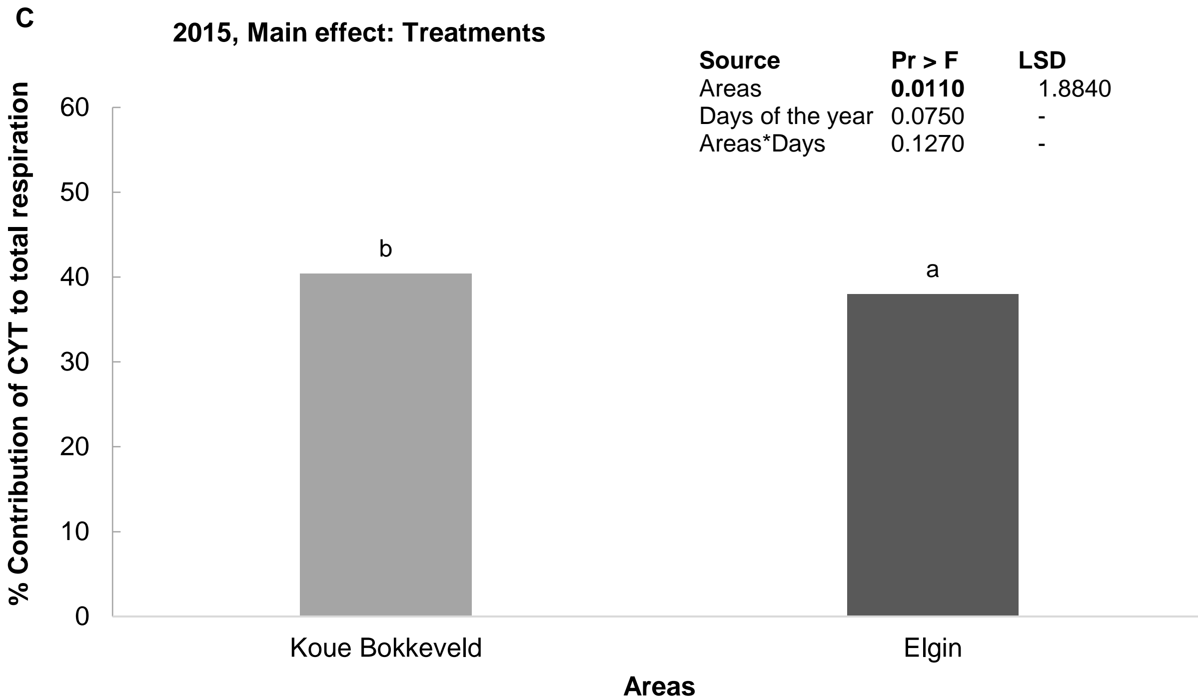


Fig. 6: Residual respiration rate (A and B) and residual contribution to the total respiration (C and D) of 'Cripps Pink' apple buds from Koue Bokkeveld (_ _ _) and Elgin (____) during the 2015 (A and C) and 2016 (B and D). Letters indicate statistical differences between the means (p=0.05). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.


A
2015
Elgin
Koue Bokkeveld
Respiration rate of CYT pathway (nmol g-1min-1)
Day of the year
Source Pr > F LSD
Areas 0.0080 -
Days of the year < 0.0001 -
Areas*Days 0.0060 2.6510


B
2016
Elgin
Koue Bokkeveld
Respiration rate of CYT pathway (nmol g-1min-1)
Day of the year
Source Pr > F LSD
Areas < 0.0001 -
Days of the year < 0.0001 -
Areas*Days 0.0010 2.0740



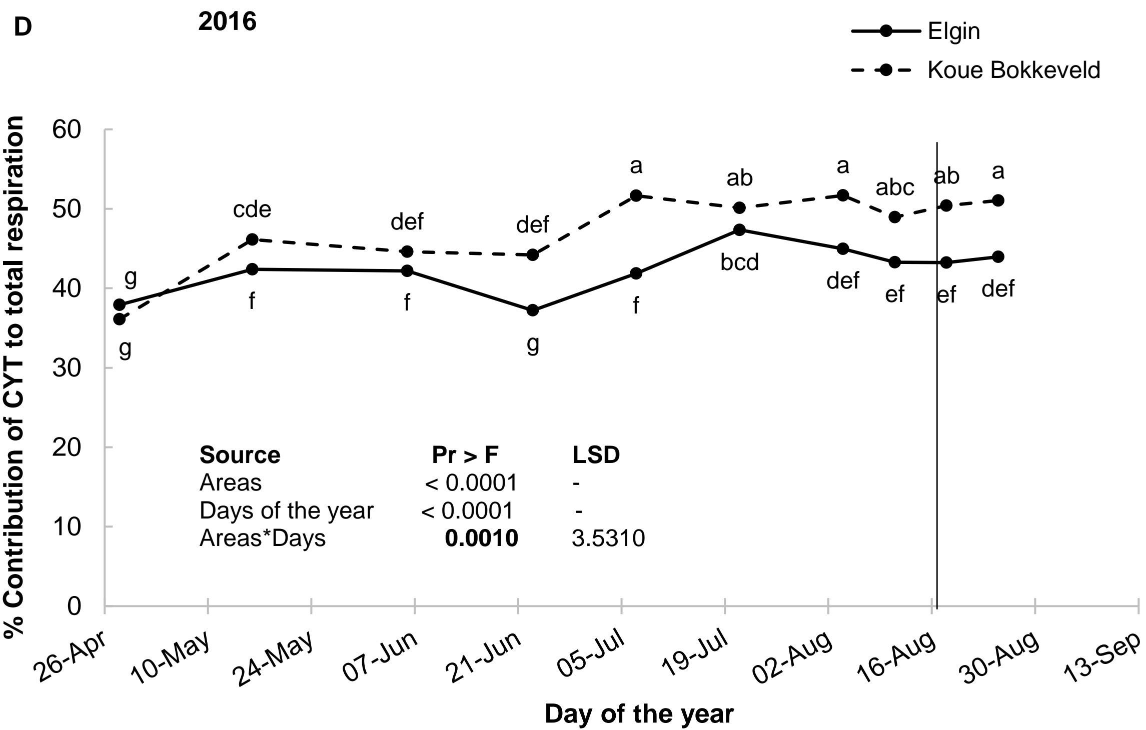
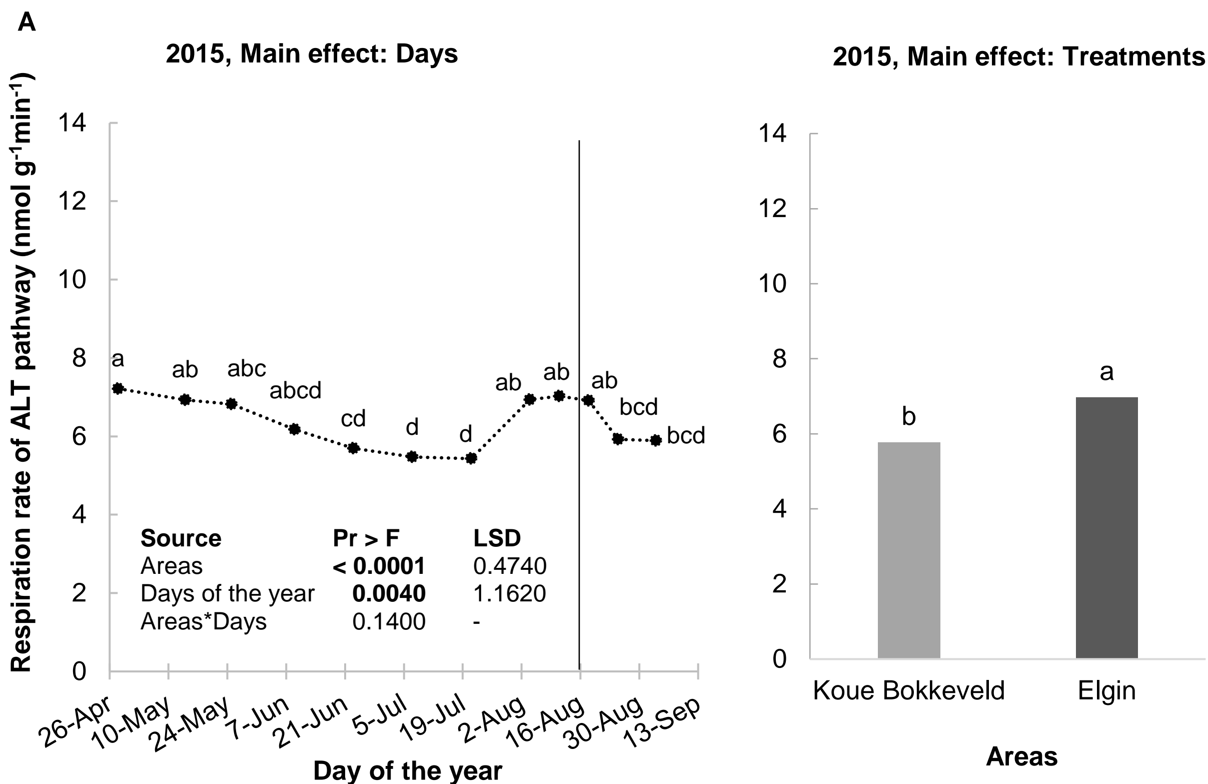
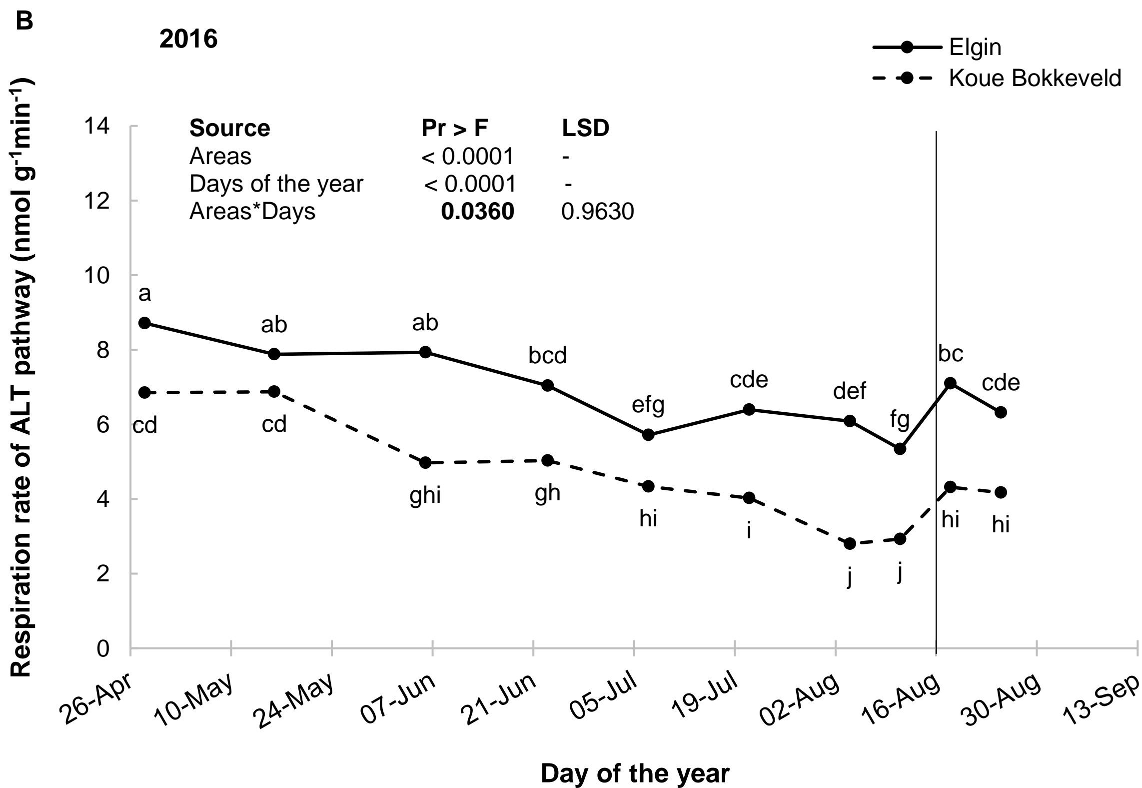


Fig. 7: CYT respiration pathway rate (A and B) and CYT contribution to total respiration (C and D) of 'Cripps Pink' apple buds from Koue Bokkeveld (_ _ _) and Elgin (____) during the 2015 (A and C) and 2016 (B and D). Letters indicate significant differences between the means (p=0.05). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.


A
2015, Main effect: Days
2015, Main effect: Treatments
Respiration rate of ALT pathway (nmol g-1min-1)
a
ab
abc
abcd
cd
d
d
ab
ab
ab
bcd
bcd
Source Pr > F LSD
Areas < 0.0001 0.4740
Days of the year 0.0040 1.1620
Areas*Days 0.1400 -
26-Apr
10-May
24-May
7-Jun
21-Jun
5-Jul
19-Jul
2-Aug
16-Aug
30-Aug
13-Sep
Day of the year
b
a
Koue Bokkeveld
Elgin
Areas


B
2016
Elgin
Koue Bokkeveld
Respiration rate of ALT pathway (nmol g-1min-1)
Source Pr > F LSD
Areas < 0.0001 -
Days of the year < 0.0001 -
Areas*Days 0.0360 0.9630
a
ab
ab
bcd
efg
cde
def
fg
bc
cde
cd
cd
ghi
gh
hi
i
j
j
hi
hi
26-Apr
10-May
24-May
07-Jun
21-Jun
05-Jul
19-Jul
02-Aug
16-Aug
30-Aug
13-Sep
Day of the year

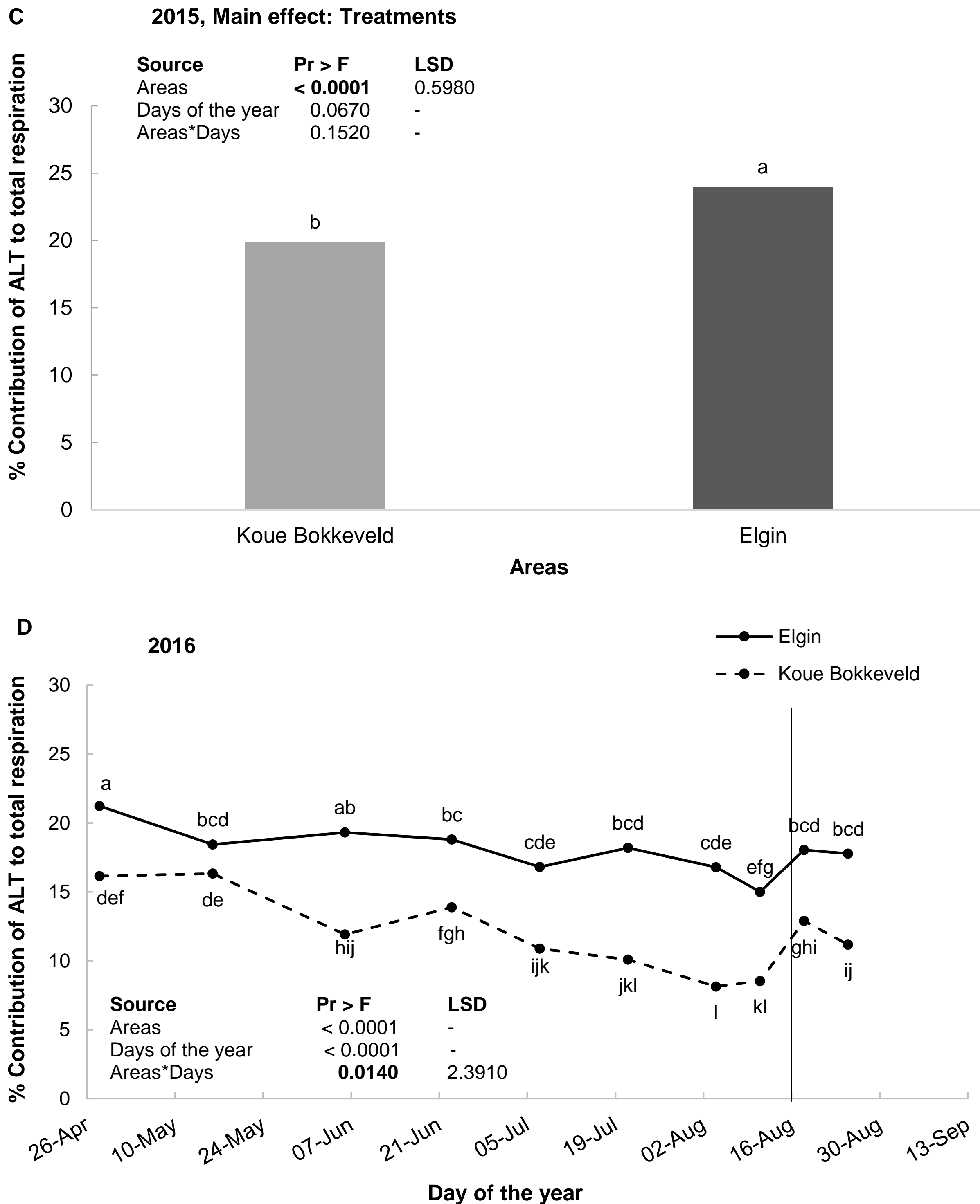


Fig. 8: ALT respiration pathway rate (A and B) and ALT contribution to total respiration (C and D) of 'Cripps Pink' apple buds from Koue Bokkeveld (_ _ _) and Elgin (____) during the 2015 (A and C) and 2016 (B and D). Letters indicate statistical differences between the means (p=0.05). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.

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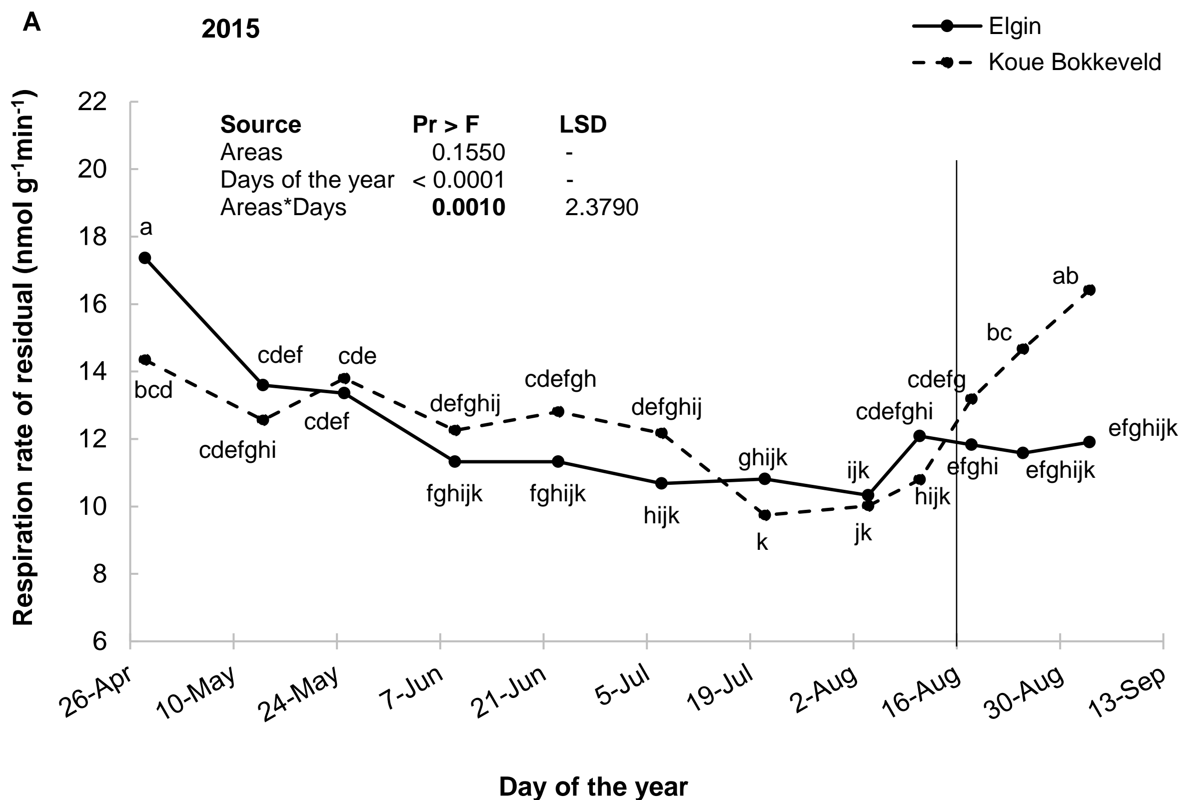

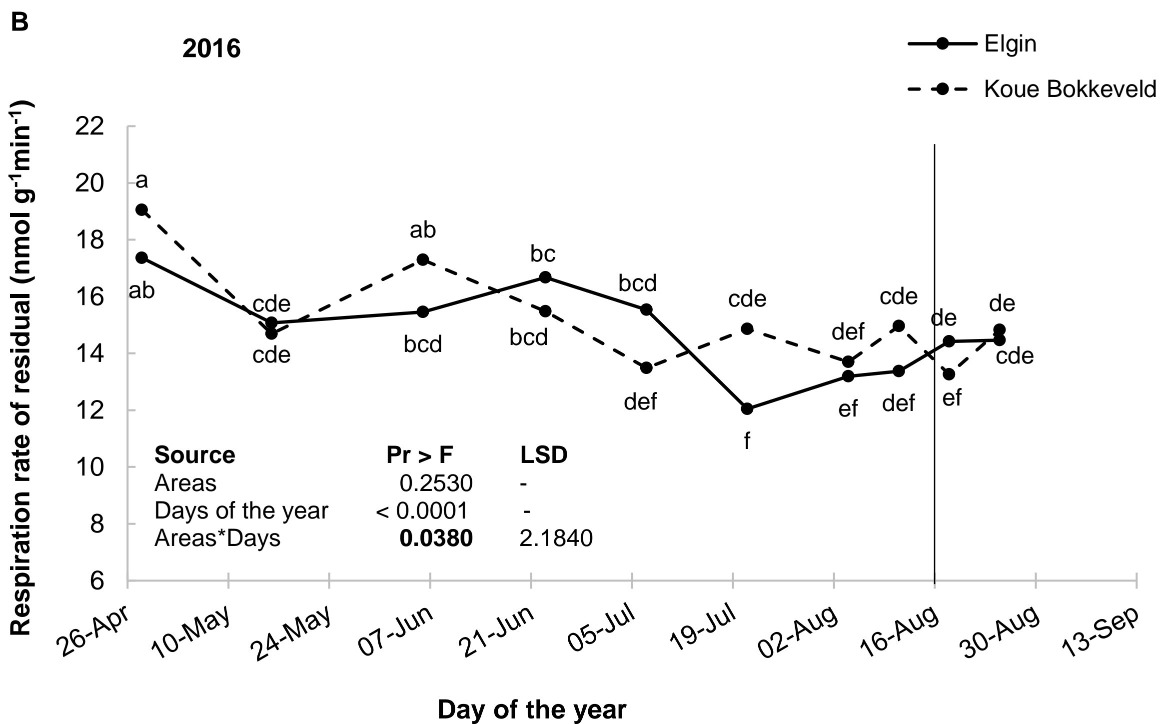

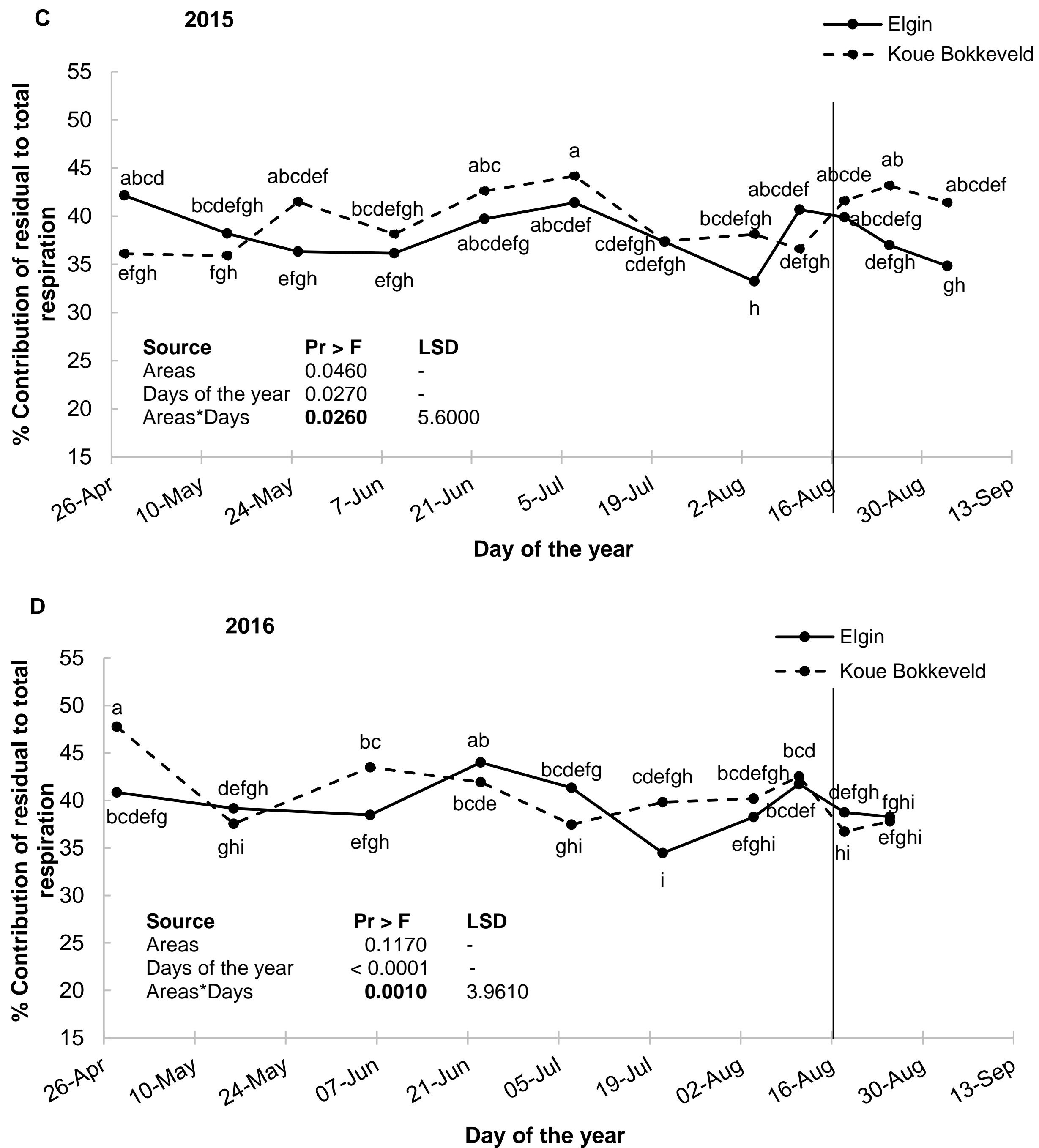

Fig. 9: Residual respiration rate (A and B) and residual contribution to total respiration (C and D) of 'Cripps Pink' apple buds sampled in the Koue Bokkeveld (_ _ _) and Elgin (____) during the 2015 (A and C) and 2016 (B and D). Letters indicate significant differences between the means (p=0.05). The vertical line is an indication of the end of the dormant phase and the start of the growth resumption phase based on temperature changes and dormancy levels.

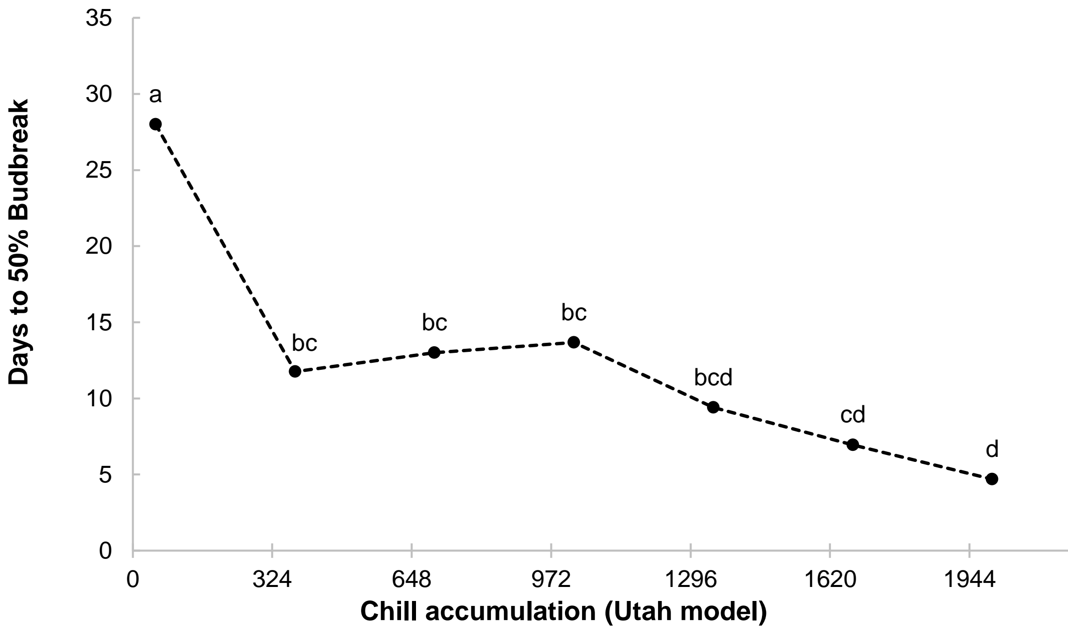

Fig. 10: Dormancy levels of 'Cripps Pink' buds that received progressively more chill units (Utah model). Letters indicate significant differences between the means (p=0.05).

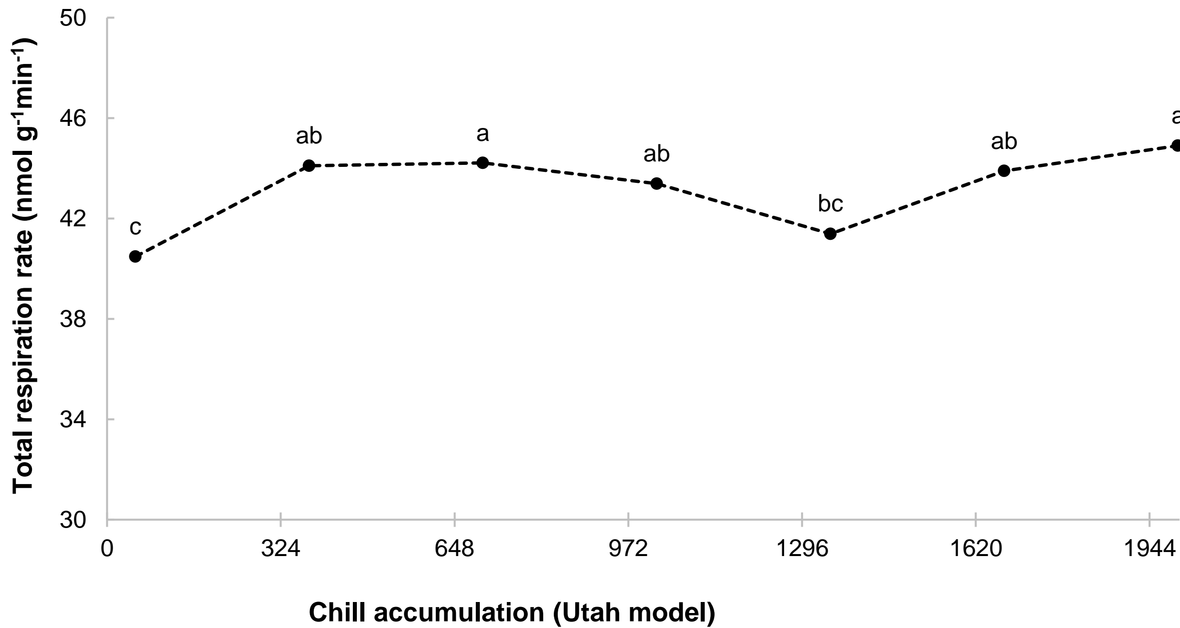

Fig. 11: Total respiration of 'Cripps Pink' dormant buds that received progressively more chill units (Utah model). Letters indicate statistical differences between the means of buds (p=0.05).

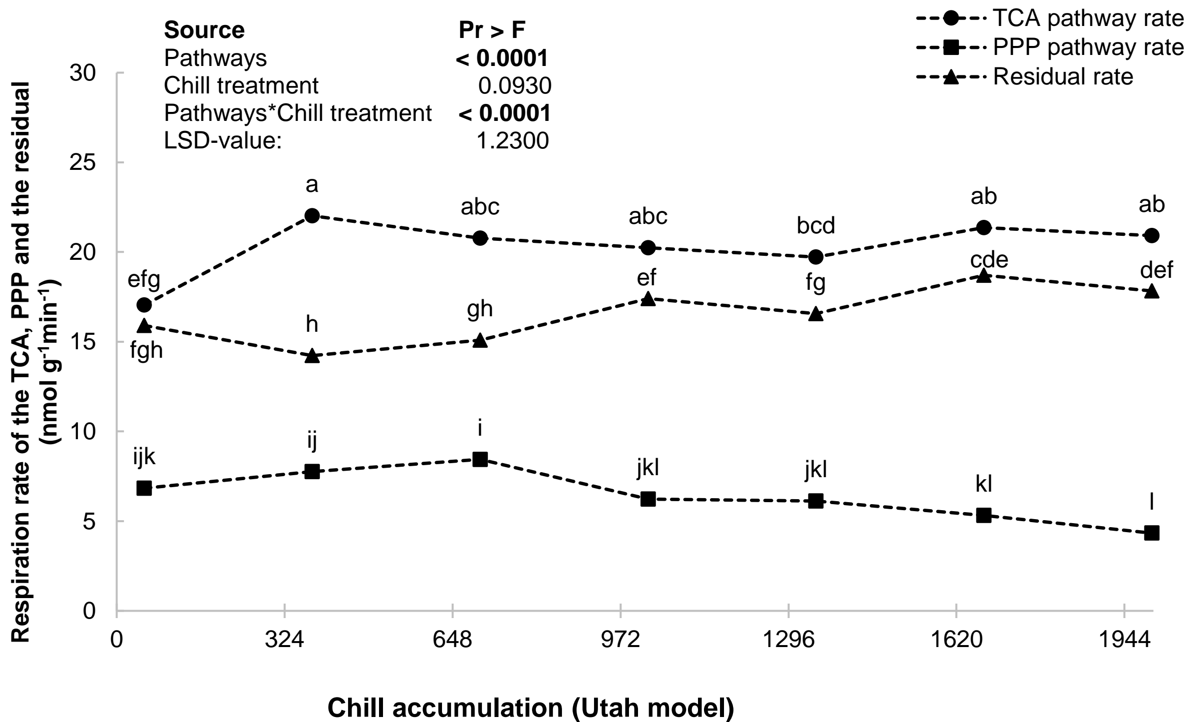

Fig. 12: Respiration of the TCA (●) and the PPP (■) pathways and the residual rate (▲) of 'Cripps Pink' buds that received progressively more chill units. Letters indicate significant differences between the means (p=0.05).

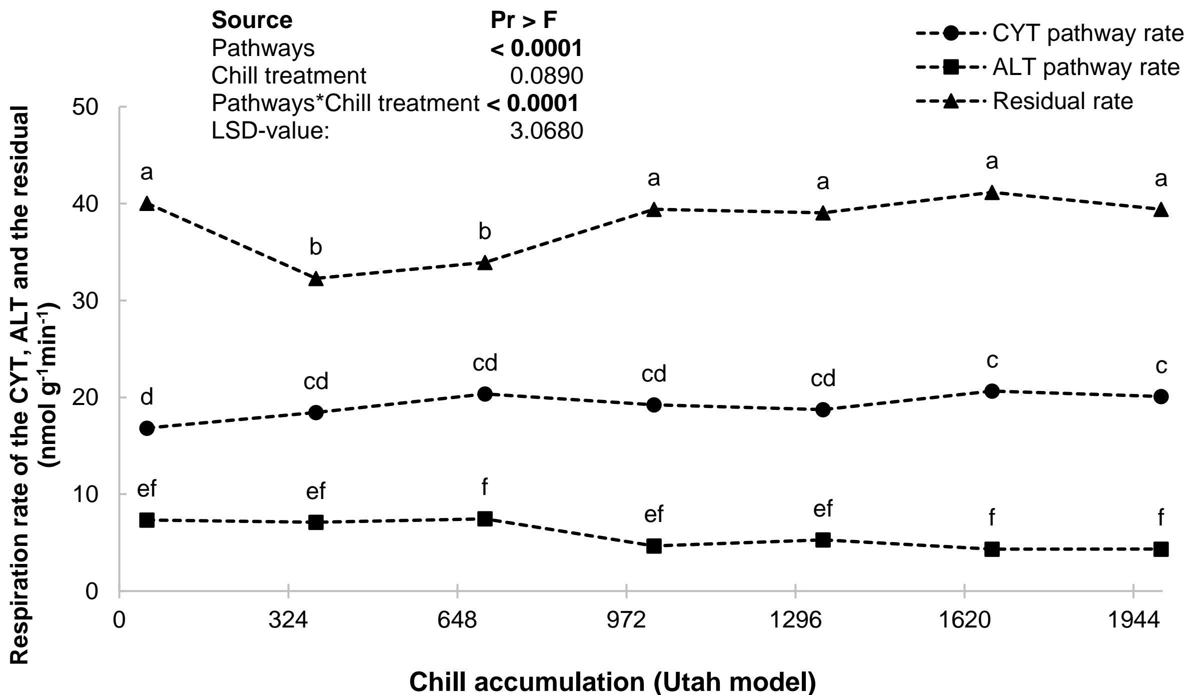

Fig. 13: Respiration of the CYT (●) and the ALT (■) pathways and the residual rate (▲) of 'Cripps Pink' buds that received progressively more chill units. Letters indicate significant differences between the means (p=0.05).

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PAPER 2

The effect of hydrogen cyanamide and oil on the respiration of 'Cripps Pink' terminal buds.

Abstract

Apple trees that do not receive adequate winter chill undergo inadequate release of endodormancy resulting in decreased budbreak, as well as uneven and delayed flowering that impact negatively on the crop. To overcome this, chemical rest breaking agents are used to artificially release the buds from endodormancy and synchronise budbreak in spring. This study investigated the effects of hydrogen cyanamide (Dormex™) and mineral oil, HCo, on the total respiration rate and four respiratory pathways (tricarboxylic acid cycle (TCA), pentose phosphate pathway (PPP), alternative pathway (ALT) and cytochrome C (CYT) pathway). The results may assist in the development of artificial ways to tagert these pathways to improve apple production in the areas with insufficient winter conditions. HCo treated terminal buds of mature 'Cripps Pink' trees were randomly collected from a commercial orchard in Elgin (warm winter area; ±700 CU (chill units) (Utah model)). The total respiration rate was determined by using a Clark-type oxygen electrode fitted to a liquid-filled chamber containing the buds. The respiration rate of the different pathways was measured using chemical inhibitors targeting the specific pathways. The results showed that the HCo treatment immediately decreased the respiration, followed by a steady increase and TCA and CYT pathways reaching a maximum at green-tip stage. The untreated buds showed a lower respiration rate with an increased use of the PPP and ALT pathways. Hydrogen cyanamide and oil seems to induce short term anoxia that changes the respiration landscape within the buds leading to an increase in TCA and CYT levels providing energy for early growth and development.

Keywords: *endodormancy, apple buds, respiration pathways, rest breaking agents*

1. Introduction

Deciduous fruit trees undergo an annual dormant state to survive unfavourable winter conditions and synchronise budbreak in spring. This growth inhibition starts at budset as paradormancy (correlative inhibition) and gradually shifts into endodormancy (inhibition within the bud itself). Endodormancy is released through sufficient chill accumulation and the buds in cold regions remain ecodormant (environmental inhibition) until increasing temperatures in spring stimulate budbreak (Lang *et al.*, 1987). Dormancy is described as a complex mechanism involving interaction between different genetic networks, physiological processes and biochemical reactions regulated by environmental factors (Cooke *et al.*, 2012; Götz *et al.*, 2014; Yamane, 2014). If the chill requirement is not met in deciduous fruit trees, it results in incomplete endodormancy release, unsynchronised budbreak and delayed foliation that negatively impact on the crop and tree architecture (Cook and Jacobs, 1999 and 2000; Atkinson *et al.*, 2013). Most of the apple producing areas in South Africa suffer from inadequate winter chill (Cook, 2010; Cook *et al.*, 2017), especially the low laying areas of the Western Cape (Cook and Jacobs, 2000; Labuschagné *et al.*, 2000). Areas such as Elgin (34ºS, 19°E; 305 m a.s.l.) has a long term mean of only 745 Utah chill units (CU) per annum (Cook and Jacobs, 2000). Global warming is forecasted to increase worldwide temperature and decrease winter chill unit accumulation even further (Midgley and Lötze, 2011). In mild climatic areas, such as Elgin, where winters are short and/or warm, the use of chemical rest breaking agents (RBAs) are vital to partially compensate for the insufficient chill accumulation, successful release of endodormancy and ensuring acceptable levels of budbreak needed to sustain profitable yields (Costa *et al.*, 2004; El-Yazal and Rady, 2012).

Although several chemical RBAs are available commercially, hydrogen cyanamide (HC) is the most researched and is used commercially over a wide range of crops including kiwifruit (McPherson *et al.*, 2001), apple (Carvajal-Millán *et al.*, 2007; El-Yazal and Rady, 2012; Amberger, 2013), grapes (Trejo-Martínez *et al*, 2009; Rubio *et al.*, 2014), peaches and nectarines (Dozier *et al.*, 1990). The use of HC in combination with mineral oil (HCo) is a common horticultural practice in South African commercial apple production areas suffering from inadequate winter chill. Mineral oil affects respiration indirectly by creating temporary hypoxia that hastens budbreak (Baker, 1970; Or *et al.*, 2000; Dami and Beam, 2004; Pérez *et al.*, 2009). HC treatment leads to various physiological changes of which its inhibition of catalase and cytochrome oxidase enzymes are the most dramatic (Amberger, 2013). As direct effects of HC treatment, catalase inhibition causes the accumulation of peroxidases (H_2O_2 and O_2^-) which lead to a sequence of oxidative stress and metabolic disorders (Moustafa and Fridovich, 1979; Nir and Lavee, 1993; Møller, 2001; Vergara

et al., 2012a and 2012b; Amberger, 2013; Horikoshi *et al.*, 2018). Furthermore, cytochrome C oxidase activity is slightly inhibited while alternative respiration increases. Amberger (2013) indicates that this action of cyanamide is immediate and reaches full activity after 48 h after which it is rapidly broken down in the plant tissue. It is then quickly decomposed (enzymatically) and metabolism returns to normal. This leads to accelerated endodormancy release and budburst (Dozier *et al.*, 1990; Mcpherson *et al.*, 2001).

The relationships among RBAs and the four main respiratory pathways during dormancy have been described in literature (McPherson *et al.* 1997; Pérez *et al.*, 2008; Tan *et al.*, 2010; Amberger, 2013; Tan *et al.*, 2010 and 2013). HCo treatment creates anaerobic conditions that affect the mitochondrial electron transport chain (ETC) directly via the cytochrome C pathway (CYT) (Amberger, 2013). This transient inhibition in electron transfer activates oxidative stress by increasing reactive oxygen species (ROS) such as peroxidases (H_2O_2 and O_2^-), which leads to a drop in the tricarboxylic acid cycle (TCA) activity and ATP production (Vergara *et al.* 2012b; Sudawan *et al.* 2016). It is reported that this decline in the activity of the main pathways of growth respiration (TCA and CYT) induces/stimulates the activity of pathways responsible for maintenance respiration, i.e. pentose phosphate pathway (PPP), alternative pathway (ALT), glycolysis and fermentation (Fernie *et al.*, 2004; Florez-Sarasa *et al.*, 2007; Amberger, 2013; Tan *et al.*, 2013). In parallel, several antioxidants are activated to cope with this transient respiratory stress (Wang and Faust, 1994). In general it is proposed that all respiration pathways and oxidative reactions which occur under hypoxic conditions lead to dormancy release and bud growth (Pérez *et al.,* 2008 and 2009; Vergara *et al.*, 2012a; Amberger, 2013, Sudawan *et al.*, 2016). Several researchers have reported the connection between the four respiration pathways and HC and/or oil treatment and their role in dormancy release. For example, the importance of the PPP pathway in NADPH production, redox state maintenance and cell growth regulation have been highlighted by Tian *et al.* (1998) and Vergara and Pérez, (2010).

This strongly suggests that HCo treatments have the capability to break endodormancy and stimulate growth. However, its ability to affect the respiration dynamics during budburst under South African warm winter conditions has not been described to date. The aim of this study was to investigate the effect of HC and oil on the total respiration rate, the respiration pathway rates and contribution of tricarboxylic acid cycle (TCA), pentose phosphate pathway (PPP), cytochrome C pathway (CYT) and the alternative oxidase pathway (ALT) to the total respiration of apple buds exposed to insufficient winter chill.

2. Materials and methods

2.1. Plant material, study site and climatic data

The study was conducted on a commercial ‘Cripps Pink’ apple orchard over two consecutive winter seasons in Elgin (34ºS, 19°E; 305 m a.s.l.), a production area in a warm winter area of South Africa with an average of ±700 CU (Utah model). A logger (Tiny Tag: Gemini Data Loggers, Chichester, UK) was placed in the orchard to record hourly temperature during the trial.

2.2. Treatment and sample collection

The trees were exposed to a commercial treatment of 0.5% (w/ v) Dormex™ (active ingredient (%) hydrogen cyanamide) and 4% (w/ v) mineral oil (HCo) (Nexus, Paarl, South Africa). Treatment dates were 3 September 2015 and 31 August 2016 for the respective years. The commercial application date of RBA takes into account historical application dates, accumulated CU, physiological development (signs of budswell) of the trees, cultivar differences and post-treatment weather conditions (Zhang and Taylor, 2011; Or *et al.*, 1999). Once the application date of the HCo was set, baseline samples were taken before the treatment and control shoots were randomly selected, tagged and covered with plastic bags to prevent contact with the treatment. Shoots were covered for no longer than 48h prior to application and again removed within 24h after treatment. One-year old shoots (±25 cm long) were randomly selected, cut, wrapped and transported on ice to the laboratory. In the laboratory the shoots were kept at room temperature with their proximal ends submerged in water. Respiration rate analysis followed within 24 hours after removal from the tree. Shoot collection was done at two/three day intervals until more than 70% green-tip stage was achieved for the treated buds in the orchard (the control buds were at <70% green-tip).

2.3. Measuring the total respiration rate

The terminal buds were removed from the shoots, weighed and cut lengthwise into four parts. The residual air inside the bud tissue was removed under vacuum and bud parts were placed in a reaction chamber filled with 2 ml of distilled water. The reaction chamber contained a Clark-type oxygen electrode linked to an OxyGraph instrument (Hansatech Instruments Ltd., Norfolk, England) that measured the oxygen content of the water at 20 ºC. Oxygen consumption was recorded per second and the respiration rate was calculated as the reduction in oxygen over three minutes after placing the bud slices into the chamber. Results are expressed as nmol per gram fresh weight (FW) per minute (nmol $g^{-1}min^{-1}$). To counter the effects of photosynthesis all assays were conducted in the dark. The assay was repeated on 12 replicates in 2015 and 8 replicates in

2016. A single replicate consisted of up to three terminal buds with an average fresh weight (FW) of 0.3 g.

2.4. Measuring the respiration rate of the individual pathways

All experiments were performed using six replicates per sample collection date for both treated and control samples. A single replicate consisted of up to three terminal buds with an average FW of 0.3 g. To measure the respiration of the four individual respiration pathways we obtained chemical inhibitors for the respective pathways. Malonic acid (Merck, Johannesburg, South Africa) was used as a competitive inhibitor of succinate dehydrogenase in the TCA cycle (Tan *et al.*, 2010 and Beevers, 1952). The PPP was inhibited using Na_3PO_4 (Sigma-Aldrich, Johannesburg, South Africa) acting as a specific inhibitor of glucose-6-phosphate dehydrogenase (Tan *et al.*, 2010; Swamy and Sandhyarani, 1985). To quantify the rate of the TCA and PPP the method of Tan *et al.* (2010) was followed by measuring the oxygen uptake of the buds in the presence of 3 M malonic acid followed by a similar measurement of residual respiration in presence of a solution of 3M malonic acid and 0.6 M Na_3PO_4.

For the CYT and ALT, pathways KCN (Sigma-Aldrich) and salicylhydroxamic acid (SHAM) (Sigma-Aldrich) were used as respective inhibitors of cytochrome C oxidase and alternative oxidase in the electron transport chain reaction (Azcbn-Bieto *et al.*, 1983; Millar *et al.*, 1995; Atkin *et al.*, 2002; Tan *et al.*, 2013). Again, similar to Tan *et al.* (2013), the oxygen uptake for CYT and ALT pathways was calculated after measuring the oxygen uptake in the presence of 2mM KCN and then in the presence of a 2 mM KCN and 30 mM SHAM solution.

2.5. Data handling and statistical analysis

Calculations similar to those of Tan *et al.* (2010) and Tan *et al.* (2013) were used to determine the O_2 consumption rate (nmol $g^{-1}min^{-1}$) and the relative contribution (% of total respiration rate) of the individual pathways. Table 1 gives a summary of the relevant calculations used. Throughout the experiment O_2 consumption was used as an indication of the respiration rate of the pathways.

To test the significance of the observed differences, a two-way analysis of variance (ANOVA) with interactions was done using XLSTAT 2017 (version, 19.01.40777). If the significance level (p-value) was <0.05 a Fisher's LSD post hoc test was performed to characterize the differences. Main effects were defined as 'Treatments' (either untreated/control shoots or HCo-treated shoots) and 'Days' (24 h time intervals post-application of the treatment).

3. Results

3.1. Phenological stages and weather conditions pre and post treatment.

At the time of RBA application, the orchard in both years had experienced budswell. RBA was applied between 26 August and 3 September and the buds were monitored until 70% of the orchard (treated trees) was at green-tip stage. In 2015, green-tip was reached 15 days after the application of RBA and in 2016 after 21 days. The daily maximum temperatures for the trial period are reported in Fig. 1. From this, it is clear that daily maximum temperatures after the application of the RBA were very different between the two years. In 2015, the two days following the application had relatively high temperatures (29.7 and 29.3 °C) with dry conditions compared to the 2016 season where the maximum temperature dropped to 16.1 and 13.1 °C for two consecutive days with rainy orchard conditions and then recovered to 25.6 °C on Day 4 compared to the relatively mild 16.8 °C in 2015. In 2015 there was a warm spell on Day 10 where the temperature reached 34.6 °C and dropped again to 19.4 °C the next day. In 2015 the control shoots were bagged 24h prior to the RBA application (Day -1) and in 2016 it occurred 48 h (Day -2) prior to RBA application. During the winter period of 2015 a total of 854 CU (Utah) were accumulated versus the 788 CU in 2016 (calculated from 1 May to 31 August in both years).

3.2. The effect of HC and mineral oil treatment on the total respiration rate.

In both years, the O_2 consumption rate of all the samples (treated and control) were between 28 and 44 nmol $g^{-1}min^{-1}$. The baseline (prior to treatment) respiration rates for the two seasons were similar at 30 and 33 nmol $g^{-1}min^{-1}$ for both treated and control buds. However, the final rate of the treated buds for the 2015 season (44 nmol $g^{-1}min^{-1}$) differed from the 2016 season (38 nmol $g^{-1}min^{-1}$) and was significantly higher compared to that of control buds at the end of assessment.

In the 2015 season there was an interaction between the main effects (treatments and days) ($p < 0.0001$), indicating that the HCo treatment changed the total respiration rate of the apple buds over time (Fig. 2A). For the first five days post treatment, the treated buds had a respiration rate lower than the control samples. After this period, the treated samples overtook the control samples (Day 7) and for the rest of the duration of the experiment the HCo treated samples showed a respiration rate significantly higher and increasing faster than the control buds. The final respiration rate of the treated samples was 38% higher at the time of budburst (70% green-tip) compared to the control.

The 2016 season (Fig. 2B) showed a similar pattern with a significant interaction between the treated and the control buds ($p < 0.0001$). Compared to the control, the respiration rate of the

treated samples was initially lower but after five days it had increased to be equal to the control. The treated and control buds remained statistically equal until Day 19 after which the treatment started to exceed the control. At Day 21 (70% green-tip stage), the treatment buds had a respiration rate 15% higher than the control.

3.3. The effect of HCo treatment on the TCA pathway, PPP pathway and residual respiration rate and their contribution to the total respiration

3.3.1 The effect of HCo treatment on the TCA pathway rate, and contribution to the total respiration.

In both years, the respiration rate of the TCA pathway was between 11 and 23 nmol $g^{-1}min^{-1}$, contributing between 37% and 58% of total respiration (Fig. 3). The initial rate (baseline samples) of the TCA of treatment and control had a similar value in both seasons. However, at the green-tip stage, higher respiration and contribution levels were observed in the treated buds, especially in the 2015 season.

The ANOVA indicated that the TCA pathway rate was significantly affected by an interaction between the main effects ($p < 0.0001$) in both seasons (Fig. 3A and B). This indicates that the HCo treatment had a differential effect on the rate of the TCA respiration pathway over the time period. In both seasons, the treated buds had a significant lower oxygen consumption rate compared to the control during the first four days post-treatment, similar to what was seen for the total respiration rate in Fig. 2. This changed rapidly after Day 5 as the HCo treatment caused a steady increase in the TCA respiration rate resulting in much higher levels at green-tip stage compared to the control buds. This difference between the treated and the control samples was more noticeable during the 2015 season where the control buds increased significantly slower than the treatment. No significant increase was detected in the control buds in 2016 between Day 5 and the green-tip stage.

When the respiration rate of the TCA pathway is expressed as a contribution (%) of total respiration, a significant interaction effect between the HCo treatment and time was observed ($p < 0.0001$) (Fig. 3C and D). It follows the same pattern, showing the treated samples initially contributing below the control but increasing and contributing more than the control buds after five days post-treatment. For most of the remaining period of the experiment the contribution of the treated samples was higher compared to the untreated samples. At the end of the trial period the HCo treated buds made a 19% and 17% higher contribution to total respiration than the control buds for the respective seasons (2015 and 2016). These results indicate that HCo does not only

increase the rate of the TCA pathway but that it also increases the contribution of this pathway to total respiration.

3.3.2. The effect of HCo treatment on the PPP pathway rate, and contribution to the total respiration.

In both years, the respiration rate of the PPP pathway varied between 4 and 12 nmol $g^{-1}min^{-1}$ contributing to between 12% and 33% of the total respiration (Fig. 4). The initial PPP rates in 2015 were higher and contributed more to the total respiration compared to the 2016 season. A similar pattern was observed during the green-tip period for the final rates of both treated and control buds with a lower contribution in the treated buds.

According to the results from the ANOVA the PPP rate was significantly affected by an interaction between the HCo treatment and time (days post-treatment) in 2015 ($p = 0.049$) and in 2016 ($p = 0.001$) (Fig. 4A and B). It should be noted that the probability value (p-value) obtained for the interaction (Treatment*Days) in 2015 is close to the cut-off value ($p = 0.05$) for the statistical model and result should therefore be interpreted as relatively weak evidence that the null hypothesis should be rejected. This is expected when considering that in 2015 the control and treated samples only differed at two occasions during the trial, at Day 1 and Day 15. It is, however, clear from the data that the control buds demonstrated a significant increase in PPP respiration from Day 1 to Day 15 whereas the treated buds lacked this increase and maintained a stable rate throughout the trial. During 2016, the difference between the treated and control samples are evident at almost all data points from Day 5 (Fig. 4B). The increase in the control buds that was evident in 2015 is not present post treatment in the 2016 results. The significant differences seen in 2016 are described by a decrease in the PPP rate of the treated buds from Day 2 onwards compared to the stable rate of the control buds. At the green-tip stage, the final rate of PPP of the treated buds was 25% lower than that of the control buds in 2015 (Fig. 4A) and 47% lower than that of the control buds in 2016 (Fig. 4B). Thus, in both seasons the treatment caused an ultimate lower PPP respiration rate at green-tip stage compared to the control.

When considering the percentage contribution of PPP to the total respiration in 2015 the weak interaction probability value observed in the respiration rate data disappears as the treatment strongly affects the percentage contribution in both seasons ($p < 0.0001$) (Fig. 4C and D). The 2015 results are again characterized by an increase in the control buds between Day 2 to Day 5 after where it remains stable while the treated samples show a decreasing contribution towards green-tip stage after initially contributing more than the control on Days 1 and 3. During the 2016 season, the PPP contribution of the control buds remained relatively stable between 20-25% after

increasing to Day 2 while the percentage PPP contribution in the treated sample increased to Day 0 and then decreased from Day 2 to below 12% at green-tip stage (Fig. 4D). In both seasons the final contribution of PPP of the treated buds were significantly lower compared to the control buds at the green-tip stage; 37.5% lower in 2015 and 45.5% lower in 2016.

3.3.3. Residual respiration and the effect of HCo treatment

The oxygen consumption that is measured in the presence of both inhibitors is termed the residual. In this trial it represents all the chemical reactions, apart from the TCA and PPP pathways, that affect the oxygen consumption of the buds. Thus, for this part of the trial, we assume that the total respiration rate is made up of the respiration rate of the TCA pathway, the PPP pathway and the residual reactions involving oxygen. Fig. 5 shows the respiration rate and contribution of the residual reactions for the treated and control buds. In both years, the respiration rate of the residual fluctuated between 7.9 and 13.9 nmol $g^{-1}min^{-1}$ and contributed to between 15% and 37% of the total respiration.

The residual rate did not show any interaction between the treatments (HCo vs control) and time post treatment in any of the years. However, significant main effects were observed in the 2015 season for both Treatment ($p = 0.036$) and Days ($p = 0.017$) (Fig. 5A). This indicted that the residual respiration rate changed in a similar fashion in all the buds over time, irrespective of the treatment, but the treated buds had on average a higher residual rate. Thus, apart from the treated buds having a slightly higher residual rate compared to the control buds in 2015, the rate in both groups (treated and control) has progressively declined until Day 5 and then increased again to levels similar to the initial levels at the end of the trial. In the 2016 season no significant differences were observed in the main effects (all $p > 0.05$) (Fig. 5B) indicating that the residual levels of the treated and control buds were similar and behaved the same over time.

The contribution of the residual rate to the total respiration is shown in Fig. 5C with only a significant main factor effect (Treatment $p = 0.527$ and Days $p = 0.030$) in 2015 and no significant effects (all $p > 0.05$) in 2016. (Fig. 5D). Both the treated and control buds showed a decreasing trend in their residual contribution from Day 0 to Day 15, with no significant difference between the mean contributions. These results indicate that the chemical reactions involved in oxygen consumption, other than the TCA and PPP pathways, were mostly unaffected by the treatment and did not interfere with the results.

3.4. The effect of HCo treatment on the CYT pathway, ALT pathway and residual respiration rate and their contribution to the total respiration.

3.4.1. The effect of HCo treatment on the CYT pathway and contribution to the total respiration.

The respiration rate of the CYT pathway varied between 9 and 21 nmol $g^{-1}min^{-1}$ contributing to between 32% and 54% of the total respiration for the respective two years (2015 and 2016) (Fig. 6). Across seasons, the overall CYT rates, as well as their contribution to the total respiration, were lower in 2015 compared to 2016. For both seasons the treated bud showed higher CYT rates and contributions at the green-tip stage.

The analysis of variance indicated that there was a significant interaction between the treatment and time (days) ($p < 0.0001$) for the CYT pathway during both seasons (Fig. 6A and B) implying that there was a differential effect on CYT rates and contribution to the total respiration over the time period in both years. In 2015 the treated samples showed a significant decrease in CYT respiration immediately after treatment with a subsequent increase to the initial rate by Day 5. Both the control and treatment showed a decrease from Day 7 to Day 9 where after both increased their CYT respiration. The treated samples increased at a faster overall rate and at green-tip stage they were 26% higher than the control buds. During the 2016 season, there were no clear trends between the CYT respiration of the treated and the control buds until 14 days post treatment. The treated samples increased their CYT respiration from Day 14 and at green-tip stage, a week later, had a rate 33% higher than the control (Fig. 6B).

The ANOVA results for the CYT contribution to total respiration started off very similar in both years at ± 45% of the total respiration (Fig. 6C and D). Significant interactions between the treatment and time (days) were observed for the CYT contribution in both seasons (2015 Treatment*Days $p < 0.0001$ and 2016 Treatment*Days $p = 0.0000$) in 2016 seasons, similar to the CYT rate results. In 2015 (Fig. 6C), the treated samples contributed below the control on Day 1 and 3 but then increased and contributed more that the control samples after Day 9 and remained relatively stable (and higher than the control) until green-tip stage. The control samples maintained a stable CYT contribution (higher than the treated samples until Day 4) up to Day 7 after which it dropped significantly and then stayed low (and below the treated samples) until the end of the trial. During the first five days post treatment of 2016 (Fig. 6D), the treated buds contributed more that the control buds on Day 0 and 5. After Day 5 the CYT contribution to total respiration of the treated samples remained above that of the control samples and from Day 9 until the end of the trial the values were always significantly higher ending on a value 10% higher

than the control. In general, the pattern of the percentage contribution of the CYT pathways was similar to that of the CYT respiration rate.

3.4.2 The effect of HCo treatment on ALT pathway and contribution to the total respiration.

The respiration rate of the ALT pathway varied between 3 and 9 nmol $g^{-1}min^{-1}$ contributing between 9% and 24% of the total respiration over the two years (Fig. 7). The initial and final rates and contributions of the two years were quite different. The initial ALT rate during 2015 season was higher than that of the 2016 season. At the green-tip stage, treated buds of both the 2015 and 2016 seasons showed lower ALT levels rates compared to the control. Results from the contribution calculations behaved in a similar manner.

The analysis of variance shows that there was a significant interaction ($p = 0.004$ and $p = 0.005$) between the HCo treatment and time (days) for the respiration rate in both of the seasons (2015 and 2016) (Fig. 7A and B). In 2015 there was no difference between the treated and control buds during the first 3 days post-treatment (Fig. 7A). By Day 5 the rate of the control group increased consistently until green-tip stage while the treated buds maintained an unchanged respiration level. At green-tip stage the treated buds had an ALT respiration rate 44.5% lower compared to the treated buds. The 2016 results were similar with the control shoots again showing a higher rate than the treated shoots from Day 9 (Fig. 7B). The rate of treated buds was 33.3% lower than the control buds at green-tip stage. In both years, the treated buds showed very little change in their ALT respiration rate throughout the trial, except for an increase from Day -2 to 0 in 2016.

The contribution of the ALT pathway also showed an interaction between the main effects ($p < 0.0001$) in both seasons with a similar outcome compared to the respiration rate results. In 2015, both treatments showed an initial drop in contribution on Day 3, but by Day 5 the control buds have recovered to the original contribution and remained stable until the end of the trial while the treated buds showed a continuous decrease in its ALT contribution from Day 0. At the end of the trial, the treated samples were 43% below the control. The following year the control buds showed little variation in its contribution throughout the trial compared to the treated buds that decreased after Day 2 and remained low. Contributions differed significantly from Day 12 so that, at green-tip stage, the treated buds were again 43% lower than the control (Fig. 7C and D).

3.4.3 Residual respiration and the effect of HCo treatment.

The CYT and ALT measurements also allowed for the measurement of residual respiration (Fig. 8). This is oxygen consumption that is measured in the presence of both inhibitors and thus represents the chemical reactions, apart from the CYT and ALT pathways, that affected the

oxygen consumption of the buds. Thus, for this part of the trial, we assume that the total respiration rate is made up of the respiration rate of the CYT pathway, the ALT pathway and the residual reactions involving oxygen (see Table 1 for details of calculations). The residual respiration rate in both years varied between 9 and 19 nmol $g^{-1}min^{-1}$ and contributed between 37% and 48% of the total respiration and in both parameters and years did not show any interaction between the main effects (all Treatment*Days $p > 0.05$). Both main effects did however show significant difference in both years (Fig. 8). In 2015 and 2016 the residual rate (Fig. 8 A and C) and the percentage contribution (Fig. 8b & D) showed that the HCo treatment caused an average increase in the respiration rate and % contribution to total respiration (2015: Rate Treatment $p = 0.004$ and % Contribution Treatments $p = 0.0001$; 2016: Rate Treatment $p = 0.015$ and % Contribution Treatments $p = 0.026$). Results showed futher that in both years the respiration rate and contribution percentage of all the treated buds from a particular year changed similarly over time (2015: Rate Days $p < 0.0001$ and % Contribution Days $p = 0.0001$; 2016: Rate Days $p = 0.004$ and % Contribution Treatments $p < 0.0001$) irrespective of the treatments. In 2015 both the treated and control buds increased their respiration rate and percentage contribution significantly from application until the end of the trial (Day 18). In 2016 both the treated and control buds showed a slight decrease in the residual respiration rate and % contribution between application and the last measurement (Day 21). Although the residual levels were quite high for both the treated and control samples, the results suggested that the HCo treatment only affected the mean average residual levels and % contribution.

4. Discussion

The weather conditions immediately (2-3 days) after the application of a rest breaking agent can influence the effectiveness of the treatment (Sheard *et al.*, 2009). Typically, rainy conditions with low temperatures may reduce/delay the desired effect as it can dilute the treatment and slow the chemical reactions and respiration rate down and decrease floral budburst (Sheard *et al.*, 2009). Similarly, higher temperatures will accelerate respiration levels (Tan *et al.*, 2013). The contrasting post-treatment orchard temperatures of the two seasons spanning this trial, might therefore explain the differences that is seen in the onset to 70% green-tip stage between the two years. The cooler post-treatment conditions (Day 0 – Day 2) in 2016 extended the onset to 21 days whereas the higher temperatures, immediately after treatment (Day 0 – Day 2) and again at 10 days post-treatment in 2015, accelerated the onset to 14 days. The increased temperatures experienced from Day 0 to Day 2 of 2015 could also explain the rise in the total respiration of the control shoots in this year. The absence of such a temperature effect in the treated buds suggests that the HCo treatment prevented such an increase, possibly due to a lack of O_2 availability. This

fits with results from Pérez *et al.* (2009) showing that HC treatment slows down oxygen uptake in the mitochondria of grapevine buds, and findings by Dami and Beam (2004) reporting a decrease in grapevine respiration due to oil acting as a physiological barrier that decreases O_2 uptake from the environment (Tan *et al.*, 2013).

When considering all the respiration results in this trial, it indicates a two-fold response to the HCo treatment. An immediate effect is seen within the first five days post treatment followed by a secondary/late effect leading up to budbreak. The immediate response to the HCo treatment may be seen as a period of treatment perception when the HCo is still active in the plant. Amberger (2013) showed that the action of cyanamide is immediate and reaches full activity within 48 hours where after it is rapidly broken down by the plant tissue. The smothering effect of oil on the external surface of a shoot is also temporary as it gets absorbed within a couple of days and the contact between the plant tissue and the external oxygen is restored (Baker, 1970). The immediate HCo reaction then sets the buds off on a biochemical route that differs from the untreated buds and brings about a secondary effect that is associated with dormancy release and earlier growth resumption (70% green-tip stage). Similarly, Trejo-Martínez *et al.* (2009) reported that HC advanced budbreak in grapevines by two weeks, and a late response to a HC treatment in 'Golden Delicious' shoots was explained by the activation of bud respiration that led to dormancy release (Carvajal-Millán *et al.*, 2007).

4.1. The 'initial effect' on bud respiration

The respiration trends in the treated compared to the control buds during the first five days post-treatment suggest that the HCo treatment affected the total respiration, TCA and CYT pathways. This was possibly due to the lack in O_2 uptake as the cytochrome C pathway, the last step in the electron transport chain, requires the binding of O_2 to the cytochrome C peripheral protein and if inhibited all preceding pathways and the total respiration rate slows down (Taiz and Zeiger, 2010). Such CYT pathway inhibition may be due to the low-oxygen effect (hypoxia) which slows down the electron transport chain and induce oxidative stress (Sweetlove *et al.*, 2010; Amberger, 2013; Toro and Pinto, 2015). Earlier studies indicated that an exposure of dormant buds to hypoxia or a HC treatment also inhibits cytochrome C oxidase (Amberger, 2013), the enzyme responsible for reducing O_2 to H_2O. This inhibition effects both the electron transport and proton (H^+) pump of complex IV (Taiz and Zeiger, 2010) by reducing the proton concentration in the intermembrane space and consequently prevent ATP synthesis (Nůsková *et al.*, 2010; Taiz and Zeiger, 2010). Additionally, the HC application inhibits the activity of catalase and thereby enhances the production of reactive oxygen species (ROS) such as H_2O_2 and O_2^- (Nir and Lavee, 1993; Nir *et al.*, 1986; Pérez and Lira, 2005; Amberger, 2013). This leads to oxidative stress in the

mitochondrial electron transport system, resulting in further ATP reduction (Tiwari *et al.*, 2002). The inhibition of the CYT pathway indirectly results in the inhibition of TCA activity, which is involved in respiration during dormancy release (Tan *et al.*, 2010 and 2013). The TCA pathway is known as the main driver for oxygen reduction to water and ATP synthesis for all electrons transmitted via the electron transfer chain (Taiz and Zeiger, 2010). The inhibition of CYT and thus TCA perhaps points towards a more important role for alternative pathways in the maintenance of respiration of plants under stress.

To survive a decrease in oxygen uptake and CYT pathway inhibition, the ALT, glycolysis, PPP and fermentation pathways are induced (Toro and Pinto, 2015). The ALT pathway is an alternative route in the electron transport chain that can receive electrons directly from the ubiquinone pool when the cytochrome C oxidase is inhibited (Taiz and Zeiger, 2010). In doing so it prevents mitochondrial oxidation by reducing oxygen to water to maintain or alleviate the redox state (Taiz and Zeiger, 2010) within the mitochondrial membrane. Through this pathway, much lower ATP is synthetised as it does not allow for the transfer of hydrogen ions across the membrane (Taiz and Zeiger, 2010). It seems that when the activity of the CYT pathway drops, the ALT activity trends higher in the control in order to compromise for the loss. Previous studies reported that the effect of stimuli such as temperature, hypoxia, H_2O_2, HC or other treatments appear to induce oxidative stress that increases the activity of ALT pathway even though their mode of action and their effectiveness may differ (Vanlerberghe *et al.*, 1995; Ribas-Carbo *et al.*, 2005; Pérez *et al.,* 2008; Vergara *et al.*, 2012a; Amberger, 2013; Tan *et al.*, 2013). In this study, the expected high trend in ALT activity in response to HCo treatment is not clearly evident in the initial stage post application, possibly because the plant was already relatively stressed by the warm winter conditions preceding the trial as is portrayed by the high ALT levels in the control samples.

Literature reports an increase in PPP activity in plant tissues under induced low temperature or RBAs (Nir and Lavee, 1993). For instance, HC treatment increases PPP by the activation of the glucose-6-phosphate dehydrogenase (G6PDH) transcript in dormant grapevine buds (Pérez *et al.*, 2009; Vergara and Pérez, 2010). Similarly, PPP levels increase under salt stress in bean roots (Liu *et al.*, 2007) or during seed dormancy release (Simmonds and Simpson, 1971). In this study the treatment showed higher PPP levels compared to the control on Day 1 only but this difference was only significant in 2015 (Fig 4C and D). The warmer conditions experienced in 2016 could have caused the control and the treatment buds to maintain a higher level of PPP in this initial post treatment stage.

4.2. The 'late effect' on bud respiration

As the HCo treated buds recovered from the anaerobic conditions brought about by the initial phase, their metabolic activities were restored. Throughout the latter part of the trial (late effect), the total respiration rate of both HCo treated and control buds maintained a similar increasing pattern in 2015 season although the rate of treated buds was higher than that of the control at the 70% green-tip stage in both seasons. Comparable results have been reported in grapevine buds where changes in the respiration rate were detected 12 days after a HC treatment, followed by a maximum level at the "half-inch green-tip" stage (Trejo-Martínez *et al.,* 2009). Also, Carvajal-Millán *et al.* (2007) reported an increase in the metabolic and respiration rate in HC treated apple buds 15 days post-treatment. Furthermore, dormant nectarine buds treated with high temperature (Tan *et al.*, 2013) and kiwifruit buds treated with RBAs (McPherson *et al.*, 1997) resulted in an increase in growth respiration prior to dormancy release.

Sufficient energy required for growth respiration and dormancy release is supplied directly via the TCA pathway (Nazaret *et al.*, 2009; Gai *et al.*, 2013). Once the mitochondrial respiratory chain is activated, a proton gradient is generated, the TCA cycle increases and produces NADH and $FADH_2$, which in turn produce the necessary energy through oxidative phosphorylation (Sweetlove *et al.*, 2010; Taiz and Zeiger, 2010). Horikoshi *et al.* (2018) found that, prior to dormancy release, most of the metabolites involved in the TCA pathway increased in Japanese pear flower buds exposed to constant chilling. They also showed that thermal fluctuations negatively affected these metabolite levels and were associated with lower budbreak. According to Gai *et al.* (2013), the ATP level in dormant peonia (*paoenia ostii*) buds was very low and the TCA enzymes and ATP synthase were only induced following an artificial chilling treatment toward dormancy release. Similarly, TCA enzyme activity increases prior to dormancy release in tubers (*Dioscorea esculenta*) and rhizomes (*Curcuma longa*) treated with chill (Panneerselvam *et al.*, 2007). These authors mentioned that the increase was detected before the appearance of visible signs of sprouting and peaked during the budbreak and green-tip stages. Wang *et al.* (1991) indicate that the TCA cycle enzyme isocitrate dehydrogenase increased during budbreak and reached a high level during the green-tip stage of thidiazuron (TDZ) treated apple buds. Tan *et al.* (2010) also found an increase in TCA activity prior to dormancy release in nectarine flower buds. At the green-tip stage in this study, TCA made up 55-60% of the total respiration, making it an important pathway during the budbreak and green-tip stages. Also, the highest total respiration and TCA levels in the treated samples were found during the green-tip stage. This again indicates that changes in TCA levels drive changes in the total respiration. Moreover, the TCA pathway is

also involved in the regulation of respiration and the synthesis of intermediates of its own cycle (Toro and Pinto, 2015).

Similar to the TCA pathway, the activity of the CYT pathway of treated buds regained its level during the 'late effect' stage after treatment. During both seasons, CYT activity in the HCo treated buds resumed, overtook the control buds and maintained high levels up to green-tip stage. Greater CYT activity occurs in the HCo treated buds and reached capacity maximum level of energy production during the green-tip stage. This data agrees with the ideas of Florez-Sarasa *et al.* (2007) who mentioned that plant growth is highly dependent on CYT activity and ATP synthesis. This high level of CYT activity, compared to the control group, suggests a link between CYT activity and dormancy release, possibly causing dormancy release, although the possibility of being merely an effect/reaction of dormancy release cannot be ignored. Similar to Tan *et al.* (2013), CYT activity increased at the later stage, prior to dormancy release, in nectarine buds treated with a high temperature stress. A higher CYT activity was associated with respiratory energy and growth processes involved in the synthesis of new plant constituents (Florez-Sarasa *et al.*, 2007).

Under growth conditions, alternative pathways seem to be involved in the maintenance respiration, which is associated with cellular structure, ion concentrations and gradients (Florez-Sarasa *et al.*, 2007). As the initial effects of the stimuli (HCo or chilling) wear off in the bud tissue, the TCA rate recovers and the PPP pathway becomes less apparent (Tan *et al.*, 2010). This is evident in the present results as the HCo treated buds maintained a lower and constant level in their PPP and ALT activity until 70% green-tip stage compared to the control buds. Similarly in grapevine buds, the ALT transcripts were observed in the control group and hardly detected in the HC treated group after 14 days post-treatment (Pérez *et al.* 2009). Previous studies show that prior to budbreak, under control condition or treatments, the PPP and ALT activity declines (Wang *et al.*, 1991, Faust and Wang, 1993; Tan *et al.*, 2010) and preserve their maintenance level. The current results suggest that the control buds, in reaction to insufficient TCA and CYT levels, maintained a significantly higher PPP activity to possibly compensate for the lack in energy production whilst increasing the ALT pathway to counter the high stress levels of incomplete endodormancy release. Thus, the HCo treatment manifested a higher level of CYT and TCA activity in the buds and therefore had no need to increase its PPP and ALT pathways. It has also been reported that the PPP and ALT pathways may be important for cell defence against high oxidative stress associated with budbreak in warm winter conditions (Vergara *et al.*, 2012a). In summary, the study results showed that the regulation of respiration and artificial dormancy

release through the use of HCo may create a physiological transition phase of hypoxia-anoxia followed by reoxygenation, which induces buds to break (Ophir *et al.*, 2009).

5. Conclusion

The environmental factor (chilling) that controls the dormancy cycle can be partially manipulated and improved in areas with insufficient winter cold. The anaerobic conditions brought about by the HCo treatment induced stress that contributed to the decrease in respiration during the five first days. This initial effect changed the respiratory landscape in the buds resulting in higher total respiration, TCA and CYT pathways, a late effect that better supports the high energy cost of budbreak. In this study, both treated and control buds were cold deprived due to insufficient chill accumulation during the preceding winter, but the HCo treatment partially accommodated for this by increasing the TCA and CYT respiration in the early stages of budbreak. Future work should include a comparison of the treated buds to buds that received sufficient field chill to assess how successful the HCo can restore the respiration dynamics compared to natural chill accumulation. Comprehensive budbreak data should also be included to illustrate the results.

6. References

Amberger, A. (2013). Cyanamide in plant metabolism. *International Journal of Plant Physiology and Biochemistry*. **5**, 1–10.

Atkin, O. K., Zhang, Q. and Wiskich, J.T. (2002). Effect of temperature on rates of alternative and cytochrome pathway respiration and their relationship with the redox poise of the quinone pool[1]. *Plant Physiology*. **128**, 212–222.

Atkinson, C.J., Brennan R. M. and Jones, H. G. (2013). Declining chilling and its impact on temperate perennial crops. *Environmental and Experimental Botany*. **91**, 48–62.

Avenant, E. and Avenant, J.H. (2014). Chill unit accumulation and necessity of rest breaking agents in South African table grape production regions. 37th World Congress of Vine and Wine and 12th General Assembly of the OIV, *BIO Web of Conferences*. **3**, 1–4.

Azcbn-Bieto, J., Lambers, H., and Day, D. A. (1983). Respiratory properties of developing bean and pea leaves. *Australian Journal of Plant Physiology*. **10**, 237–245.

Baker, J.M. (1970). The Effects of oils on plants. *Environmental Pollution*. **I**, 27–44.

Beevers, H. (1952). Malonic acid as an inhibitor of maize root respiration. *Plant Physiology*. **27**, 725–735.

Carvajal-Millán, E., Gardea, A.A., Rascón-Chu, A., Orozco, J.A., Ponce de León, N., Márquez-Escalante, J.A., Romo Chacón, A. and Guerrero Centro, V.M. (2007). Respiratory response of apple buds treated with budbreaking agents. *Thermochimica Acta*. **457**, 109–112.

Cook, N.C. and Jacobs, G. (1999). Sub-optimal winter chilling impedes development of acrotony in apple shoots. *HortScience*. **34**, 1213–1216.

Cook, N.C. and Jacobs, G. (2000). Progression of apple (*Malus x domestica* Borkh.) bud dormancy in two mild winter climates. *Journal of Horticultural Science*. **75**, 233–236.

Cook, N.C. (2010). Apple Production under Conditions of Sub-Optimal Winter Chilling in South Africa. *Acta Horticulturae.* **872**, 199–204.

Cook, N.C., Calitz, F.J., .Allderman, L.A., Steyn, W.J., Louw, E.D. (2017). Diverse patterns in dormancy progression of apple buds under variable winter conditions. *Scientia Horticulturae.* **226**, 307–315.

Cooke, J.E.K., Eriksson, M.E. and Junttila, O. (2012). The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. *Plant Cell Environment*. **35**, 1707–1728

Costa, C., Stassen, P.J.C. and Mudzunga, J. (2004). Chemical rest breaking agents for the South African pome and stone fruit industry. *Acta Horticulturae*. **636**, 295–302.

Dami, I. and Beam, B. (2004). Response of grapevines to soybean oil application. *American Journal of Enology and Viticulture.* **55**, 269–275.

Dozier, W.A., Powell, A.A., Caylor, A.W., McDaniel N.R., Carden E.L. and McGuire J.A. (1990). Hydrogen cyanamide induces budbreak of peaches and nectarines following inadequate chilling. *HortScience*, **25**, 1573–1575.

El-Yazal, M.A and Rady, M.M. (2012). Changes in nitrogen and polyamines during breaking bud dormancy in Anna apple trees with foliar application of some compounds. *HortScience*. **136**, 75–80

Faust, M. and S.Y. Wang. (1993). Biochemical events associated with resumption of growth in temperate zone fruit trees. *Acta Horticulturae*. **329**, 257–264.

Fernie, A.R., Carrari, F. and Sweetlove F. L. (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology*. **7**, 254–261

Florez-Sarasa, I.D., Bouma, T.J., Medrano, H., Azcon-Bieto, J. and Ribas-Carbo, M. (2007). Contribution of the cytochrome and alternative pathways to growth respiration and maintenance respiration in *Arabidopsis thaliana*. *Physiologia plantarum*. **129**, 143–151.

Gai, S., Zhang, Y., Liu C., Zhang, Y. and Zheng G. (2013). Transcript profiling of *Paoenia ostii* during artificial chilling induced dormancy release identifies activation of GA pathway and carbohydrate metabolism. *PloS One.* **8**, e55297–e55307.

Götz, K.P., Chmielewski, F.M., Homann, T., Huschek, G., Matzneller, P. and Rawel, H.M. (2014). Seasonal changes of physiological parameters in sweet cherry (*Prunus avium* L.) buds. *Scientia Horticulturae.* **172**, 183–190.

Horikoshi, H.M., Sekozawa, Y., Kobayashi, M., Saito, K., Kusano, M., Sugaya, S. (2018). Metabolomics analysis of 'Housui' Japanese pear flower buds during endodormancy reveals metabolic suppression by thermal fluctuation. *Plant Physiology and Biochemistry*. **126**, 134–141.

Labuschagné, I.F., Louw, J.H., Schmidt, K. & Sadie, A. (2002). Genotypic variation in prolonged dormancy symptoms in apple progenies. *HortScience*. **37**, 157–163.

Lang, G.A. (1987). Dormancy a new universal terminology, *HortScience*, **22**, 817–820.

Liu, Y.G., Wu, R.R., Wan, Q., Xie, G.Q. and Bi, Y.R. (2007). Glucose-6- phosphate dehydrogenase plays a pivotal role in nitric oxide involved defense against oxidative stress under salt stress in red kidney bean roots. *Plant Cell Physiology*. **48**, 511–522.

McPherson, H.G., Snelgar, W.P., Manson, P.G. and Snowball, A.M. (1997). Bud respiration and dormancy of kiwifruit (*Actinidia deliciosa*). *Annals of Botany*. **80**, 411–418.

McPherson, H.G., Richardson, A.C., Snelgar, W.P. and Currie, M.B. (2001). Effects of hydrogen cyanamide on budbreak and flowering in kiwifruit (*Actinidia deliciosa* 'Hayward'). *New Zealand Journal of Crop and Horticultural Science*. **29**, 277–285.

Midgley, S.J.E. and Lötze, E. (2011). Climate change in the western cape of South Africa: trends, projections and implications for chill unit accumulation. *Acta Horticulturae*. **903**, 1127–1134.

Millar, A.H., Atkin O.K., Lambers H., Wiskich J.T. and Day, D.A. (1995). A critique of the use of inhibitors to estimate partitioning of electrons between mitochondrial respiratory pathways in plants. *Physiology Plant*. **95**, 523–532

Møller, I.M. (2001). Plant mitochondria and oxidative stress: electron transport, NADPH turnover and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology*. **52**, 561–591.

Moustafa, H.H. and Fridovich, I. (1979). Intracellular production peroxide of superoxide radical and of hydrogen by redox active compounds. *Archives of Biochemistry and Biophysics*. **196**, 385–395.

Nazaret, C., Heiske, M., Thurley, K., Jean-Pierre Mazat, J-P. (2009). Mitochondrial energetic metabolism: A simplified model of TCA cycle with ATP production. *Journal of Theoretical Biology*. **258**, 455–464.

Nir, G., Shulman, Y., Fanberstein, L. and Lavee, S. (1986). Changes in the activity of catalase (EC 1.11.1.6) in relation to the dormancy of grapevine (*Vitis vinifera* L.) buds. *Plant Physiology*. **81**, 1140–1142.

Nir, G. and Lavee, S. (1993). Metabolic changes during cyanamide induced dormancy release in grapevines. *Acta Horticulturae*. **329**, 271–274.

Nůsková, H., Vrbacký, M., Drahota, Z. and Houštěk, J. (2010). Cyanide inhibition and pyruvate-induced recovery of cytochrome c oxidase. *Journal of Bioenergy and Biomembrane*. **42**, 395–403

Ophir, R., Pang, X., Halaly, T., Venkateswari, J., Lavee, S., Galbraith, D. and Or, E. (2009). Gene expression profiling of grape bud response to two alternative dormancy release stimuli expose possible links between impaired mitochondrial activity, hypoxia, ethylene-ABA interplay and cell enlargement. *Plant Molecular Biology.* **71**, 403–423.

Or, E., Nir G. and Vilozny, I. (1999). Timing of hydrogen cyanamide application to grapevine buds. *Vitis*. **38**, 1–6.

Or, E., Vilozny, I., Eyal, Y. and Ogrodovitch, A. (2000). The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. *Plant Molecular Biology*. **43**, 483–494.

Panneerselvam, R., Jaleel, C.A., Somasundaram, R., Sridharan, R. and Gomathinayagam, M. (2007). Carbohydrate metabolism in *Dioscorea esculenta* (Lour.) burk tubers and *Curcuma longa L*. rhizomes during two phases of dormancy. *Colloids and Surfaces Biointerfaces*. **59**, 59–66.

Pérez, F.J. and Lira, W. (2005). Possible role of catalase in post-dormancy budbreak in grapevines. *Journal of Plant Physiology*. **162**, 301–308.

Pérez, F.J., Vergara, R. and Rubio, S. (2008). H_2O_2 is involved in the dormancy-breaking effect of hydrogen cyanamide in grapevine buds. *Plant Growth Regulation*. **55**, 149–155.

Pérez, F.J., Vergara, R. and Or, E. (2009). On the mechanism of dormancy release in grapevine buds: a comparative study between hydrogen cyanamide and sodium azide. *Plant Growth Regulation*. **59**, 145–152.

Ribas-Carbo, M., Taylor, N.L., Giles, L., Busquets, S., Finnegan, P.M., Day D.A., Lambers, H., Medrano, H., Berry, J.A. and Flexas, J. (2005). Effects of water stress on respiration in soybean leaves. *Plant Physiology*. **139**, 466–473.

Rubio, S., Donoso, A. and Pérez, F.J. (2014). The dormancy-breaking stimuli "chilling, hypoxia and cyanamide exposure" up-regulate the expression of α-amylase genes in grapevine buds. *Journal Plant Physiology*. **171**, 373–381.

Sheard, A.G., Johnson, S.D. and Cook N.C. (2009). Effect of timing and concentration of rest breaking agents on budburst in 'Bing' sweet cherry under conditions of inadequate winter chilling in South Africa. *South African Journal of Plant and Soil*. 26 (2), 73-79.

Simmonds, J.A. and Simpson, G.M. (1971). Increased participation of pentose phosphate pathway in response to after ripening and gibberelic acid treatment in caryopses of *Avenafatua*. *Canadian Journal of Botany*. **49**, 1833–1840.

Sudawan, B., Chang, C.S., Chao, H.F., Ku, M.S.B. and Yen, Y.F. (2016). Hydrogen cyanamide breaks grapevine bud dormancy in the summer through transient activation of gene expression and accumulation of reactive oxygen and nitrogen species. *BMC Plant Biology*. **16**, 202.

Swamy, P.M. and Sandhyarani, C.K. (1985). Contribution of the pentose phosphate pathway and glycolytic pathway to dormancy breakage and germination of peanut (*Arachis hypogaea* L.) seeds. *Journal of Experimental Botany*. **37**, 80–88.

Sweetlove, L.J., Beard, K.F.M., Nunes-Nesi, A., Fernie, A.R. and Ratcliffe, R.G. (2010). Not just a circle: flux modes in the plant TCA cycle. *Trends in Plant Science*. **15**, 462–470

Taiz, L. and Zeiger, E. (2010). Plant Physiology. 5nd ed. Sinauer Associates Inc., Sunderland, M.A. Chapter 11, Respiration and and lipid metabolisme.

Tan, Y., Gao, D. S., Li, L., Chen, X. D. and Xu, A. H. (2010). Respiratory response of dormant nectarine floral buds on chilling deficiency. *Agricultural Sciences in China*. **9**, 1448–1454.

Tan, Y., Li, L., Leng, C., Li, D., Chen, X. and Gao, D. (2013). Respiratory response of dormant nectarine vegetative buds to high temperature stress. *Journal of Integrative Agriculture*. **12**, 80–86.

Tharaga, P.C. (2014). Impacts of climate change on accumulated chill units at selected fruit production sites in South Africa. MSc Thesis, Bloemfontein University.

Tian, W.N., Braunstein, L.D., Pang, J., Stuhmeier, K.M., Xi, Q., Tian, X. and Stanton, R.C. (1998). Importance of glucose-6-phosphate dehydrogenase activity for cell growth. *Journal of Biological Chemistry*. **273**, 609–617.

Tiwari, B.S., Belenghi, B., Levine, A. (2002). Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition and programmed cell death. *Plant Physiology.* **128**, 1271–81.

Toro, G. and Pinto, M. (2015). Plant respiration under low oxygen. *Chilean Journal of Agricultural Research*. **75**, 57–70.

Trejo-Martínez, M.A., Orozco, A., Almaguer-Vargas, G., Carvajal-Millán, E. and Gardea, A.A. (2009). Metabolic activity of low chilling grapevine buds forced to budbreak. *Thermochimica Acta.* **481**, 28–31.

Vanlerberghe, G.C., Day, D.A., Wiskich, J.T., Vanlerberghe, A.E. and McIntosh, L. (1995). Alternative oxidase activity in tobacco leaf mitochondria. *Plant Physiology.* **109**, 353–361.

Vergara, R. and Pérez, F.J. (2010). Similarities between natural and chemically induced bud-endodormancy release in grapevine (*Vitis vinifera* L.). *HortScience*. **125**, 648–653.

Vergara, R., Parada, F., Rubio, S. and Pérez, F.J. (2012a). Hypoxia induces H_2O_2 production and activates antioxidant defence system in grapevine buds through mediation of H_2O_2 and ethylene. *Journal of Experimental Botany*. **63**, 4123–4131.

Vergara, R., Rubio, S. and Pérez, F.J. (2012b). Hypoxia and hydrogen cyanamide induce budbreak and up-regulate hypoxic responsive genes (*HRG*) and *VvFT* in grapevine-buds. *Plant Molecular Biology*. **79**, 171–178.

Wang, S.Y., Jiao, H.J. and Faust, M. (1991). Changes in metabolic enzyme activities during Thidiazuron-induced lateral budbreak of apple. *HortScience*. **82**, 231–236.

Wang, S.Y. and Faust M. (1994). Changes in the antioxidant system associated with budbreak in 'Anna' apple (*Malus domestica* Borkh.) Buds. *Journal of the American Society for Horticultural Science*. **119**, 735–741.

Yamane, H. (2014). Regulation of bud dormancy and budbreak in Japanese apricot (*Prunus mume* Siebold and Zucc.) and Peach [*Prunus persica* (L.) Batsch]: *A Summary of Recent Studies. Journal of the Japanese Society for Horticultural Science*. **83**, 187–202.

Zhang, J. and Taylor, C. (2011). The dynamic model provides the best description of the chill process on 'Sirora' pistachio trees in Australia. *HortScience.* **46**, 420–425.

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Table

Table.1: Calculation of the respiratory parameters (Tan *et al.,* 2010 and 2013)

Abbreviation	Parameter	Measurement
R	Total O_2 uptake of buds	O_2 uptake in water/buffer
$Res_{tca\&ppp}$	Residual respiratory rate	O_2 uptake in the presence of MA and Na_3PO_4
R_{tca}	Rate of TCA	R minus respiratory rate in the presence of MA
R_{ppp}	Rate of PPP	R minus $Res_{tca\&ppp}$ and R_{tca}
Con_{tca}	%Contribution of TCA	Dividing R_{tca} with R x 100
Con_{ppp}	%Contribution of PPP	Dividing R_{ppp} with R x 100
$Res_{cyt\&alt}$	Residual respiratory rate	O_2 uptake in the presence of KCN and SHAM
R_{cyt}	Rate of CYT	R minus respiratory rate in the presence of KCN
R_{alt}	Rate of ALT	R minus $Res_{cyt\&alt}$ and R_{cyt}
Con_{cyt}	%Contribution of CYT	Dividing R_{cyt} with R x 100
Con_{alt}	%Contribution of ALT	Dividing R_{alt} with R x 100

Figures

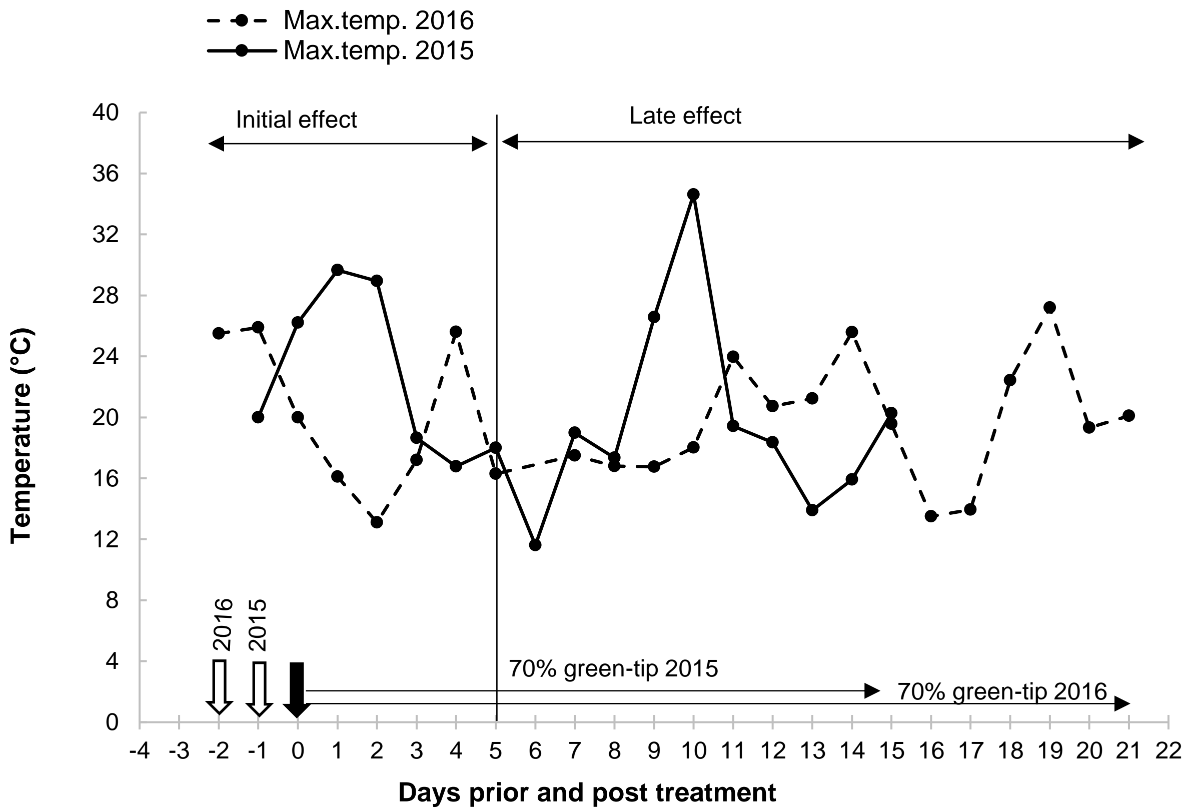


Fig.1: Daily maximum temperatures during 2015 (—) and 2016 (- - -). Negative values represent days prior to treatment. Day 0 is the day of treatment and positive values indicate days post treatment. Open arrows indicate bagging of control shoots and shaded arrow indicates the removal of the bags for both seasons. Horizontal arrows indicate time to 70% green-tip stage of the treated buds. The vertical line indicates the end of the initial response and the start of the longer term effect.

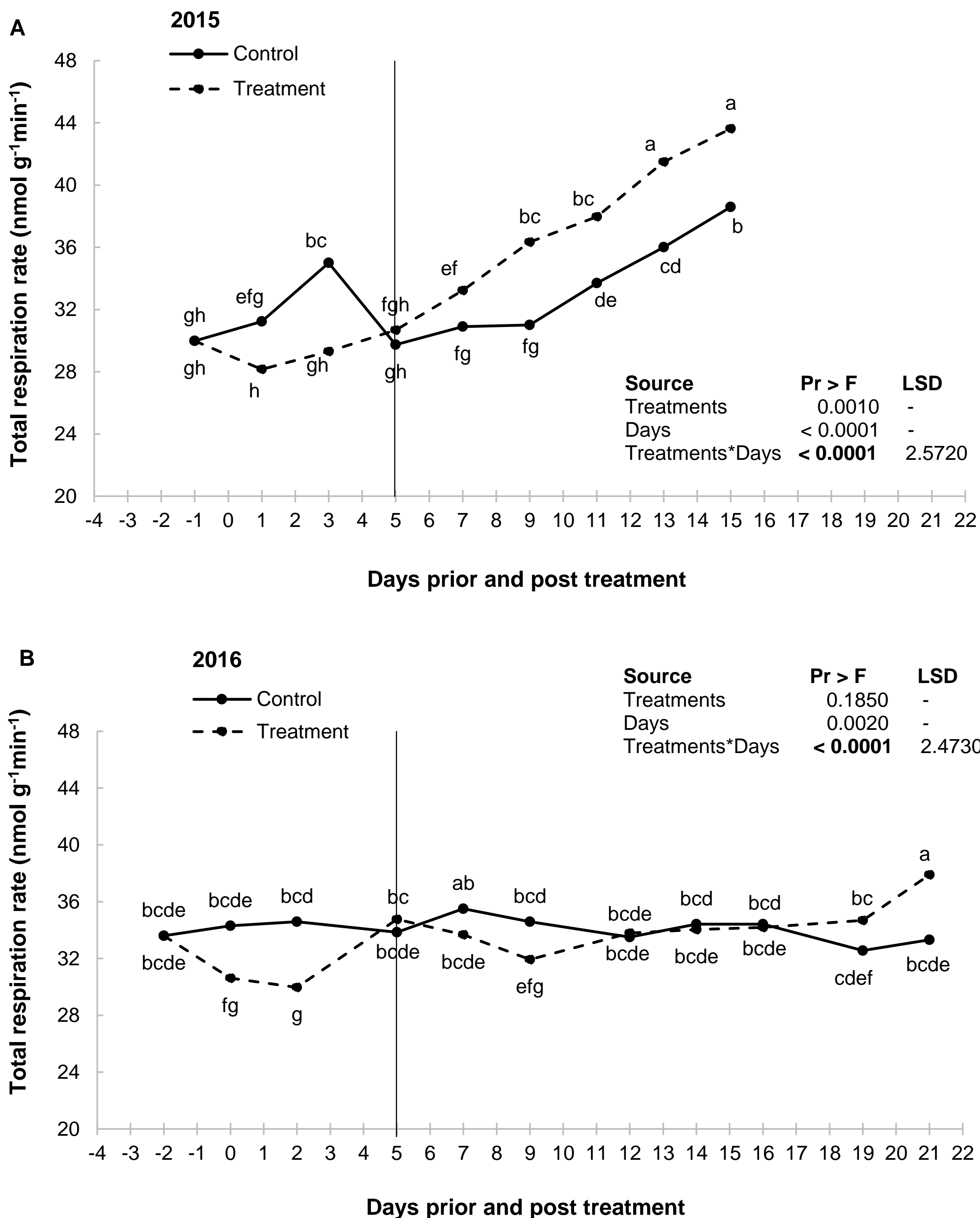
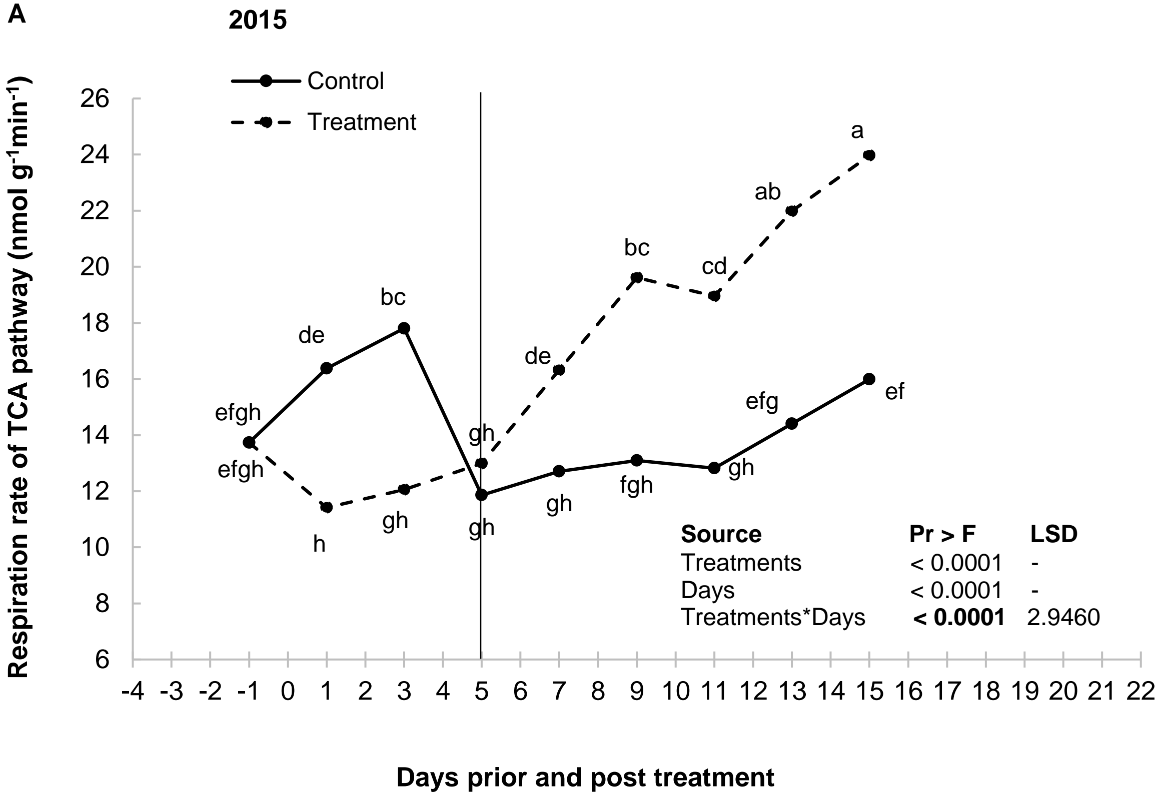
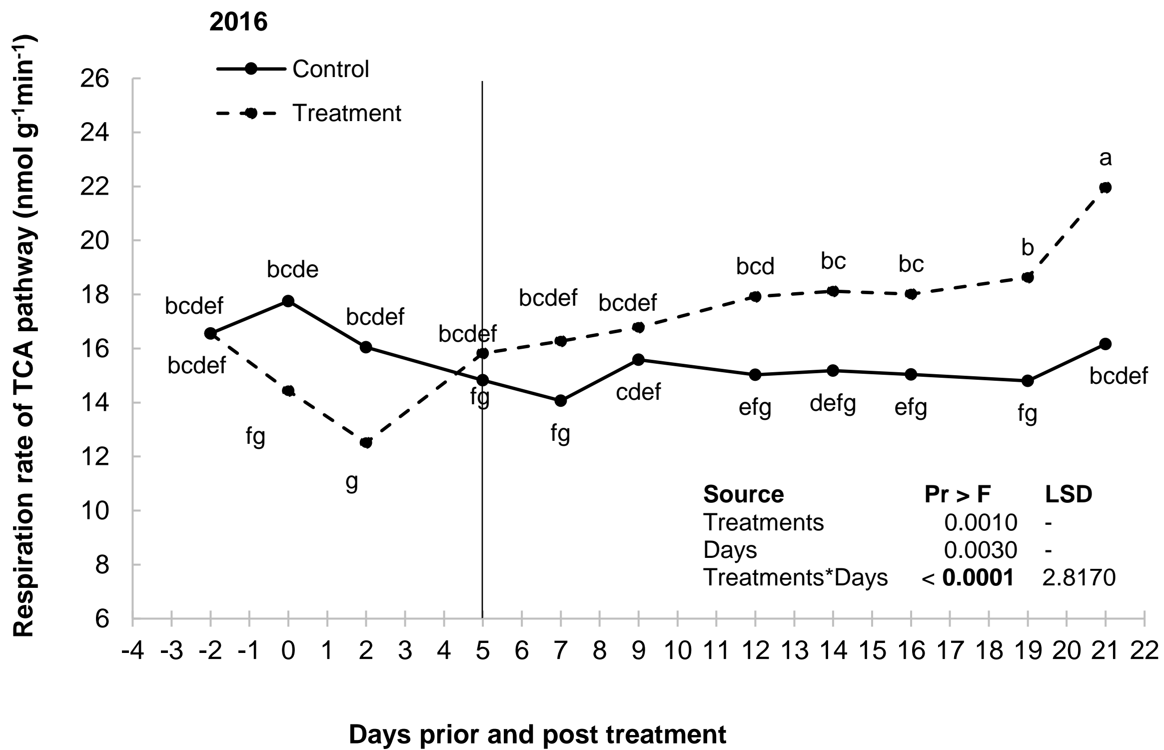


Fig. 2: Oxygen consumption rates of 'Cripps Pink' apple buds treated with HC and oil during 2015 (A) and 2016 (B). Day 0 is the day of treatment. Negative values represent days prior to treatment and positive values days post treatment. Letters indicate significant differences (*p*=0.05). The vertical line indicates the end of the initial response and the start of the longer term effect.


A
2015
Control
Treatment
Respiration rate of TCA pathway (nmol g-1min-1)
Days prior and post treatment
efgh
de
bc
gh
gh
gh
h
efgh
de
bc
cd
ab
a
ef
efg
gh
fgh
Source Pr > F LSD
Treatments < 0.0001 -
Days < 0.0001 -
Treatments*Days < 0.0001 2.9460


B
2016
Control
Treatment
Respiration rate of TCA pathway (nmol g-1min-1)
Days prior and post treatment
bcdef
bcde
bcdef
bcdef
bcdef
fg
g
fg
bcdef
bcdef
bcd
bc
bc
b
a
cdef
efg
defg
efg
fg
bcdef
Source Pr > F LSD
Treatments 0.0010 -
Days 0.0030 -
Treatments*Days < 0.0001 2.8170

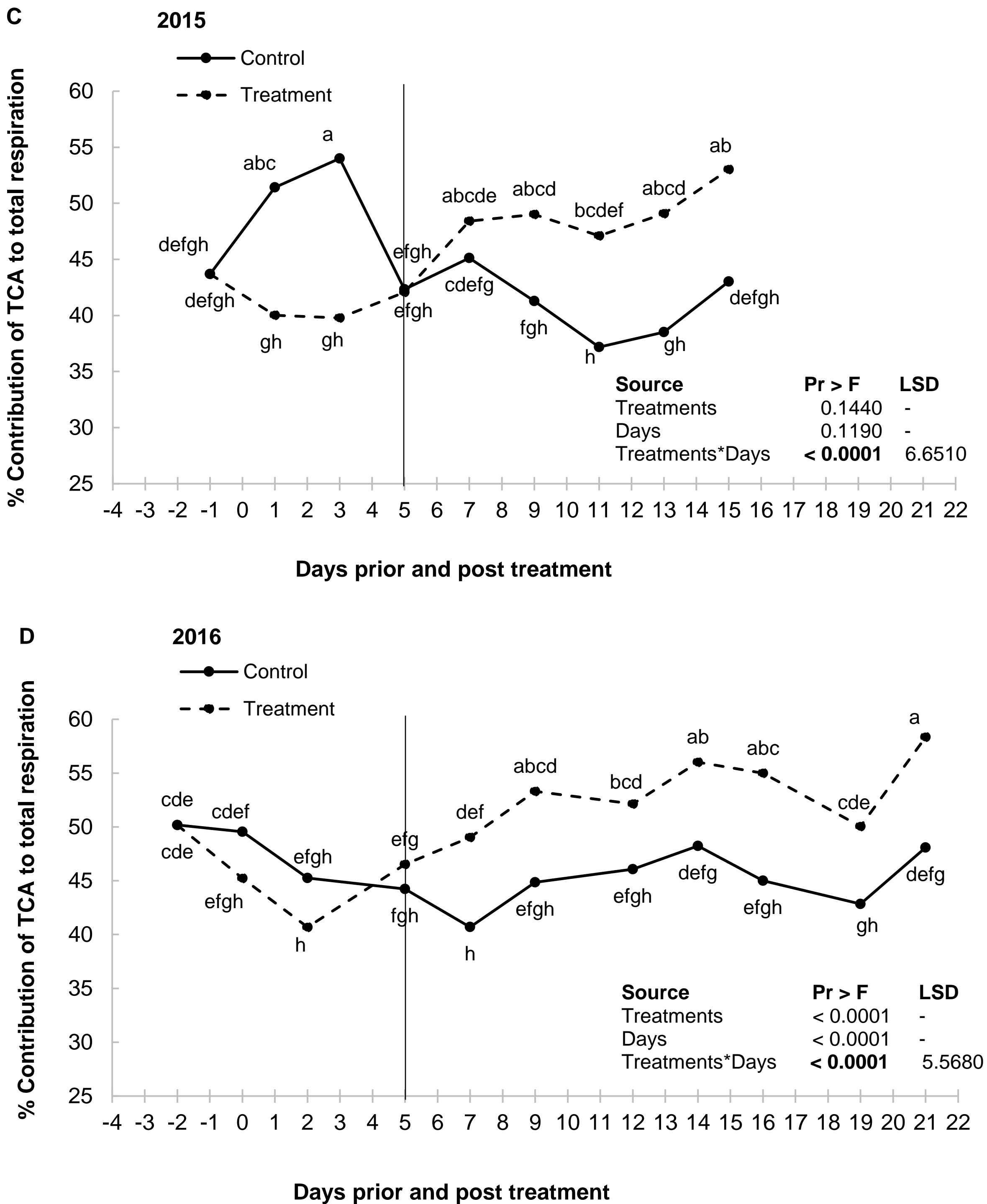


Fig. 3. TCA pathway rate (A and B) and TCA contribution rate (C and D) of 'Cripps Pink' apple buds treated with HC and oil during 2015 (A and C) and 2016 (B and D). Day 0 is the day of treatment. Negative values represent days prior to treatment and positive values days post treatment. Letters indicate significant differences (p=0.05). The vertical line indicates the end of the initial response and the start of the longer term effect.

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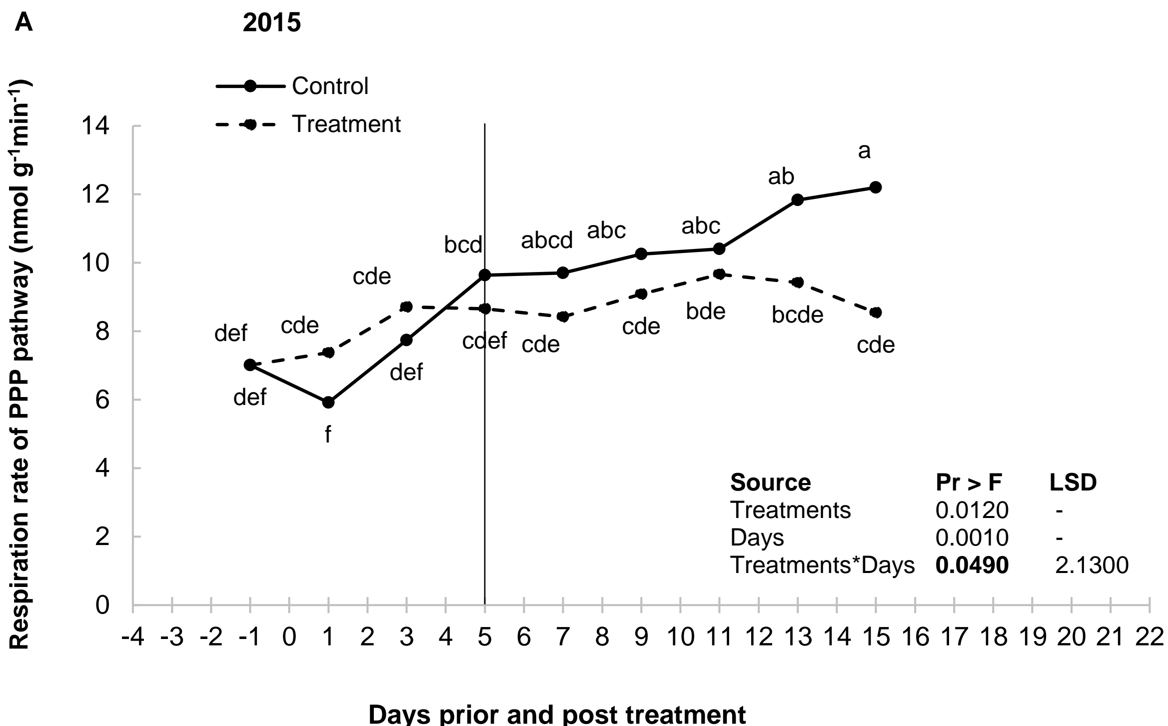
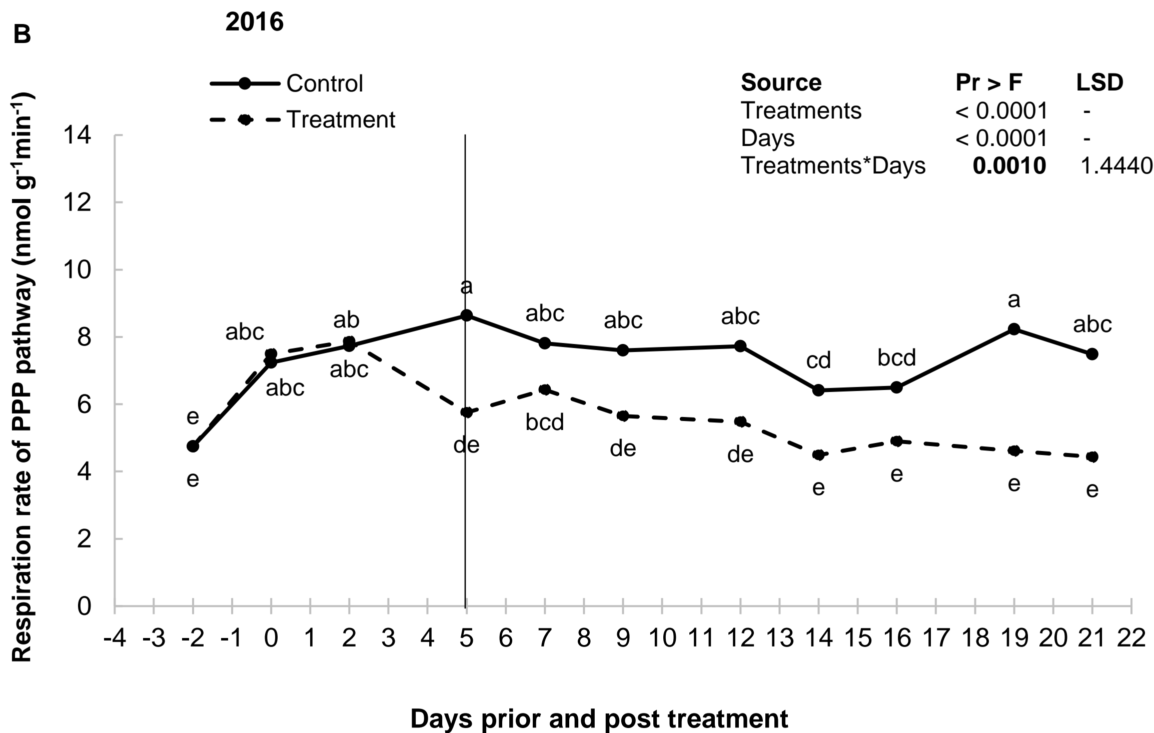




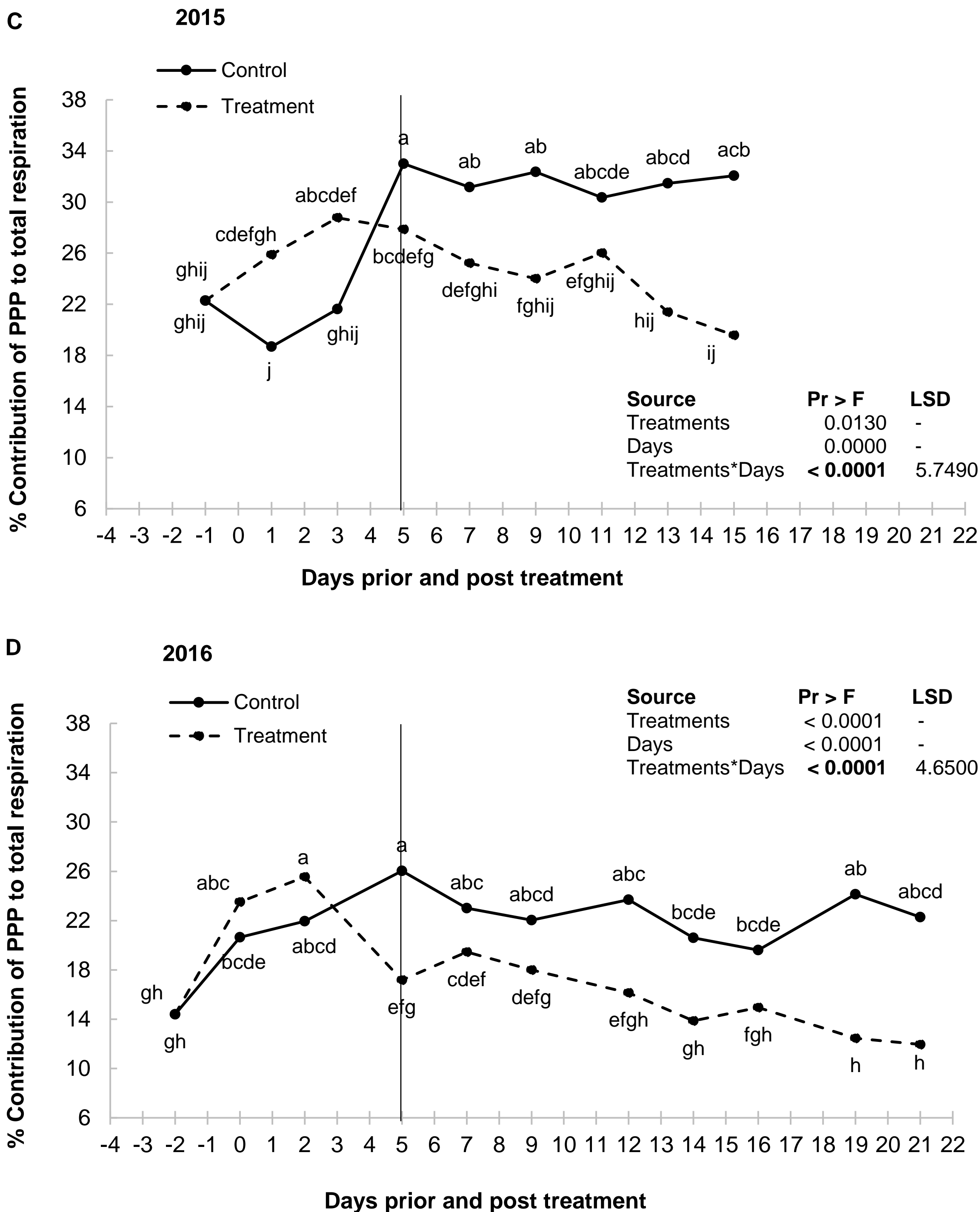
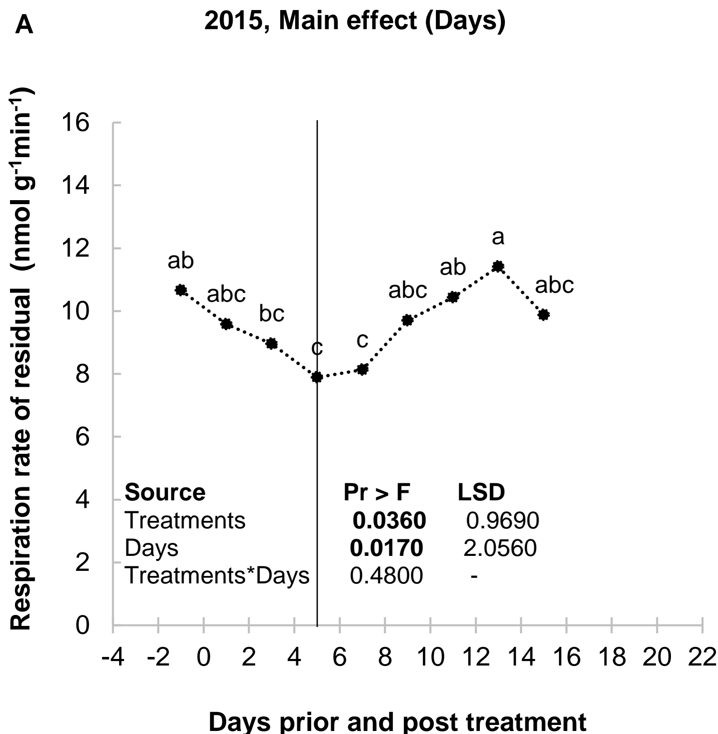
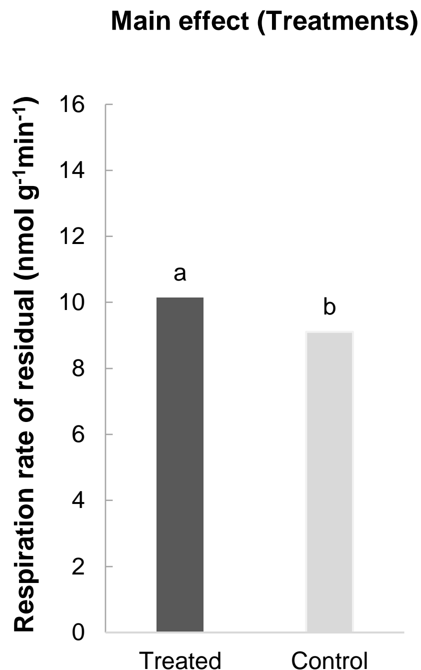
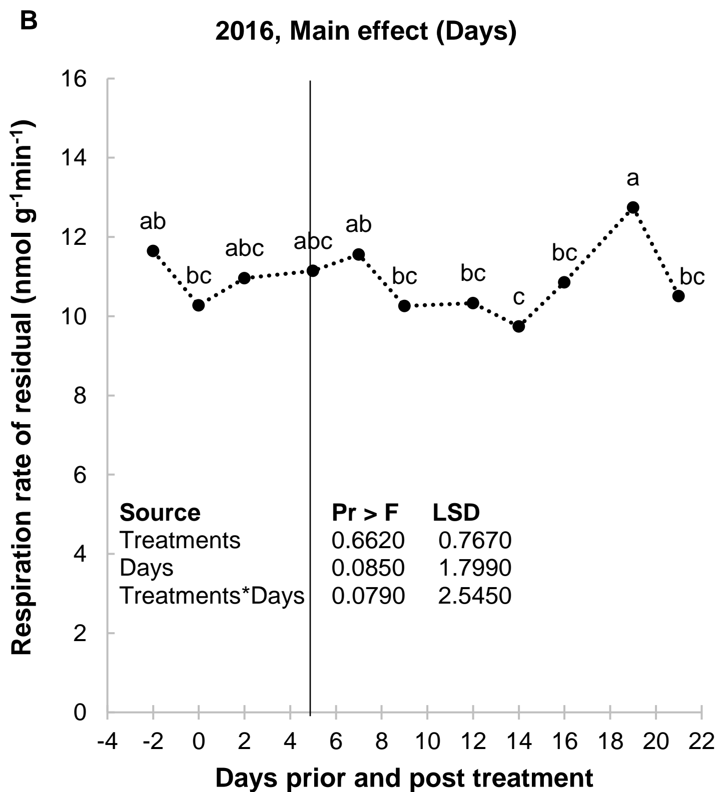
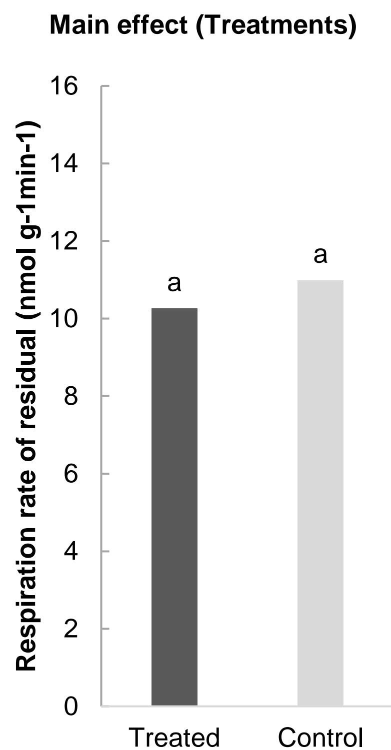


Fig. 4. PPP pathway rate (A and B) and PPP contribution rate (C and D) of 'Cripps Pink' apple buds treated with HC and oil during 2015 (A) and 2016 (B). Day 0 is the day of treatment. Negative values represent days prior to treatment and positive values days post treatment. Letters indicate significant differences (*p*=0.05). The vertical line indicates the end of the initial response and the start of the longer term effect.


A
2015, Main effect (Days)
Respiration rate of residual (nmol g-1min-1)
Days prior and post treatment
ab
abc
bc
c
c
abc
ab
a
abc
Source
Treatments
Days
Treatments*Days
Pr > F
0.0360
0.0170
0.4800
LSD
0.9690
2.0560
-


Main effect (Treatments)
Respiration rate of residual (nmol g-1min-1)
a
b
Treated
Control


B
2016, Main effect (Days)
Respiration rate of residual (nmol g-1min-1)
Days prior and post treatment
ab
bc
abc
abc
ab
bc
bc
c
bc
a
bc
Source
Treatments
Days
Treatments*Days
Pr > F
0.6620
0.0850
0.0790
LSD
0.7670
1.7990
2.5450


Main effect (Treatments)
Respiration rate of residual (nmol g-1min-1)
a
a
Treated
Control

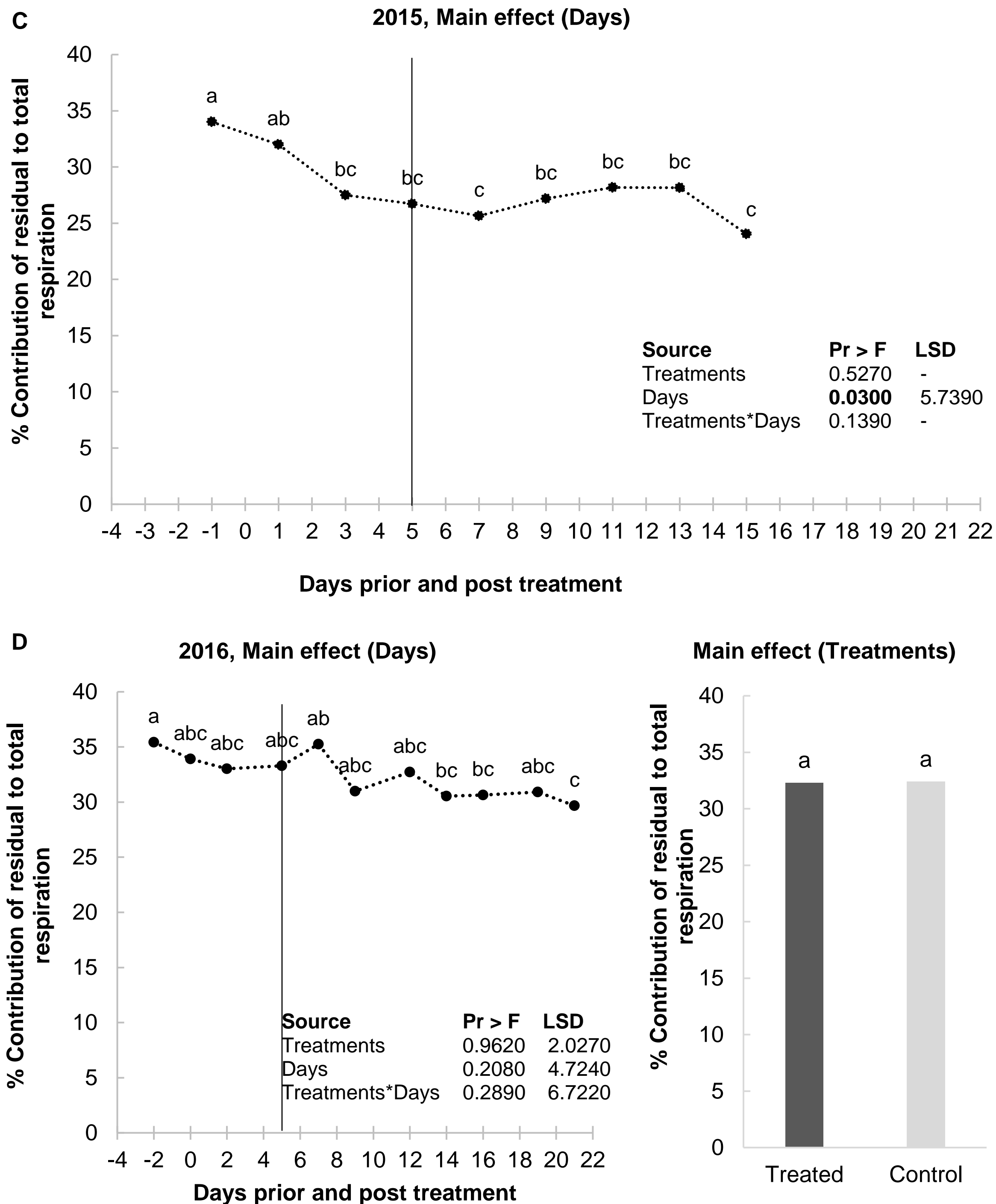
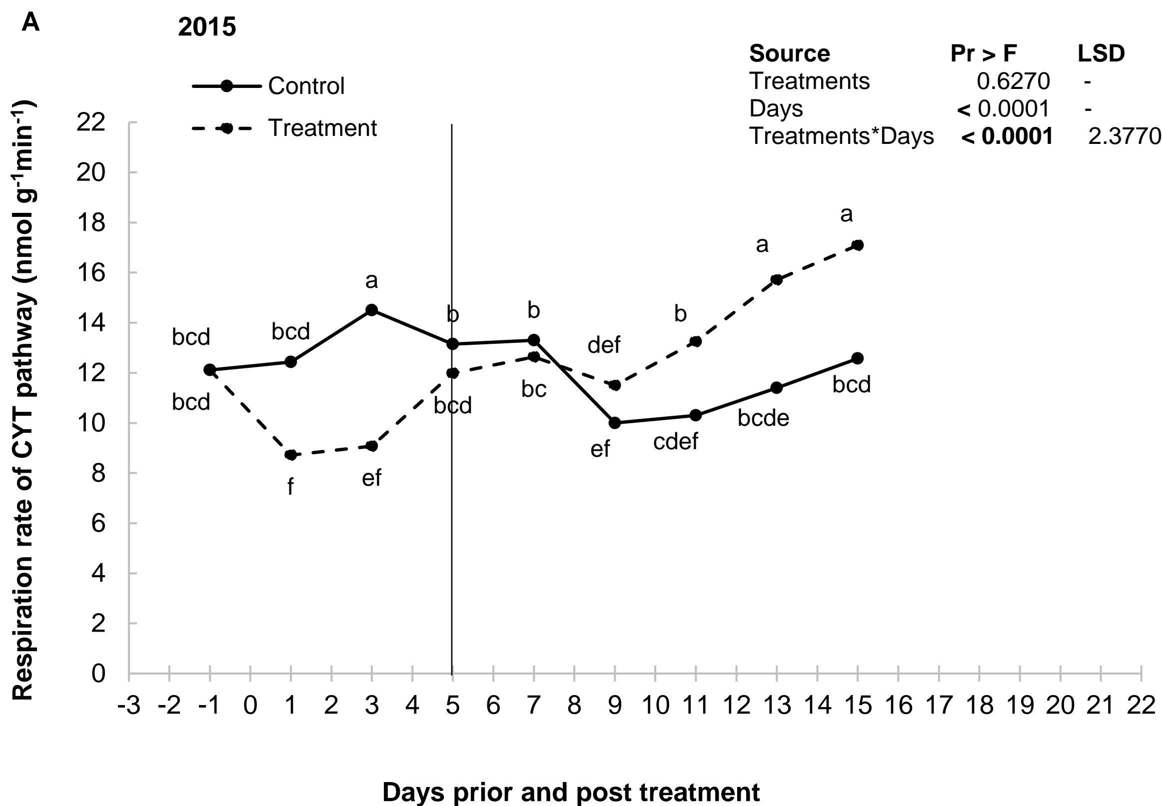
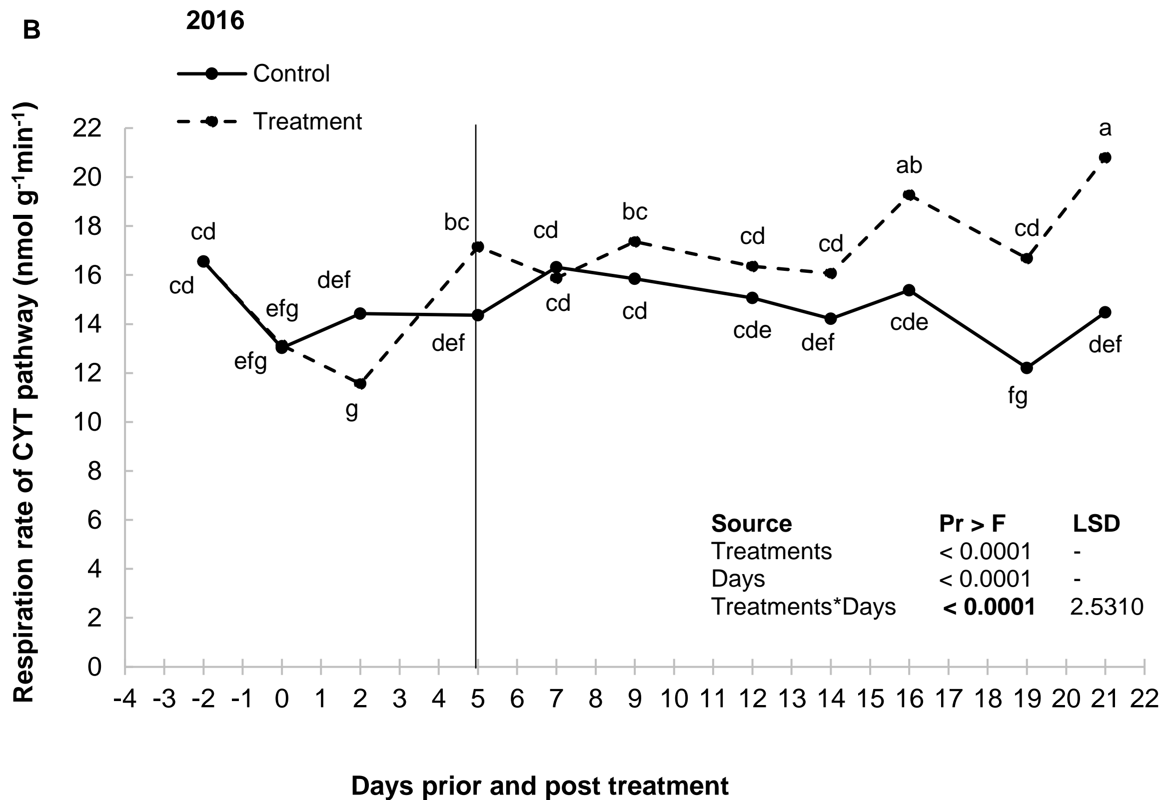


Fig. 5. Residual rate (A and B) and residual contribution rate (C and D) of 'Cripps Pink' apple buds treated with HC and oil during 2015 (A) and 2016 (B). Day 0 is the day of treatment. Negative values represent days prior to treatment and positive values days post treatment. Letters indicate significant differences (*p*=0.05). The vertical line indicates the end of the initial response and the start of the longer term effect.


A
2015
Control
Treatment
Source Pr > F LSD
Treatments 0.6270 -
Days < 0.0001 -
Treatments*Days < 0.0001 2.3770
Respiration rate of CYT pathway (nmol g-1min-1)
Days prior and post treatment


B
2016
Control
Treatment
Source Pr > F LSD
Treatments < 0.0001 -
Days < 0.0001 -
Treatments*Days < 0.0001 2.5310
Respiration rate of CYT pathway (nmol g-1min-1)
Days prior and post treatment

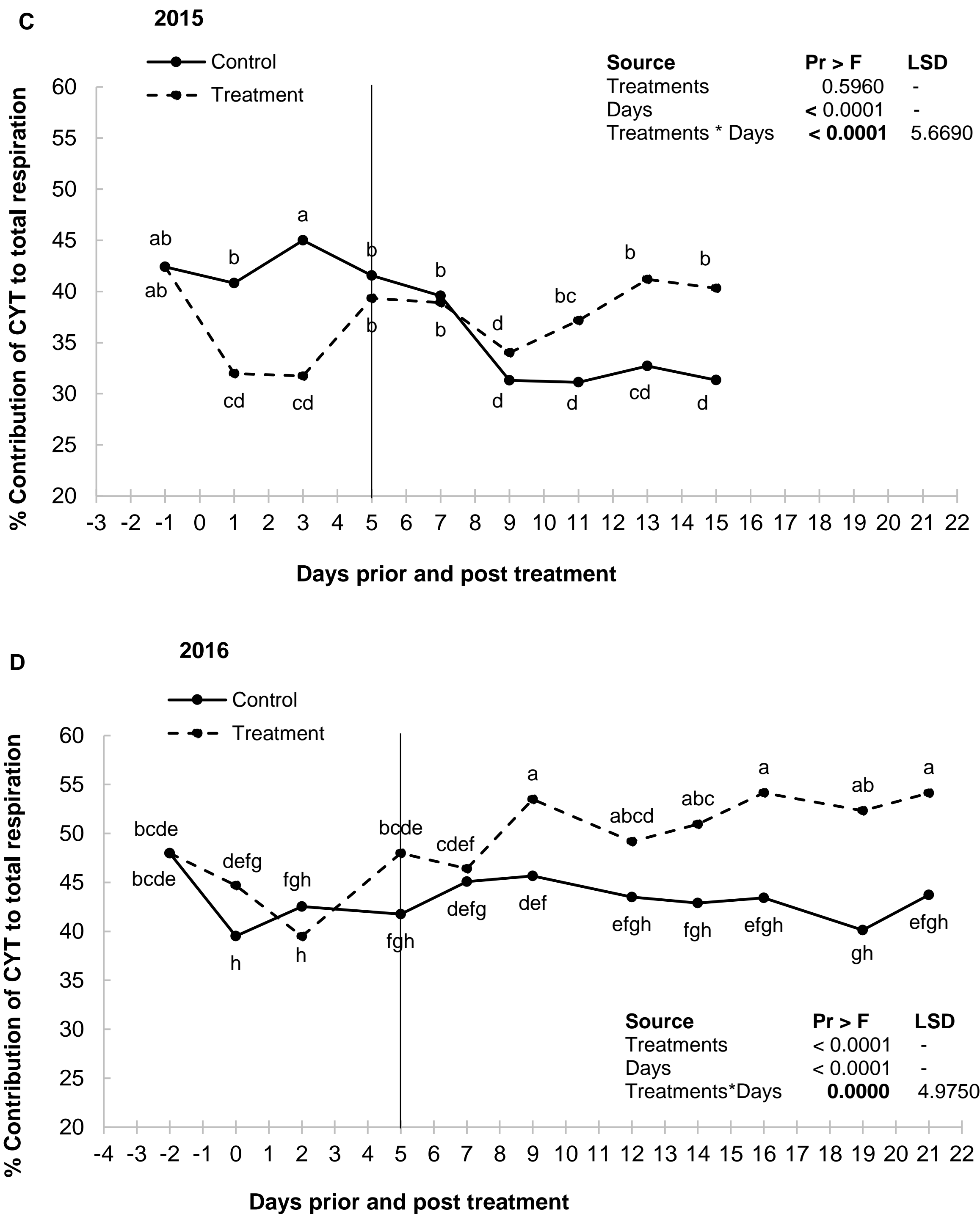


Fig. 6. CYT pathway rate (A and B) and CYT contribution (C and D) of 'Cripps Pink' apple buds treated with HC and oil during 2015 (A) and 2016 (B). Day 0 is the day of treatment. Negative values represent days prior to treatment and positive values days post treatment. Letters indicate significant differences (*p*=0.05). The vertical line indicates the end of the initial response and the start of the longer term effect.

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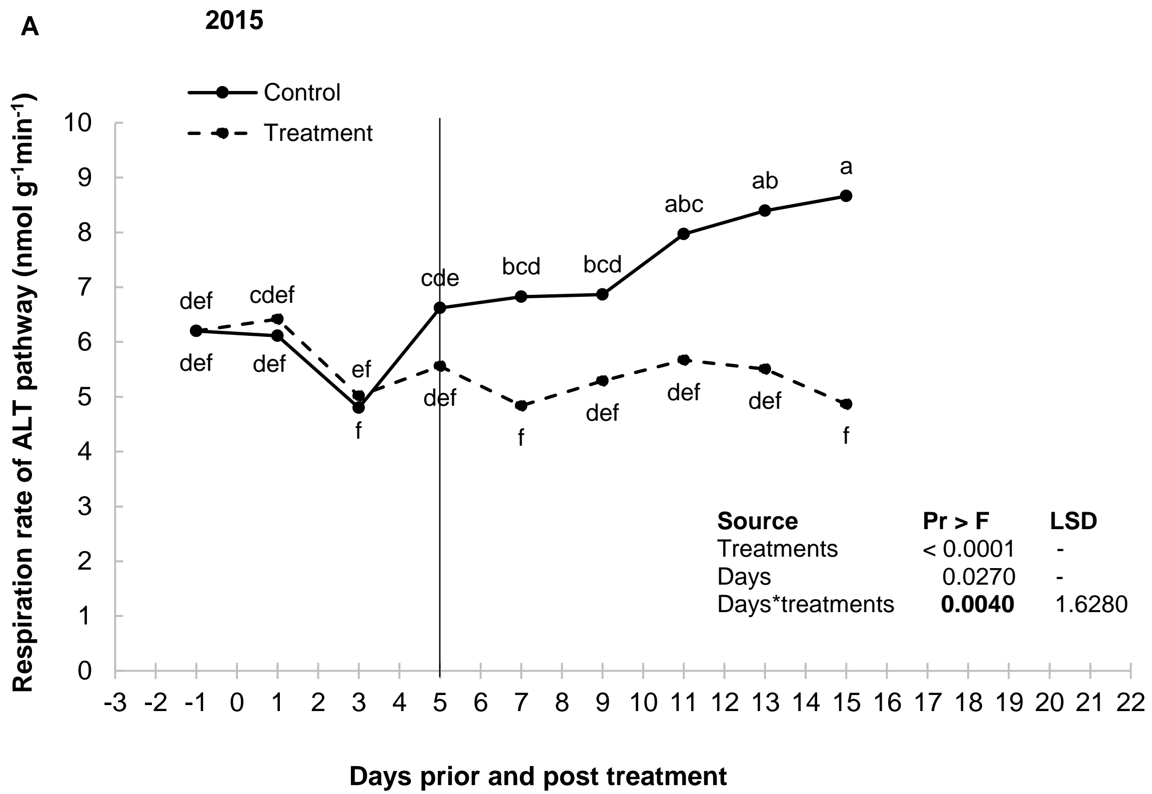
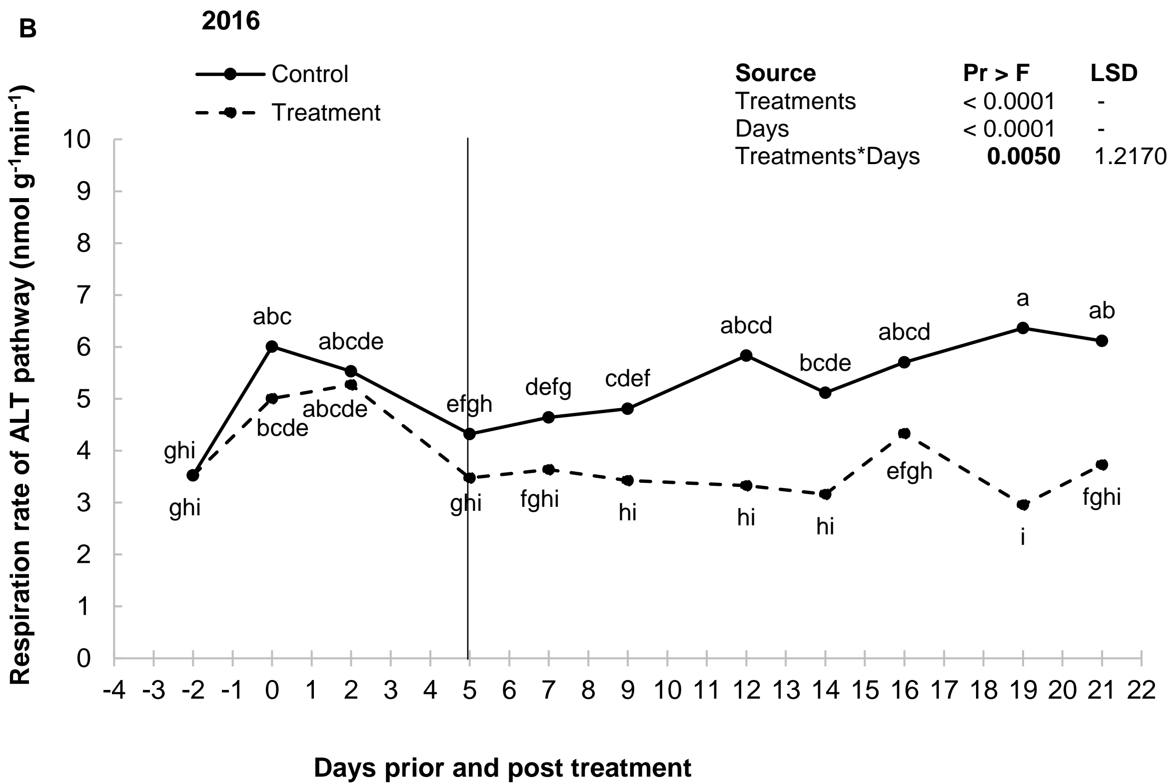




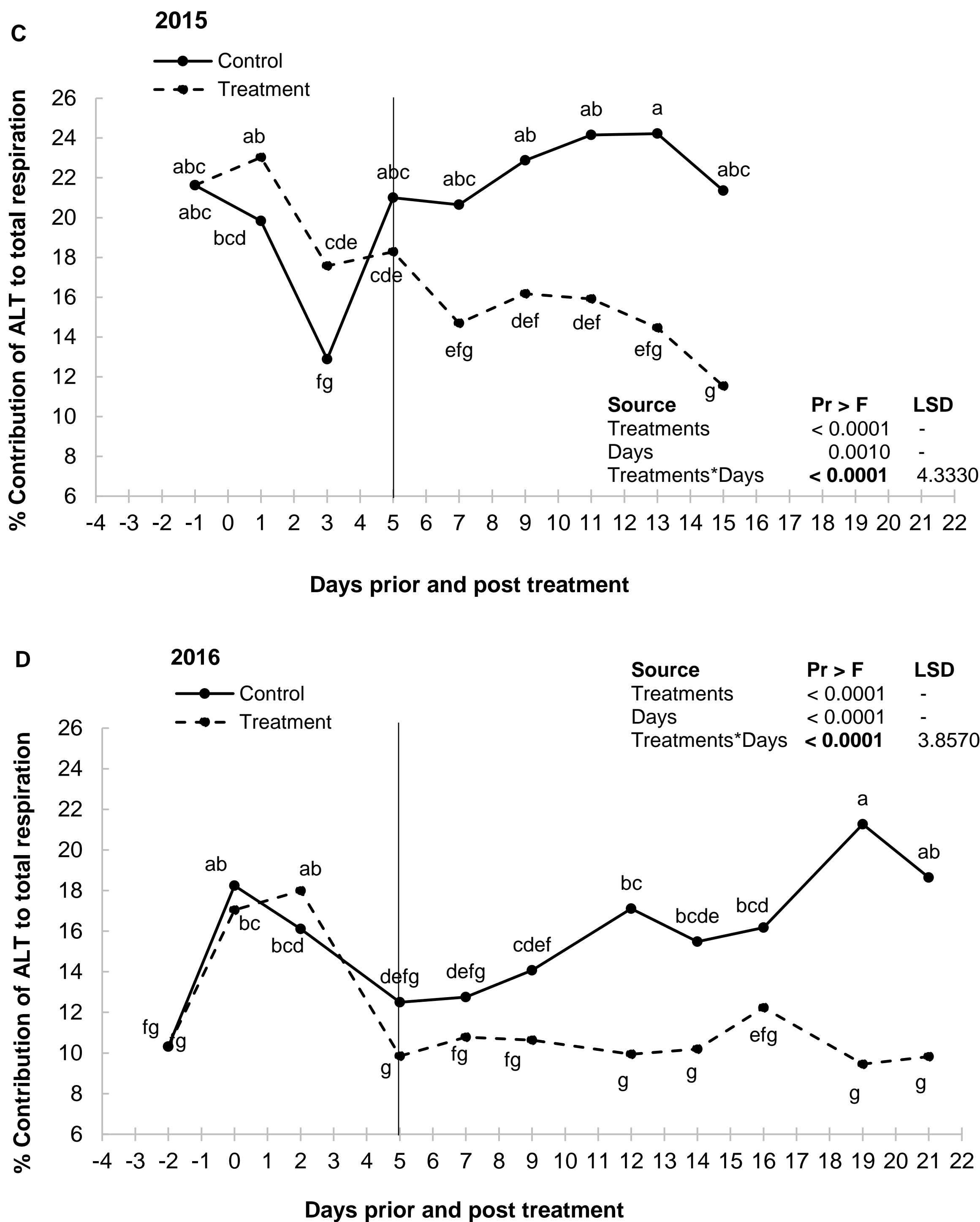
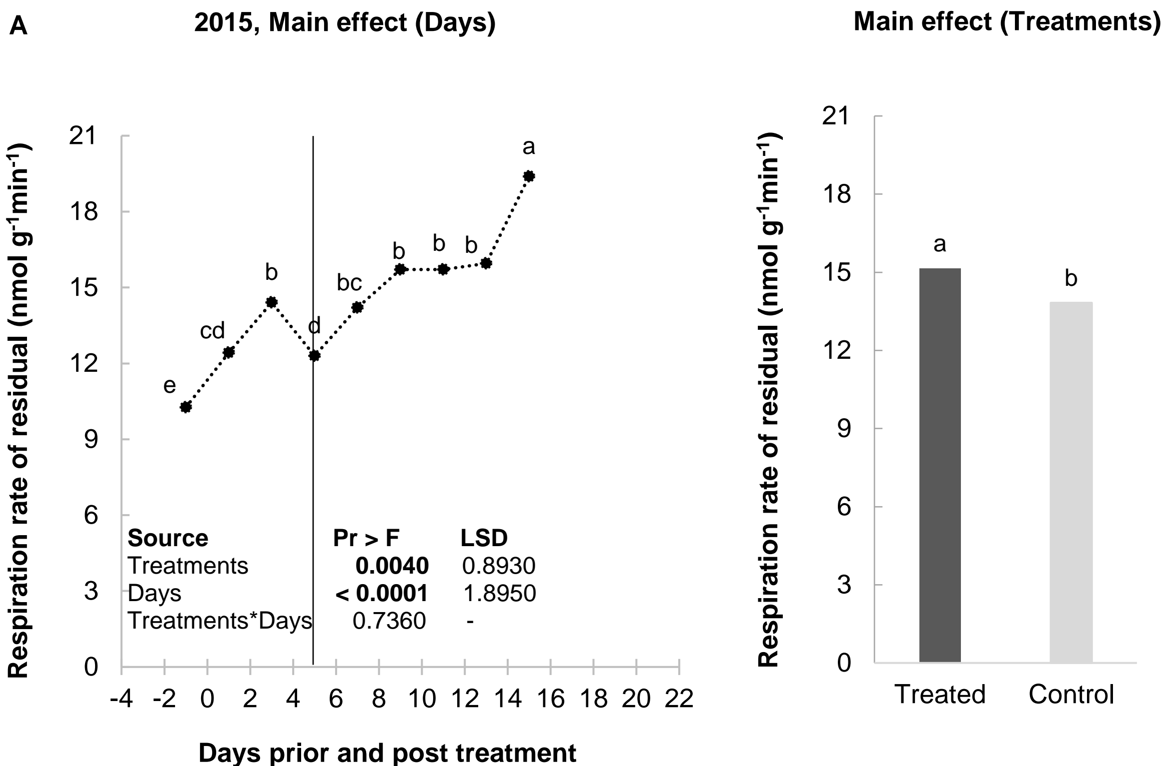
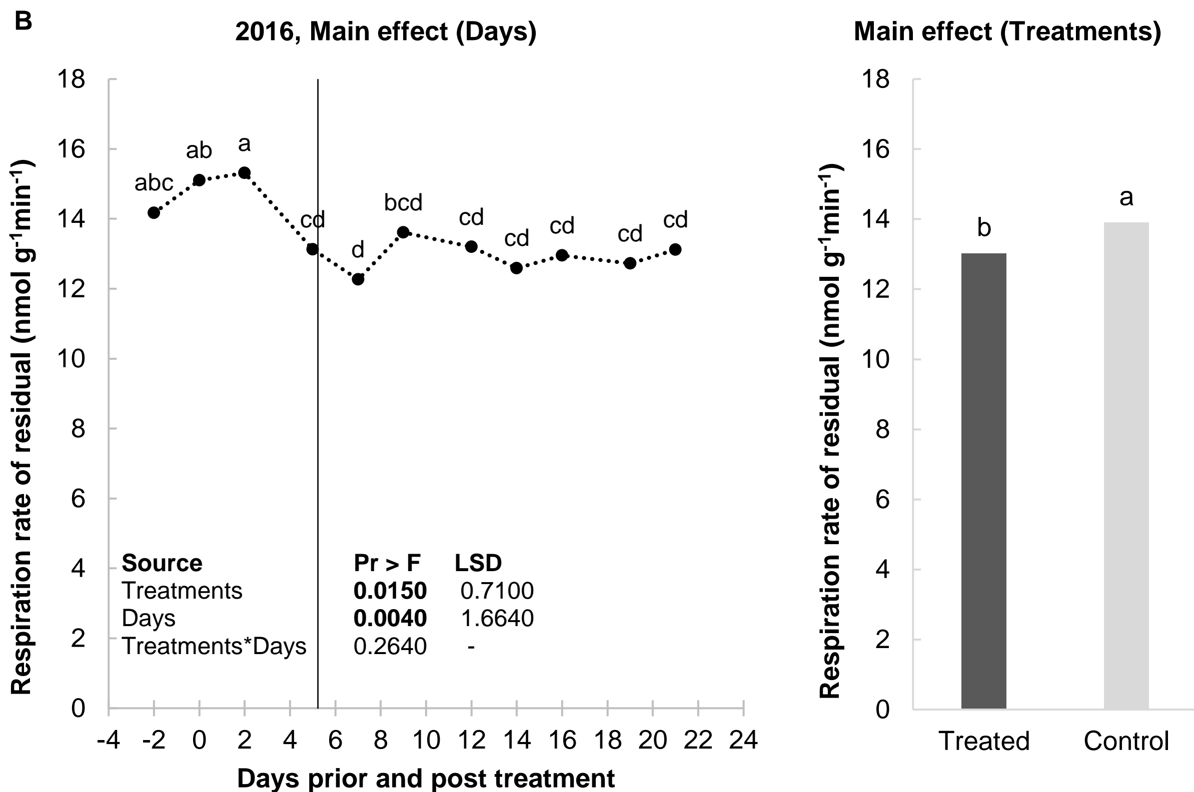


Fig. 7. ALT pathway rate (A and B) and ALT contribution rate (C and D) of 'Cripps Pink' apple buds treated with HC and oil during 2015 (A) and 2016 (B). Day 0 is the day of treatment. Negative values represent days prior to treatment and positive values days post treatment. Letters indicate significant differences (*p*=0.05). The vertical line indicates the end of the initial response and the start of the longer term effect.


A
2015, Main effect (Days)
Main effect (Treatments)
Respiration rate of residual (nmol g-1min-1)
e
cd
b
d
bc
b
b
b
a
Source
Treatments
Days
Treatments*Days
Pr > F
0.0040
< 0.0001
0.7360
LSD
0.8930
1.8950
-
Days prior and post treatment
Treated
Control


B
2016, Main effect (Days)
Main effect (Treatments)
Respiration rate of residual (nmol g-1min-1)
abc
ab
a
cd
d
bcd
cd
cd
cd
cd
cd
Source
Treatments
Days
Treatments*Days
Pr > F
0.0150
0.0040
0.2640
LSD
0.7100
1.6640
-
Days prior and post treatment
Treated
Control

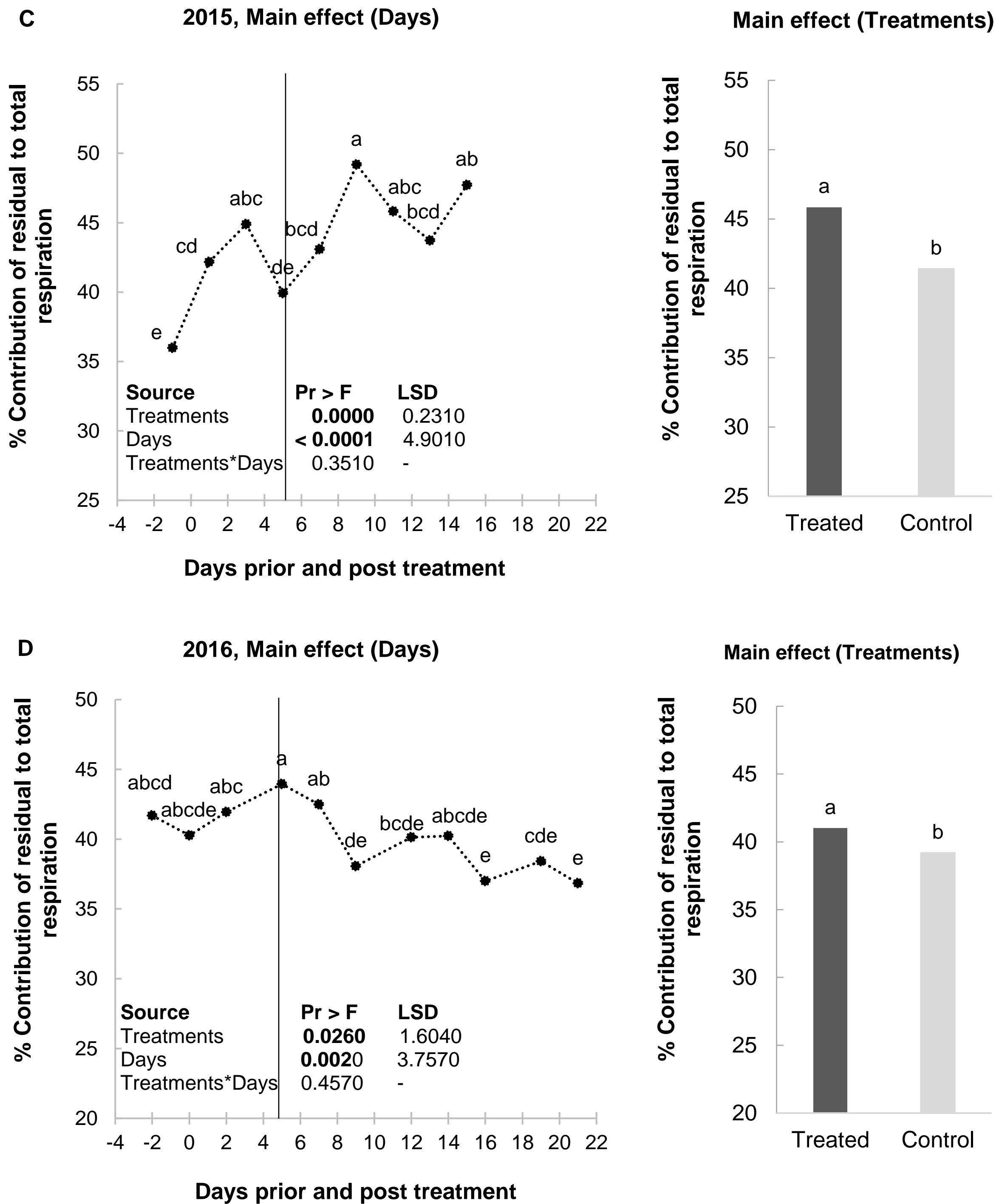


Fig. 8. Residual rate (A and B) and residual contribution rate (C and D) of 'Cripps Pink' apple buds treated with HC and oil during 2015 (A) and 2016 (B). Day 0 is the day of treatment. Negative values represent days prior to treatment and positive values days post treatment. Letters indicate significant differences (p=0.05). The vertical line indicates the end of the initial response and the start of the longer term effect.

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PAPER 3

The effect of insufficient winter chill on the lipids of dormant 'Cripps Pink' apple buds.

Abstract

Temperate deciduous fruit trees undergo irregular budbreak when autumn and winter temperatures are too warm to fulfil their chilling requirement. In warm climates, such as the Western Cape region of South Africa, the lack of sufficiently cold winters affects the crop by decreasing budbreak, and resulting in uneven and delayed foliation and bloom. Environmental conditions affect lipid composition, physical state and function that can lead to cell adaptation. This study was conducted to investigate the effect of insufficient winter chill on the polar and neutral lipids, total free phospholipids and sterols concentration in dormant 'Cripps Pink' apple buds. Terminal apple buds from mature trees were collected from autumn until the end of winter in Elgin (warm area, ±700 CU) and Koue Bokkeveld (colder area, ±1400 CU) during 2015 and 2016. The lipids from both areas were quantified using gas chromatography, while free sterols and free phospholipids were measured spectrophotometrically. The major fatty acids (FA) found in the polar and neutral lipid fractions were similar for both areas in both years. Linoleic acid and linolenic acid were predominant (between 28 – 37% and 32.2 – 45.5% of the total FA concentration, respectively). Buds from the warm region showed a lower degree of C18 desaturation during the dormant period with higher percentages of oleic acid and lower percentages of linoleic and linolenic acid compared to the colder region. This was also evident in the significantly lower (C18:3 +C18:2)/C18:1 ratios during the winter period and especially during growth resumption. A lower unsaturated:saturated FA ratio and a higher free sterol:phospholipid ratio was evident throughout the dormant phase in the warm area. The findings suggest that milder winter conditions favoured the saturated and monounsaturated fatty acids by reducing the desaturation processes. This in turn, may lead to decreased membrane fluidity and permeability hampering growth resumption in the following spring.

Keywords: *apple buds, deficient winter chill, endodormancy, polar lipids, phospholipids, sterols*

1. Introduction

Bud dormancy is an essential adaptive trait for survival, growth and development of temperate deciduous fruit trees. Dormancy is a complex biological phase that is not completely understood. The dormant period is defined as the absence of visible growth in plant tissues that contain meristems and occurs in three stages, i.e. paradormancy, endodormancy and ecodormancy (Lang *et al.*, 1987). Shoot growth stops in mid-summer (paradormancy) followed by bud formation and acclimation during early autumn. Growth inhibition persists and as the cooler temperatures of autumn promote cold hardiness the buds enter a state of endodormancy as winter approaches. Once the chilling requirement has been satisfied endodormancy is rapidly released and the buds progress into a state of ecodormancy where buds are physiologically ready for growth resumption (Rohde and Bhalerao, 2007; van der Schoot and Rinne, 2011). Insufficient winter chill delays the entrance into and exit from dormancy and if the chilling requirement is not satisfied, budbreak occurs at a slow and irregular rate (Cook and Jacobs, 2000). It is known that chill accumulation controls dormancy progression and that the composition of cell membranes in temperate plants changes in response to seasonal and environmental conditions (Theocharis *et al.*, 2012). Cold temperatures are known to trigger the modification of the composition of cell membranes and induce physiological and biochemical changes (Cooke *et al.*, 2012; Theocharis *et al.*, 2012). Therefore, cell membranes are sensors that act as the main sites for perceiving and processing environmental temperatures, or other adverse effects, thus dictating whether a plant cell survives or not (Mittler *et al.*, 2012; Theocharis *et al.*, 2012).

Cell membranes also regulate movement of substances into or out of a cell (Van Meer *et al.*, 2008), a role that is mediated by the functional units of the cell membranes, i.e. polar phospholipids. These molecules are arranged in a bilayer with their hydrophobic (non-charged, nonpolar) tail group forming the inner part of the membrane, while the hydrophilic (charged, polar) head group forms the outer part of the membrane (Furt *et al.*, 2011). Cell membranes may also contain other glycerolipids, as well as sphingolipids, but the main characteristic of all of these lipids are that their tail groups consist of different fatty acids (FAs). These FAs may be saturated or unsaturated and the fluidity of a cell membrane is affected by the degree of FA saturation. Most FAs are made up of 16 or 18 carbon (C) chains that may contain one to three *cis* double bonds. The most prominent saturated FAs (SFAs, with no double bonds) are palmitic (16:0) and stearic (18:0) acid. In higher plants, SFAs can be desaturated to monounsaturated FAs (MUFAs, with one double bond), e.g. stearic acid, can be desaturated to oleic acid (18:1) and MUFAs can be further desaturated to form polyunsaturated FAs (PUFAs), e.g. linoleic acid (18:2, with two *cis*-double bonds) and linolenic acid (18:3, with three *cis*-double bonds). In addition, very long chain

fatty acids (VLCFAs, >C18) are known to occur in higher plants, where they mostly form part of the neutral lipids (Ohlrogge and Browse, 1995, Murata and Wada, 1995). These lipids are also known as triacylglycerols (TAG) or lipid reserves and serve primarily as storage of reduced carbon (energy). Although neutral lipids have no structural function, they undergo similar unsaturation of FAs as polar lipids (Ohlrogge and Browse, 1995). Cell membranes can also be enriched with sterols and proteins, which are involved in diverse biological, structural and physiological functions (Ohlrogge and Browse 1995). In addition, phospholipids present in cell membranes play important roles in plant growth, stress acclimatisation and signalling (Simon, 1974; Xue *et al.*, 2009). It has also been shown that the lipid content of membranes can vary significantly across plant species (Uemura and Peter, 1994) and within the organs of a specific plant species (Wu *et al.*, 2005) and can be significantly altered by environmental conditions (Palta *et al.*, 1993).

It has been shown that intermittent winter chilling not only causes abnormal endodormancy release, but also alters lipid composition by disrupting phospholipid packing and reducing the number of phospholipid molecules in peach buds (Erez *et al.*, 1997). Furthermore, an earlier study associated a decline in cold hardiness with a reduction in the phospholipid concentration and degree of saturation, as well as an increase in the sterol/ phospholipid ratio and membrane fluidity in mulberry (*Morus bombysis*) bark (Yoshida, 1986). Other studies report that sufficient winter chill conditions induce a sequence of desaturation in the FAs increasing the polyunsaturated FA, linolenic acid, to preserve membrane fluidity, stability and permeability during growth resumption (Wang and Faust, 1990a and 1990b). It seems that a fluid and permeable "membrane landscape" is essential for growth resumption. Despite indications that chilling plays a role in the lipid composition of cell membranes, limited information is available on the lipid composition of apple bud cell membranes when exposed to insufficient chill. Therefore, the aim of this study was to investigate the effect of insufficient winter chill on the FA composition of lipids, as well as the total free sterol and phospholipid concentrations in 'Cripps Pink' apple buds throughout the dormant period.

2. Material and Methods

2.1. Plant material and study site

As described in Paper 1, 'Cripps Pink', a medium chill (450 - 800 positive chill units, ARC-Infruitec, 1997 as per Tharaga, 2014) apple cultivar of high commercial importance in South Africa, was used. Shoots were collected from Elgin (34.2°S, 19.0°E, 305 m.a.s.l.) and the Koue Bokkeveld (33.4° S, 19.5° E, 945 m.a.s.l.) in the Western Cape region of South Africa during the dormancy periods of 2015 and 2016. The Koue Bokkeveld is described as a cold winter area with ±1400

Utah CU (sufficient chill) accumulation compared to Elgin, a warm winter area, with ±700 Utah CU accumulation (insufficient chill) (Midgley and Lötze, 2011) during the period of May to September. Three commercial orchards from each area were selected and temperature loggers (Tiny Tag, Gemini Data Loggers, Chichester, UK) were placed in the orchards to record hourly temperature that was converted to Utah chill units (Richardson *et al*., 1974). The orchards were between 11 and 19 years old with trees of similar size, training system and farming practices. All trees had M793 as a rootstock and similar soil types. Historically the first signs of growth resumption (budbreak and green-tip stage) in the warmer area (Elgin) occurs on average 10-14 days later than the colder (Koue Bokkeveld) area.

2.2. Dormancy progression curves from field conditions

As presented in Paper 1, one-year-old shoots (40 cm) were collected randomly from each orchard every 14 days and every seven days during the last month of the dormancy period. Samples were collected over two consecutive seasons (2015 and 2016) starting at the end of April and ending at the time of application of commercial rest breaking agent in late August in the warmer Elgin area. The shoots were defoliated (if necessary) and labelled in the field and brought to the laboratory where they were sorted into three replicates of ten shoots each.

Subsequently, 3 cm was cut off the proximal end of each shoot to remove possible air embolisms and the shoots were placed into 5 L white plastic buckets (three bundles per bucket) containing 1 L of water and 5% sodium hypochlorite (5 ml sodium/L H_2O household bleach). Forcing experiments were performed according to Cook and Jacobs (1999) and Dennis (2003), where by the shoots were placed in a growth chamber with a constant temperature (25 °C) and continuous illumination (215 μmol $m^{-2}s^{-1}$ PAR (photosynthetically active radiation), 93% “cool-white” fluorescent and 7% incandescent light source). The water and bleach mixture was changed every second or third day and the proximal 5 mm of each shoot was removed weekly. Terminal buds were monitored twice per week and the time it took for 50% of the terminal buds in each bundle to bloom was recorded. Dormancy progression curves were constructed by plotting the number of days it took to 50% budbreak (BB) for every sampling point. When a shoot took more than 10 days to 50% BB, it was considered as endodormant as suggested by Cook and Jacobs (1999). The depth of dormancy was considered to increase with an increase in the number of days to 50% BB, while 50% BB within less than 10 days was considered the complete release of endodormancy. The means of the replicates were compared via an ANOVA followed by Fischer's Least Significant Difference test if means were found to differ at a significance level of $p<0.05$. Data analysis was done using XLStat Institute 2017 (version: 19.01.40777 (32 bit)).

2.3. Lipid composition measurements from field conditions

2.3.1. Sample collection

To determine if insufficient chill affected the lipid composition of apple buds, one-year-old shoots (20 to 25 cm in length) were harvested randomly from each orchard (*n* = 35 shoots per orchard) during the dormancy periods of 2015 and 2016. In 2015, sample collection was done every 14 days from 28 April to 2 August and thereafter on a weekly basis up until 1 September before the application of rest breaking agent in the warmer Elgin area. In 2016, sample collection started on 28 April and shoots were collected every 21 days until 6 June, every 14 days from 6 June until 3 August and every 7 days from 3 August until 25 August before the application of rest breaking agent in the warmer Elgin area. Following collection, the shoots were wrapped in plastic bags and transported to the laboratory. The terminal buds from each area were excised and pooled into triplicate replicates (*n* = 35 buds per replicate), frozen in liquid nitrogen within 24 hours after collection and stored at -80 °C until further use.

2.3.2. Lipid extraction

To determine the lipid content of the field collected and 'lab-chilled buds' (detail to follow in section 2.4), each of the samples were milled with a ceramic mortar and pestle in liquid nitrogen and stored at -80 °C until further analysis. The total lipids were extracted from the frozen milled samples using the method of Folsch *et al.* (1957) as modified by Jooste (2014). This extraction approach fractionates a mix of membranes from various cellular organelles, such as mitochondria, tonoplasts and chloroplasts. All extractions were conducted at room temperature using glassware and Teflon lined caps to minimise plastic contamination of lipid extracts (Christie, 1993). Since prolonged air contamination during lipid extraction induces autoxidation that attacks free radicals (Christie, 1986), glass reaction vessels that contained the lipid extracts were handled in limited atmospheric air. Autoxidation and lipid degradation by enzymes during the extraction process was reduced by adding butylated hydroxytoluene (BHT) (Sigma- Aldrich, Johannesburg, South Africa) to solvents as a synthetic antioxidant. To extract lipids from the buds, 6 mL of a methanol-BHT mixture (100 % methanol [Merck, Johannesburg, South Africa]; 0.01 % [w/ v] BHT) was added to 0.5 g of each frozen sample and vortexed. The internal standard was prepared by mixing 0.6 g of heptadecanoic acid (Sigma- Aldrich) with 500 mL HPLC grade hexane (Merck) and of this standard 200 µL was added to each sample. Subsequently, 12 mL of chloroform (Merck) containing 0.01% (w/ v) BHT was added to the samples and vortexed. Each extract was then filtered using a vacuum pump and Büchner funnel (Merck) and the extraction tubes were cleaned with 12 mL chloroform: methanol (2:1, v/ v) mixture with 0.01 % (w/ v) BHT. Following the addition

of 7.5 mL of a 0.88 % (w/ v) potassium chloride (KCl) (Merck) solution to each sample, the tubes were shaken to allow the two phases to separate. Thereafter, vacuum aspiration was used to remove the top, aqueous layer and the bottom chloroform layer was evaporated to complete dryness under vacuum using a Savant (Thermo Savant, Inc., Waltham, USA). The concentrated lipids were dissolved with 2 mL of a chloroform-BHT mixture (100 % chloroform; 0.01 % [w/ v] BHT) and stored at -80 ºC until further analysis.

2.3.3. Polar lipids, neutral lipids and free fatty acids

To determine the fatty acid content of neutral and polar lipids, each of the total lipid extracts were first fractionated into free, neutral and polar lipids according to the methods of Kaluzny *et al.* (1985), Christie (1989), Pietsch and Lorenz (1993), Laffargue *et al.* (2007), as adapted by Jooste *et al.* (2014). To achieve this 6 mL (500 mg) NH_2 cartridges (Chromabond columns, Düren, Germany) were fitted to a Visiprep™24 vacuum manifold (Supelco, Bellefonte, USA) and washed twice with 2 mL HPLC-grade hexane (Merck, Johannesburg, South Africa) under a vacuum of -20 kPa. Thereafter, 300 µL of each total lipid extract was applied to a separate cartridge and allowed to run through the column under vacuum. First, the neutral lipids (NLs) were eluted four times from the cartridges by running 1 mL of a chloroform: isopropanol (Merck) (2:1, v/ v) solution through the cartridges and collecting the eluent in kimix test tubes (Lasec, Cape Town, South Africa). Thereafter, the free fatty acids (FFA) were eluted again four times with 1 mL of a diethyl ether (Merck): acetic acid (Merck) (98:2, v/ v) solution. After each of the 1 mL elution steps, the FFA fraction was collected in a kimix tube and kept for later analysis as it contained the internal standard (n-Heptadecanoic acid). Lastly the polar lipids (PLs) were eluted four times with 1 mL of HPLC-grade methanol (Sigma-Aldrich, Johannesburg, South Africa) and collected in kimix tubes. After each of the lipid classes were separated, the solvents were evaporated through by vacuum aspiration at medium temperature (40 ºC).

After the fractions were dry, they were methylated to form fatty acid methyl esters (FAMEs) for gas chromatography (GC) analysis. To achieve this, 0.4 mL of a 10 % (w/ v) boron trifluoride (BF_3) (Sigma-Aldrich, Johannesburg, South Africa) in a methanol solution was added to the three fractions (NLs, FFA and PLs) of each sample, followed by a heating each sample at 100 °C on a heating block for 5 min and cooling the samples to room temperature in a water bath. The FAMEs were then extracted by adding 2 mL of HPLC-grade hexane and 1 mL deionized H_2O to each sample, followed by centrifugation at 20 ˚C for 5 min @ 3000 rpm. The FAMEs were immediately quantified, by transferring 1 mL of the top hexane layer of each sample to a gas chromatography (GC) vial (Chemetrix, Johannesburg, South Africa). Thereafter, a 2 µL aliquot was subjected to GC analysis using a model 7890A GC instrument (Model 7890A, Agilent Inc., Wilmington, USA)

fitted with a flame ionization detector (FID) and automatic sampler (Model 7683B, Agilent Inc., Wilmington, USA) by split injection (5:1) with a split vent flow rate of 68.3 mL min^{-1} and septum purge flow rate of 3 mL min^{-1}. Helium was used as a carrier gas at a column flow rate of 1.4 mL min^{-1} and a column inlet pressure of 270 kPa. Both the injector and detector were at 260 ºC. Fatty acid separations were done on an HP-Innowax capillary column (30 m × 0.25 I.D. and 0.25 µm film thickness, coated with 100% polyethylene glycol, J&W Scientific, Folsom and Agilent Technologies Inc., Wilmington, USA). The oven temperature was programmed from 150 °C (isothermal for 1 min) to 170 ºC at 1.2 ºC min^{-1}, from 170 ºC to 180 ºC at 5 ºC min^{-1} and from 180 ºC to 250 ºC (isothermal for 6 min) at 20 ºC min^{-1}. Data were collected and integrated using the GC Chemstation software (Rev. B.04.02 (96), Agilent Technologies, Waldbronn, Germany). Quantification of the unknown FAMEs was achieved by setting up a standard curve of the Supelco 37 component FAME Mix and subjecting the samples to a similar GC analysis as described above and calculating the ratio between the obtained values and the internal standard (unmethylated n-Heptadecanoic acid), contained in the free fatty acid fraction.

2.3.4. Total free sterols

The quantification of total sterols from the bud samples was done in triplicate at room temperature using the method of Courchaine *et al.* (1959) as adapted by Jooste *et al.* (2014) with minor modifications. During the preliminary assays, a number of trials were done in order to adjust sample size according to the absorbance range values. Prior to analysis, a ferric chloride in phosphoric acid solution (2.5 g $FeCl_3.6H_2O$ [Merck, Johannesburg, South Africa]; 100 mL concentrated phosphoric acid [Merck]) was prepared, of which 8 mL was added to 100 mL concentrated sulphuric acid (Merck) to obtain the ferric chloride colour reagent. Subsequently, a 100 µL aliquot of each total lipid extract (see section 2.3.3) was evaporated to dryness in a stream of nitrogen in a fume hood, after which it was dissolved in 6 mL glacial acetic acid (Merck). In addition, a standard curve of stigmasterol (Merck) with a concentration range of 0 – 1.5 mg mL^{-1} was prepared in glacial acetic acid. Thereafter, 4 mL of the ferric chloride colour reagent was added to 6 mL of each of the standards and to 6 mL of each sample. The resulting mixtures were vortexed and incubated for colour development to occur. Of each mixture, 1 mL was transferred to plastic cuvettes (Lasec, Cape Town, South Africa) and the amount of total free sterol was determined by measuring the absorbance at 550 nm using a UV/VIS spectrophotometer (Varian, 50 Bio-UV-Visible Spectrophotometer, Mulgrave, Australia). The total sterol concentration present in each sample of both seasons (2015 and 2016) was calculated from the stigmasterol standard curve and was expressed as mg stigmasterol equivalents g^{-1} FW.

2.3.5. Total free phospholipids

The concentration of the total free phospholipids present in buds from both areas (Koue Bokkeveld and Elgin) and seasons (2015 and 2016) was quantified according to the adapted method of Ames (1966) and Duck-Chong (1979) as modified by Jooste *et al.* (2014) with minor modifications. All measurements were done at room temperature and all glassware were scrupulously cleaned (free of phosphate) because of the sensitivity of the method. To measure the total phospholipids in each sample, 30 µL of 10% (w/ v) $Mg(NO_3)_2.6H_2O$ (Sigma- Aldrich, Johannesburg, South Africa) in 95% methanol was added to 25 µl of each total lipid extract (see section 2.3.3). In addition, a sodium phosphate standard curve ranging from 0 – 0.125 mg/ml was prepared and 30 µL of the same mixture was added to each of the tubes. Subsequently, the liquid in the tubes was evaporated and the tubes were dried in a heating block for 5 minutes at 95 °C in a fume hood. The resulting precipitate was heated with a Bunsen burner in a fume hood until the brown fumes disappeared. The pyrophosphate reaction, which formed during burning, was subsequently hydrolysed to phosphate by adding 300 µL of 0.5 M HCl (Merck, Johannesburg, South Africa) to each sample. The samples were then heated in a heating block at 100 °C for 15 min and immediately cooled by immersion in a room temperature water bath. After the tubes had cooled, 700 µL of a 1:6 (v/ v) mixture of 10% (w/v) ascorbic acid (Sigma-Aldrich) and 0.42% (w/v) ammonium molybdate tetrahydrate (Laboratory Supplies, Johannesburg, South Africa) in 1 N H_2SO_4 (Merck) were added to each sample and to the standard curve designated tubes. Following incubation at 45 ºC for 20 min on a heating block, 1 mL of each sample was added to a plastic cuvette (1cm path length) and the absorbance was read at 820 nm using a 50 Bio-UV-Visible spectrophotometer (Varian, 50 Bio-UV-Visible Spectrophotometer, Mulgrave, Australia). The quantity of total free phospholipids present in each sample was determined from the sodium phosphate standard curve and was expressed as µmol g^{-1} FW.

2.4. Dormancy progression curves and lipid measurements from 'lab-chilled buds'

To compare these findings to buds that received a controlled amount of chill, we included buds that were exposed to increasingly more CU under artificial conditions. These buds are subsequently referred to as 'lab-chilled buds'. On 25 April 2016, shoots were randomly collected from the same orchard used as described in 2.1 in the Koue Bokkeveld, defoliated and brought to the laboratory. The shoots (with 53 Utah CU already accumulated in the field) were folded in moist paper and plastic before placed into a cold room at 4˚C where it accumulated 24 Utah CU every 24 hours (Richardson *et al.*, 1974). Shoots were removed from the cold room every two weeks (from 324 until 1997 Utah CU) and processed.

The dormancy progression and lipid composition of the buds were tested as indicated above (sections 2.2 and 2.3). Three replications (ten shoots per replication) were used for dormancy progression and three replications (n = 35 terminal buds per replicate) for the lipid composition assays.

2.5. Calculations and statistical analyses

The percentage of individual FAs in the NL and PL fractions were calculated and the different FA groupings and ratios are expressed to make it comparable to similar studies from literature (Wang and Faust 1988, 1990; Ruiz *et al.*, 2004; Jooste, 2012). The total percentage of saturated fatty acids (%SFA) was obtained by adding up all the individual percentages of saturated FAs present in the total fraction (PL or NL). Saturated fatty acid chains contain only single bonds (e.g. palmitic acid (16:0), stearic acid (C18:0)) while the unsaturated chains have one or more *cis* double bonds (e.g. oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3)). Similarly, the total percentage unsaturated fatty acids (%UFA) was determined by adding up all the individual percentages of the unsaturated FAs present in the total fraction (PL or NL). From this the UFA: SFA ratio for both the PL and NL fractions was calculated. The percentage monounsaturated fatty acids (%MUFA) were obtained by adding the percentages of all the FAs that had only one double bond (e.g. oleic acid (C18:1n9c)), while the percentage polyunsaturated fatty acids (%PUFA) was calculated by adding up the percentages of all the FAs that had two or more double bonds (e.g. linoleic acid (C18:2n6c), linolenic acid (C18:3n3)) and from this the MUFA: PUFA ratio was calculated for the PL and NL fractions. Lastly, the unsaturation ratios of the C18 FAs (C18:3+C18:2)/ (C18:1), (C18:3)/ (C18:1+C18:2) and (C18:3/C18:2) in the PL and NL fractions, were calculated similarly to Wang and Faust (1988 and 1990b). Only results from the PL fraction will be discussed in this chapter as they contain membrane lipids that are considered to be the matrix for metabolic events, selective permeable barriers to macromolecules and solutes, regulate fluidity and transducing signals (Furt *et al.*, 2011). Of these, the FAs (18:1, 18:2, 18:3, 16:0) make up 90% of the glycerolipids of plant membranes (Ohlrogge and Browse, 1995). Results from the neutral lipid fraction (main part of the stored lipid pool) are included in Appendix A as additional information and will not be discussed.

To describe the effect of the different regions throughout the trial period a two-way ANOVA (including possible interaction between the two areas (Elgin and Koue Bokkeveld) and the day of the year) was performed across the sample collection points within each season at a significance level of 5%. Where a significant interaction (Area*Days) was detected ($p < 0.05$), differences among the means were compared using a *post hoc* Fischer's Least Significant Difference test. If the interaction was found to be non-significant ($p > 0.05$) the main effects, i.e. Area and Days, are

presented independently. All statistical analyses were performed using the XLSTAT package (Version 19.01.40777, XLSTAT Institute).

3. Results

3.1. Temperature and chill accumulation during the trial

As presented in Paper 1 of this dissertation, the climatic data confirmed differences between the two contrasting areas in both years. According to the Utah chill model, the Koue Bokkeveld received 1537 and 1338 CU and Elgin 869 and 645 CU in 2015 and 2016, respectively (Fig.1A and B). Chill accumulation started earlier in the Koue Bokkeveld and by May in both years, it had already accumulated more chill compared to Elgin in the same period. The average temperatures for both years confirmed that the lower laying Elgin region had warmer winter conditions compared to the Koue Bokkeveld. Average daily temperature declined throughout winter and was at its lowest in July in both areas in both years after which it increased again towards spring. Overall, the average temperatures and CU accumulation across seasons showed that 2016 was a warmer year in both areas. Phenological stages such as leaf drop and growth resumption occurred on average 12-14 days later in Elgin compared to the Koue Bokkeveld (arrows in Fig.1A and B). In both years, growth resumption was associated with significant increases in bud weight (data not shown) and occurred between 22-24 August in the Koue Bokkeveld and 5-7 September in Elgin although it is generally accepted that growth stimulating reactions can occur in the bud tissue as soon as temperature conditions are favourable, thus long before the visible signs of growth resumption.

3.2. Dormancy progression curves from field conditions

As described in Paper 1, a significant interaction was detected between the two areas and the time period (days) (both years $p < 0.0001$), in 2015 (Fig. 2A) and 2016 (Fig. 2B). Shoots collected from the Koue Bokkeveld took longer to reach 50% budbreak at the end of April 2015 and were already in deep endodormancy (Fig. 2A) at the start of the trial. This level was maintained until mid-June after which it decreased significantly over a short period and by mid-August 2015 the endodormancy of the Koue Bokkeveld buds had been released completely (below 10 days to 50% budbreak). In contrast, buds from Elgin never attained the same deep endodormancy during the winter period and remained at between 10 and 21 days to 50% budbreak during the entire trial period. A maximum dormancy level of 42 days to 50% budbreak was reached in the Koue Bokkeveld compared to a maximum of only 21 days in Elgin. Elgin buds showed signs of release form this low level of dormancy by the end of August.

In 2016, similar to 2015, the buds from the Koue Bokkeveld reached a maximum dormancy level (42 days to 50% budbreak) by mid-May (Fig. 2B). This level was maintained until the end of June when the depth of dormancy started to decrease. In contrast, the buds from Elgin reached a maximum level (34 days to 50% budbreak) by mid-June, three weeks later than buds from the Koue Bokkeveld. This maximum endodormancy level of the Elgin buds in 2016 was higher than the maximum level (21 days to 50 % budbreak) reached in 2015. In both areas dormancy levels declined at the same rate to reach complete release by mid-Aug.

In brief, the dormancy progression of buds from the Koue Bokkeveld was very similar over the two years and reached deeper dormancy levels early in the season compared to buds from Elgin. The dormancy curves of Elgin (the warmer area) differed significantly between the two seasons with 2015 showing a shallower endodormancy compared to 2016 although the latter being a warmer season.

3.3. Lipid concentration and FA composition

During the two seasons, the neutral to polar lipid ratio (NL:PL) were between 1.01 and 1.51 (Fig. 3). The NL concentration in the Koue Bokkeveld and Elgin regions was significantly higher than the corresponding PL concentration for all the sampling dates. No significant interaction was detected between day of the year and area in either season. However, both main effects showed significant differences in both years (Fig. 3). During 2015, the ratio increased from the beginning of the trial until mid-May, then remained stable until the beginning of August when it increased and remained at this higher ratio during the growth resumption phase (Fig. 3A). The average ratio for 2015 was higher in the Koue Bokkeveld buds compared to the Elgin buds (Fig. 3B). In 2016, a similar slow increasing trend of the ratio was observed until the beginning of August as it decreased by mid-August and then maintained this level during the growth resumption phase (Fig. 3C). The average ratio was again significantly higher in the Koue Bokkeveld buds compared to the Elgin buds (Fig. 3D).

The lipid concentrations of the neutral and polar fractions, across the two seasons, were between 1.43– 2.51 mg g^{-1} FW for the NL and 1.02 – 2.04 mg g^{-1} FW for the PL (Fig. 4). In both seasons no interactions were found between the variables for the NL (Fig. 4). In both areas the NL concentration showed an increasing pattern until the beginning of July, then remained stable before declining during the growth resumption phase (Fig. 4A). The average NL concentration was higher in the Koue Bokkeveld compared to Elgin (Fig. 4B). The PL fraction in the buds from the Koue Bokkeveld and Elgin was significantly affected by the interaction between areas and day of the year (Fig. 4A). The buds from both areas maintained a relatively constant PL level

during the dormant phase (Koue Bokkeveld at a higher level in late April, early May and early June) and started to decline significantly in mid-August in the Koue Bokkeveld buds followed by Elgin a week later. Both areas ended the trial on similar levels that were the lowest recorded for the season. Similar to 2015, the NL concentration increased until mid-May in 2016 and remained stable until the growth resumption phase. However in 2016 there was no decrease in NL concentration at the growth resumption stage. (Fig. 4C). The Koue Bokkeveld levels on average was again higher than Elgin (Fig. 4D). A significant interaction was detected in the PL levels (Fig. 4C) in 2016. Although the PL concentration was relatively stable in both areas, the levels in the Koue Bokkeveld were significantly higher except for the first sampling point on 26 April, 23 June and 18 Aug. At the end of the trial the concentration in the Elgin buds was 16 % lower compared to the Koue Bokkeveld.

In the PL fraction, six FAs were detected in both seasons namely, palmitic acid (16:0), stearic acid (C18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3) and arachic acid (C20:0) (Fig. 5). Amongst these, the three predominant FAs were linolenic acid, linoleic acid and palmitic acid in both areas and years. The same six FAs were also detected in the NL fraction of the buds collected from both areas and in addition, four VLCFAs, i.e. heneicosanoic acid (C20:1), eicosatrienoic acid (C20:3n3), behenic acid (C22:0) and lignoceric acid (C24:0) were detected. Changes in the NL FA and their saturation levels are not central to this paper and the data is presented in Appendix B (Fig. 1 to Fig. 6) of this dissertation. As mentioned before, this paper will only focus on the changes of the individual FA from the PL fraction.

3.4. Effect of winter chill on FA composition and saturation

3.4.1. Changes in individual FAs

3.4.1.1. Palmitic acid (C16:0)

Across samples and areas in both years, palmitic acid made up between 12.7 – 19.5% of the FA in the polar fraction. A significant interaction was detected between the areas and day of the year, in both 2015 and 2016 (Fig. 5A and B). Except for an increase observed on 21 June 2015 (Fig. 5A), the percentage palmitic acid in the Elgin buds decreased slowly from the onset of the winter until September. Likewise, the percentage palmitic acid in buds from the Koue Bokkeveld declined from the beginning of June until mid-July 2015. This decline, however, was faster than that observed in the buds from Elgin, especially from 7 June, and reached its lowest contribution on 19 July when it was 3.8% lower than the initial value. The percentage contribution subsequently increased until the beginning of August to reach a level similar to the Elgin buds by the end of the

trial. During the 2016 season, the percentage palmitic acid also decreased throughout the season in buds from both areas (Fig. 5B). A gradual decrease was observed in the Koue Bokkeveld buds until the end of the trial whereas the percentage in the Elgin buds remained constant during growth resumption. Palmitic acid in the buds from the Koue Bokkeveld was 8% lower than in the Elgin buds at the end of the trial.

3.4.1.2. Stearic acid (C18:0)

In both years, stearic acid in the treated and control buds measured between 6.2 – 8.4% of the total polar traction (Fig. 5C, D and E). The percentage stearic acid present in the buds from the Koue Bokkeveld and Elgin was significantly affected by the interaction between areas and day of the year in the 2015 season (Fig. 5C). Despite some slight fluctuations in both areas in 2015, the percentage stearic acid decreased until 25 May then remained constant in the Koue Bokkeveld buds while in Elgin it increased until 8 June before remaining stable until the end of the trial. The buds from Elgin had a significantly higher stearic acid percentage than the Koue Bokkeveld buds throughout the 2015 trial period except for four occasions measured in July where the Koue Bokkeveld percentage was equal to that of Elgin. The Koue Bokkeveld stearic acid percentage decreased again after July and was 20% lower compared to buds from Elgin at the end of the trial. No significant interaction was detected in 2016, but both main effects showed significant differences (Fig. 5D, E). Throughout the season the stearic acid percentage remained relatively constant in the buds (Fig. 5D) from both areas, only rising slightly at the last sampling point. Similar to 2015, stearic acid made a greater contribution to the PL pool, in the Elgin (8%) compared to the Koue Bokkeveld buds (7%) (Fig. 5E).

3.4.1.3. Oleic acid (C18:1n9c)

Across sample dates and areas, oleic acid made up between 1.8 – 9.1% of the FA in the polar fraction (Fig. 5F and G). There was a significant interaction between the areas (Elgin and Koue Bokkeveld) and the day of the year in both seasons. In general, in both 2015 and 2016, the percentage oleic acid was higher in buds collected from Elgin than in those collected from the Koue Bokkeveld. In 2016, these differences were significant throughout the trial while in 2015 differences were mostly significant from early June onwards. During the 2015 season, the percentage oleic acid in both areas decreased from the onset of the trial (end of autumn) until the beginning of June (Fig. 5F), where after it remained constant until the beginning of August. From mid-August (start of growth resumption) the percentage oleic acid increased in Elgin buds, while it decreased in the Koue Bokkeveld buds and by the end of the trial the percentage contribution in the Elgin buds was 2.5% higher compared to the Koue Bokkeveld buds. In the 2016 season,

the percentage oleic acid also showed an overall decline in the buds from both areas but this time until the beginning of July, where after it remained relatively constant until the end of the trial (Fig. 5G). Although the pattern in the two areas was similar in 2016, the percentage oleic acid was 1.3% higher in the Elgin buds at the end of the trial.

3.4.1.4. Linoleic acid (C18:2n6c)

Linoleic acid made up between 28 – 37% of the polar fraction for both areas during both years of the trial (Fig. 5H and I) and was significantly influenced by the interaction between areas and day of the year. Apart from minor fluctuations, the percentage linoleic acid remained relatively constant in the Elgin buds from the start to the end of the 2015 trial. Buds from the Koue Bokkeveld showed a percentage contribution generally significantly higher with an increasing trend until mid-August when it decreased rapidly to a percentage 2.4% lower than that of the Elgin buds (Fig. 5H) prior to spring. In 2016, the percentage linoleic acid in the buds from both areas increased gradually from the onset until the end of June (Fig. 5I). Although the percentage of linoleic acid declined from thereon until the end of the trial, it remained higher at the end of the trial than at the beginning in both areas. Similar to 2015, buds from the Koue Bokkeveld showed a significantly higher contribution of linoleic acid to the total PL pool than that from Elgin throughout the trial.

3.4.1.5. Linolenic acid (C18:3n3)

In both seasons, linolenic acid made up the largest portion of the FA and was between 32.2 – 45.5% of the total amount found in the polar fraction (Fig. 5J and K). A significant interaction was detected between areas area and day of the year in both seasons (Fig. 5J and K). The Elgin buds exhibited a slight increase in the percentage linolenic acid throughout the 2015 season. In the buds from the Koue Bokkeveld, the linolenic acid percentage contribution also showed a gradual slight increase throughout the winter but from mid-August the percentages increased rapidly and was 5.8% higher than the Elgin buds at the beginning of spring (Fig. 5J). Similarly, in 2016, the percentage linolenic acid increased slightly throughout the winter in both areas with small differences between the two areas (Fig. 3K). The spike seen in the Koue Bokkeveld buds at the end of 2015 was not observed in 2016 and both areas ended on similar percentages at the beginning of spring.

3.4.1.6. Arachic acid (C20:0)

The arachic acid contribution to the PL pool was between 0.9 – 2.7% and the lowest of the FAs (Fig. 5L and M). There was a significant interaction between area and day of the year (Fig. 5L and M). In general, the percentage contribution of arachic acid increased during the study period

in the buds from both areas in both years. In the 2015 season, the percentage arachic acid increased significantly in the Elgin buds from the end of April until the end of June, then remained constant from June to July and subsequently increased again until August (Fig. 5L). In the Koue Bokkeveld buds, the percentage increased from the beginning of June until mid-July after which it remained relatively constant until the end of the trial where it was 1.3% lower than the Elgin buds. In 2016, the percentage arachic acid increased steadily in buds from both areas but levelled off in the Elgin buds by the beginning of July until the end of the trial. The buds from the Koue Bokkeveld only levelled off by mid-August and was 0.18% higher than the Elgin buds by the end of the trial (Fig. 5M).

3.4.2. Effect of winter chill on the saturation of the fatty acids

3.4.2.1. SFA and UFA percentages *in the treated and control buds*

The SFA (palmitic, stearic and arachic acid) concentration made up between 22 – 29% of the polar fraction. The %SFA present in buds from the Koue Bokkeveld and Elgin was affected by the interaction between the two main effects in the 2015 season (Fig. 6A), while in 2016 no interaction was found but both main effects were significant (Fig. 6B and C). As palmitic acid is the largest contributor (64%) to the SFA grouping in 2015, the pattern of the %SFA is very similar to that of palmitic acid and showed similar percentage between the two areas expect for three sampling points (end of June and in July 2015) where the Elgin buds had significantly higher percentage compared to the Koue Bokkeveld buds (Fig. 6A). In the 2016 season, the %SFA in the buds from both areas declined from the onset of the trial until the beginning of June when it remained constant until the end of the experiment (Fig. 6B). On average, a significantly higher %SFA was detected in Elgin buds compared to the Koue Bokkeveld buds (Fig. 6C) in 2016. In this season, palmitic acid accounted for only 27.5 % of the SFA grouping and palmitic acid was the main FA contributor followed by stearic acid.

As the SFA and the UFA are calculated together to form 100 % of the FAs, the UFA patterns will be mirror images of the SFA patterns with similar statistical analysis as described in the previous section. The UFA are the larger of the two groupings and make up between 71% and 78% of the total FA pool (Fig. 6D, E and F). In both years the major FAs contributing towards the UFA in the PL fraction were linolenic acid (32 – 45%) and linoleic acid (28 – 37%).

Because of the magnitude of the UFA grouping (>75 %), the UFA/SFA ratio showed a very similar pattern to the %UFA observed in the 2015 and 2016 seasons (Fig. 6D, E and F) and to prevent duplication is presented in Appendix A (Fig. 1A and B) as reference and not discussed again in

detail. In summary the UFA/SFA pattern showed a ratio between 2.4 and 3.4 for the areas in both years.

3.4.2.2. MUFA and PUFA percentages in the treated and control buds

As oleic acid is the only MUFA detected in the polar fraction of the buds, the complete MUFA grouping consisted of only oleic acid. This means that the statistical analysis, pattern and trends for MUFA were identical to that of oleic acid that was already presented in Fig. 6F and 4G and therefore not discussed again. See Appendix A, Fig. 2A and 2B for reference.

As the PUFA grouping is made up of only linoleic and linolenic acid and the oleic acid percentages are very low, the PUFA and the UFA groupings have very similar patterns in both areas and both years. The UFA results were already presented in Fig. 6D, E and F the PUFA results are presented in Appendix A (Fig. 2C and D) and not reported on in detail.

As the oleic acid contribution (and therefore the MUFA grouping contribution) to the total polar fraction was very low (below 10% in both areas and years) compared to the PUFA contribution (higher than 63% in both areas and years), the expression of the MUFA/PUFA ratio resulted in relatively small values with a trend almost identical to that of oleic acid already presented in Fig. 6F and G. The MUFA/PUFA results are therefore not reported in detail and presented in Appendix A (Fig. 2F and G).

3.4.2.3. Saturation ratios of the C18 FAs in the treated and control buds

In both years, the range of the (C18:3+C18:2)/C18:1 ratio during the experiment was between 7 – 39 (Fig. 7A and B). This ratio was affected by an interaction between area and day of the year in both years (both $p < 0.0001$) (Fig. 7A and B). In 2015, the (C18:3+C18:2)/C18:1 ratios started off low and increased gradually in the buds from both areas from the end of April until the beginning of June when they remained relatively constant until the end of July before increasing again until mid-August. The ratio then remained stable in the Koue Bokkeveld while decreasing in the Elgin buds (Fig. 7A). From 8 June until the end of the trial, the ratio was significantly higher in the Koue Bokkeveld. In the growth resumption phase the ratio in the Elgin buds declined and by the end of the trail, it was 58% lower compared to the Koue Bokkeveld. In 2016, the initial pattern was similar with the (C18:3+C18:2)/C18:1 ratio increasing in the buds from both areas from the end of April until the beginning of July. The ratios from the Koue Bokkeveld were significantly higher than Elgin from the end of June until mid-August. During growth resumption, the ratios (in both areas) remained constant (Fig. 7B). At the end of the trial the Koue Bokkeveld ratio was significantly higher (33%) compared to Elgin. The increasing ratios found in the buds

from both areas and in both seasons during the winter period indicate that a process of unsaturation is taking place amongst the fatty acids causing increasing percentages of linoleic (C18:2n6c) and linolenic (C18:3n3) acids at the expense of oleic acid (C18:1n9c).

The C18:3/C18:2 ratio was found to range between 0.82 – 1.58 throughout of the trial (Fig. 7C and D). The interaction between the main effects was significant for both years (Fig. 7C and D). The ratio remained relatively constant in the buds for both areas from the end of April until the end of July in 2015 although the level was significantly higher in the Elgin buds compared to the Koue Bokkeveld buds (Fig. 7C). Beyond August and throughout growth resumption, the ratio of the Elgin buds continued to remain unchanged, but the ratio in the buds from the Koue Bokkeveld increased significantly to overtake the Elgin ratio and reach a ratio 20% higher than the Elgin buds by the end of the trial. This sudden increase of this ratio indicates a rapid unsaturation of linoleic acid (C18:2n6c) to linolenic acid (C18:3n3) just before budbreak that is absent in the warmer Elgin region. In 2016, the C18:3/C18:2 ratio from both areas decreased at first until 24 May and then gradually increased until mid-August after which both areas maintained their ratios until the end of the trial. The ratios in the buds from the Koue Bokkeveld were always significantly lower (bar 19 July and 23 Aug) compared to the ratios in Elgin and did not show the sudden increase at the end of August as observed in 2015 (Fig. 7D).

In both years, the C18:3/(C18:1+C18:2) unsaturation ratio was between 0.78 – 1.48 during the trial (Appendix A, Fig 3A and B). The trends of the C18:3/(C18:1+C18:2) ratio are very similar to the C18:3/C18:2 trends because the percentages of the (C18:1+C18:2) grouping and C18:2 are almost equal since the contribution of C18:1 is low. To avoid duplication, the C18:3/(C18:1+C18:2) ratios are presented in Appendix A (Fig 3A and B) and not discussed in detail here.

3.5. Effect of winter cold on the free sterols and phospholipids

The free sterol concentration varied between 3.4 – 4.3 mmol g^{-1} FW in both years (Fig 8A, B). Except for a significant decrease observed from April to May 2015, the total free sterol concentration gradually and significantly increased with the progression of the season until the beginning of August and then remained stable during the rest of the period (Fig. 8A). On average, a higher concentration of total free sterols was measured in the buds from the Elgin area than in those from the Koue Bokkeveld (Fig. 8B). In 2016, the total free sterol concentration increased progressively from the onset of the trial until the end of July, whereafter it remained constant until the end of the experiment (Fig. 8C). Similar to 2015, a higher free sterol concentration was detected in the Elgin buds when compared to the Koue Bokkeveld buds (Fig. 8D).

Across seasons and areas, the total free phospholipid concentrations fluctuated between 16.6 – 22.5 µmol g^{-1} FW (Fig. 8F, E and G). In 2015, apart from a significant reduction observed from April to May, the total free phospholipid concentration was relatively constant during the whole season (Fig. 8E). A higher concentration was present in the Koue Bokkeveld buds compared to Elgin (Fig. 8F). During 2016, a significant interaction occurred between area and day of the year (Fig. 8G). The concentration in the Elgin buds remained constant until August, but then gradually decreased towards the end of the season. In contrast, the concentration in the Koue Bokkeveld buds increased from mid-May until mid-July and then remained constant until the end of the season. The concentration were significantly higher from 19 July onwards in the Koue Bokkeveld buds than in the Elgin buds ending on a level 21% higher than Elgin.

The free sterol/free phospholipids ratio was between 185 – 300 in both seasons and areas (Fig. 8H and I). In both years, there was a significant interaction between the main factors (Fig. 8H, I). During the 2015 season, the free sterol/free phospholipids ratio remained relatively unchanged in the Koue Bokkeveld buds from the start to the end of the trial (Fig. 8H), while in the Elgin buds the ratio remained constant until mid-July and then increased up to the end of July. Thereafter, it remained constant until the end of the trial where it had a ratio 37% higher than the Koue Bokkeveld. During 2016, the ratio in the Elgin buds showed a similar pattern by remaining constant until early July, then increasing and maintaining this higher level until the end of the trial while the buds from the Koue Bokkeveld remained relatively constant throughout the season. At the end of the trial in 2016 the free sterol/free phospholipids ratio in the Elgin buds were 29% higher than in buds from the Koue Bokkeveld.

3.6. Dormancy progression and lipid composition of the lab-chilled buds

The dormancy progression curve of the shoots that were treated with artificial cold (lab-chilled) showed that the number of days to 50% budbreak decreased from 28 days at the onset of the experiment (53 CU accumulated) to 12 days by the time that the buds had accumulated 377 CU (Fig. 9). The number of days to 50% budburst remained at 12 up to the end of the trial (when 1997 CU had accumulated).

3.6.1. Composition and saturation of the FAs in the PL fraction of the lab-chilled buds

3.6.1.1. Individual FAs

The individual FAs composition in the lab-chilled buds are presented in Fig. 10. Throughout the trial, palmitic acid (C16:0) was the major SFA present in the lab-chilled buds and varied between 15 – 20% of the total polar lipids. Increased artificial chilling caused a gradual decrease in palmitic

acid from 53 CU to 1673 CU, followed by a slight increase from 1673 CU to 1997 CU. The percentage stearic acid (C18:0) in the lab-chilled buds made up 7 – 8% of the polar fraction and remained constant during the first 377 CU accumulation and then decreased until 1349 CU where after it remained constant until the end of the trial. The oleic acid (C18:1) measured between 3 – 7% and its percentage declined from the onset of the trial (53 CU) up to an accumulation of 377 CU and then remained relatively constant. Linoleic acid (C18:2) was the major PUFA present in the lab-chilled buds and varied between 31 – 37% over the trial period. Its percentage increased gradually in the buds as the chilling exposure increased; however, the percentage contribution decreased when chilling exceeded 1349 CU. Linolenic acid (C18:3) was equally prevalent making up 33 – 37% of the polar fraction. Its percentage increased until 701 CU. It then declined until 1349 CU accumulated and thereafter it remained constant. Although the initial percentage contribution of arachic acid was low (1 – 2%), it increased in the buds during the accumulation of the first 1673 CU after which the percentage remained constant until the end of the experiment.

3.6.1.2. FAs groupings and the unsaturation of C18 FAs

Because of the high contribution of linoleic and linolenic acid, the lab-chilled buds contained a higher %UFA (74 – 76%) than %SFA (24 – 29%) in the PL fraction (Fig. 11A). As these two groupings make up the total PL fractions, their patterns will be mirror images. In general, the %UFA increased (and the %SFA decreased) as chill units accumulated. The UFA/SFA ratio was between 2.5 and 3.1 during the trial and showed a sudden increase during the accumulation of the first 377 chill units, and then a more gradual increase up to 1349 CU.

The unsaturated PLs of the lab-chilled buds were mostly polyunsaturated, since the %PUFA (65 – 73%) was higher than the %MUFA (2 – 7%) (Fig. 11B). The %PUFA increased during the accumulation of the first 701 CU and remained constant until the end of the experiment (1997 CU). In contrast, the %MUFA declined rapidly from the onset of experiment (53 CU) until 701 CU accumulated and then increased gradually until 1673 CU were accumulated with a gradual decrease until the end of the trial. Because of the big difference in the %MUFA and %PUFA in the buds, the trend of the MUFA/PUFA ratios were influenced mainly by the PUFA content and the MUFA/PUFA ratio varied between 0.03 – 0.11. Thus, in contrast to the %PUFA trend, the MUFA/PUFA ratio decreased rapidly during the accumulation of the first 377 CU, the decline continued at a slower rate until 701 CU accumulated and then increased slightly until the end of the trial at 1997 CU accumulation.

The saturation ratios of the C18 FAs groupings were largely driven by the changes in the linoleic and linolenic percentages (Fig. 11C). The (C18:3+C18:2)/(C18:1) ratio (18-33 during the trial)

increased significantly during the accumulation of the first 701 chill units after which it decreased until 1673 CU accumulated only to increased again until the end of the experiment (1997 CU). The C18:3/(C18:1+C18:2) ratio (between 1.09 and 0.96) also increased during the first 377 CU accumulation and then remained constant until 701 CU accumulated. This was followed by a decrease as CU accumulated up to 1340 CU after which it increased again and remained stable until 1997 CU accumulated. Because of the relatively low percentages of C18:1, the C18:3/C18:2 ratio (0.87 to 1.01) followed a pattern similar to the C18:3/(C18:1+C18:2) ratio increasing at first and then decreasing until 1349 CU accumulated followed by an increase until the end of the trial.

3.6.2. Total free sterols and phospholipids present in the lab-chilled buds

The total free sterol concentration measured in the lab-chilled buds fluctuated between 3.2 – 4.9 mmol g^{-1} FW (Fig 11A). It had an increasing trend from 53 CU to 701 CU accumulation then remained constant until the end of experiment (1997 CU accumulation). The total free phospholipid concentration (Fig. 12B) remained within 16 – 19 µmol g^{-1} FW as more CU accumulated with only one significant increase at the 701 CU level. The free sterol/free phospholipid ratio was driven by the large total free sterol values and therefore had a similar trend to the free sterol concentration (Fig. 12A). The ratio ranged between 189 and 284 (Fig. 12C) and showed an increase early in the CU accumulation (377 CU) followed by a stable performance during the rest of the trial.

4. Discussion

The climatic conditions recorded in the Elgin and Koue Bokkeveld areas of the Western Cape area during this study were typical to that previously observed for these areas (Cook and Jacobs, 2000; Halgryn *et al.,* 2001; Midgley and Lötze, 2011). In general, Elgin experienced a warmer winter than the Koue Bokkeveld in both years as was the case in Cook and Jacobs (2000) whom used the same study sites. The differences in temperature and CU accumulation between the two locations may be related to altitude and geographical location, since Elgin is located at a lower altitude (305 m a.s.l.) than the Koue Bokkeveld (945 m a.s.l.) (Cook *et al.*, 2017). It is known that CU accumulated in apple trees is strongly dependent on winter temperatures (Rai *et al.*, 2015) and it is not surprising that in the present study the accumulation of CU in the Koue Bokkeveld started earlier and increased faster compared to Elgin in both seasons. Across seasons, the 2016 winter period was warmer according to the average temperature and had lower amounts of CUs in both areas (645 and 1338 CU compared to 2015 values of 869 and 1537 CU for Elgin and Koue Bokkeveld, respectively). Similar to the Koue Bokkeveld buds,

Differences observed in the dormancy progression of the two areas and between the two seasons could be as a result of the differences in climatic conditions and chill accumulation. In the colder 2015, buds from the colder Koue Bokkeveld region were already in deep dormancy at the onset of the trial at the end of April, reached a maximum during June and were released from dormancy by the end of the winter. As expected, this high level of dormancy was not reached in the warmer Elgin area but no differences were detected in the dormancy release between the two areas. This abnormal dormancy pattern was typical and comparable to the 'Golden Delicious' and 'Granny Smith' results from the same locations (Cook and Jacobs, 2000). Interestingly, in the warmer 2016, buds from Elgin showed a dormancy progression curve similar but lower to buds from the Koue Bokkeveld (2015 and 2016) and reached a higher maximum dormancy level than in the 2015 season. This pattern was unexpected as dormancy levels tend to be shallower in warmer seasons under South Africa conditions (Cook *et al.*, 2017). Similar to the Koue Bokkeveld buds, the lab-chilled buds (collected from the Koue Bokkeveld on 25 April 2016) were already dormant before a substantial amount of chill accumulated (53 CU). This dormancy level decreased considerably in the terminal buds and remained low after the accumulation of 377 CU agreeing with Halgryn *et al.* (2001) who indicated that the chilling requirement for 'Cripps Pink' may be relatively low.

From the temperature and dormancy progression results presented in this study, two physiological phases became evident. Similar to the respiration study (Paper 1 of this dissertation), a dormant phase was observed from the onset of the experiment (end of April) until mid-August, followed by a growth resumption phase from mid-August until the end of the trial. From mid-August onwards, temperatures start to increase steadily in both areas in both years and the dormancy progression curves showed that in both areas 50% of the terminal buds took less than ten days to start growing. Both of these results suggest conditions that are conducive to the transition from a dormant state to a state of active growth. These are general estimates as the Elgin area could be up to 10 days later than the Koue Bokkeveld. We refer to these two distinct phases as the 'dormant' phase (period of continued decrease in temperature and an increase in chill accumulation) and the 'growth resumption' phase (period of continued increase in temperature and activation of chemical reactions and enzymes associated with growth). The lipid results generated in this experiment will be discussed using these two phases as reference points.

When considering the neutral (NL) and polar lipid (PL) fractions during the winter, it was observed that the NL was higher than PL and their ratio increased with the decrease in temperature across the dormant phase. This increase in NLs concentration is expected as the demand for energy during dormancy is low as chemical reactions slow down. NLs are also known to be used for the

storage of unused reduced carbons. The higher level of NLs during the dormant phase was also more pronounced in the colder production area compared to the warmer area where chemical reactions are more likely to continue during the (milder) winter and thus less carbon will be stored. According to the respiration data of this dissertation (Paper 1), higher activities of PPP and ALT pathways were observed in the area with milder winter temperatures. Similarly, the increase in NLs was less in the warmer of the two season resulting in a lower NL concentration (and energy reserve) at the end of dormancy. Piispanen and Saranpää (2004) indicated that FAs of NLs in silver birch wood can be affected by temperature or seasonal variation. The growth resumption phase is believed to be energy demanding; this is also evident in the drastic decrease in the NLs levels from mid-Aug 2015 onwards as cell division recommences and taps into the stored energy resources. Generally, an increase is expected in the PL concentration in spring, as cell division is associated with growth resumption and budbreak, especially if cold accumulation was sufficient (Wang and Faust, 1990b). This was not obvious in the current trial in that the PL contribution to total FA declined during growth resumption, especially in 2015.

Dormant phase

It is known that the cell membrane is modified during cold acclimation under natural conditions (Wang and Faust, 1990a and 1990b). It is thus not surprising that winter chill altered the membrane composition of dormant 'Cripps Pink' apple buds in the present study. The finding that palmitic acid, linoleic acid and linolenic acid were the main PL FAs in buds from both areas, while stearic, oleic and arachic acid contributed less to the FA pool is in accordance with work done on dormant apple buds (Wang and Faust, 1988, 1990b) and dormant apple shoots (Wang and Faust, 1990a). In the current study, apple buds had more palmitic acid compared to other SFAs. Its percentage contribution generally decreased as chill accumulated in both seasons regardless of milder conditions while stearic acid seemed to be unaffected by the climatic area. This disparity in the SFAs composition in response to winter condition was also found by Wang and Faust (1990a and 1990b) who showed that palmitic acid in apple buds decreased during the dormant period while stearic acid remained low and constant. Although the percentage oleic acid was lower in the colder area compared to the warm area in this study, it declined during autumn and winter to a stable, significantly lower level before the end of the dormant phase. This decrease coincided with the concomitant increase of linoleic and linolenic acid in both areas. It has been shown that during the dormant phase in apple buds, a reduction in the percentage of USFAs or MUFAs is associated with an increase in the amount of PUFAs (Wang and Faust, 1990a and 1990b; Murata and Wada, 1995). In the current study, this desaturation was more evident in the colder winter area as the percentage contribution from linoleic acid, a PUFA, was higher in the

Koue Bokkeveld buds compared to buds from Elgin; this was the main difference between buds from the two areas. In general, low temperatures are known to increase the percentage of PUFAs (Kodama *et al.*, 1995). Liao *et al.* (1996) also reported changes in FAs composition and polyunsaturation of linolenic acid due to the development of cold hardiness in Japanese pear. Other studies indicated that the polyunsaturation is due to the chill accumulation in dormant blackberry buds (Izadyar *et al.*, 1999), apple buds and shoots (Wang and Faust, 1988; Wang and Faust, 1990a and 1990b) and peaches (Gibson *et al.*, 2004) where UFAs were desaturated into linolenic acid under growth temperatures. Desaturation, especially to PUFA molecules, is oxygen sensitive during fatty acid (phospholipids) peroxidation (Spiteller, 2003). Linoleic and linolenic acid are thought to allow the cell to adapt to adverse conditions such as low temperature, and it has been shown that as their concentration increases, there is a rise in membrane fluidity (Murata and Wada, 1995; Upchurch, 2008). It is logical that under chilling temperatures, membrane lipids change their fatty acyl chains to at least two double bonds to keep their selective permeability and reduce injuries.

The higher %SFA detected in the Elgin buds during the dormant phase may be due to the higher percentage contribution of SFAs such as palmitic acid and stearic acid found in the buds. In general, membrane lipids with higher %SFA tend to cause rigidification through the tighter packaging of the FAs causing less fluidity of the membrane (Simon,1974; Cook and McMaster, 2002). This could possibly be due to the effect of insufficient winter chill and this low level of desaturation in the membranes could influence their functionality. A high proportion of UFAs in the membranes is generally a major aspect in detecting cold hardiness, the plants' response to cold stress (Upchurch, 2008) and improves membrane fluidity (Yoshida, 1984; Liao *et al.*, 1996). Membrane fluidity refers to the movement dynamic of lipids within the membrane bi-layers and allows for the transport of ions and metabolites and the functioning of associated proteins. If the lipid movement within the bi-layer decreases the transport and functionality is also hampered or slowed (Mikami and Murata, 2003). The %UFA increase in membrane lipids alongside the decrease in temperature demonstrates the degree of desaturation associated with the cold tolerance (Nishida and Murata, 1996) as was found in the lab-chilled buds that progressively accumulated more CU. The lower UFA to SFA ratio found in the Elgin buds indicates a lower fluidity of the membranes as it is known that an increase in this ratio indicated a more fluid and permeable membrane (Yoshida, 1984; Nishida and Murata, 1996; Jooste *et al.*, 2004) and would then potentially result in lower electron transport and integral protein functionality. The ratio was much higher in buds from the cold winter area compared to warm area. Similarly, Yoshida (1984) found an increase in the ratio in parallel with cold deacclimation in mulberry (*Morus bombysis*) bark.

Although the %MUFA was low, it showed a rapid decline followed by a constant percentage during the dormant phase. It was also lower in the Koue Bokkeveld buds compared to the Elgin buds. In general, the presence of at least one double bond in *cis* configuration can cause a bend within the carbon chain and influence the FAs packing and enhance fluidity. Previous studies indicate that cells that contained only MUFAs and SFAs were susceptible to low temperature and highlighted the importance of the second double-bond (Murata and Wada, 1995; Nishida and Murata, 1996). In the current data, MUFAs were converted or desaturated into PUFAs, possibly to enhance membrane adaptation and viability in low temperature conditions to maintain membrane fluidity, permeability and flexibility even though the buds are susceptible to peroxidation (Munro *et al*., 2004). The differences in %PUFA increase between the two areas possibly mirrored the differences in temperature experienced during winter. Normally, the polyunsaturation occurs in higher plants as a response to the tolerance to low temperatures or stress (Sakamoto and Murata 2002). The MUFA/ PUFA ratio decreased over both winter seasons and also in the lab-chilled buds. Monteiro *et al.* (2013) reported this MUFA to PUFA ratio to be the peroxidation index. Membranes with higher ratio are resistant to the oxidation and lipid peroxidation under stress conditions while a lower ratio indicated susceptibility of the membrane lipids. The higher MUFA/ PUFA ratio and lower UFA to SFA ratio in Elgin buds during winter seasons revealed a lower tendency to oxidation when compared to the Koue Bokkeveld buds.

The (C18:3+C18:2)/ (C18:1) ratio increased and was higher in the Koue Bokkeveld buds, similar to results reported by Wang and Faust (1990b). However, the (C18:3/ C18:2) ratio remained constant in buds from both areas as well as in the lab-chilled buds during the dormant phase, which supports Izadyar and Wang's (1999) findings in dormant blackberry buds. This suggests that the ratios indicate the progressive effect of chill accumulation on the modification and desaturation of FAs during this dormant phase.

Free sterols are one of the key components of plasma membranes that control fluidity and permeability of phospholipid bilayers in higher plants (Grunwald, 1971 and 1978; Schaller, 2004; Dufourc, 2008). Earlier studies reported that in young stem wood of silver birch, free sterols were higher during winter, declined during spring and early summer when the temperature gradually increases and later increased again during autumn when the trees became dormant (Piispanen and Saranpää, 2004). Free phospholipids are known as essential components of all biological membranes, their structure and composition in plant cells vary with host environment, stress or physiological conditions (Simon, 1974). The major phospholipid FAs increase in apple throughout the winter season and similar increases areare found with cold acclimation in mulberry (*Morus bombysis*) bark (Yoshida, 1984). The interaction of free sterols and free phospholipids is known

to affect membrane function and their ratio can serve as an index of fluidity and permeability (Grunwald, 1971, Quinn, 1981). Yoshida (1984) also reported that the ratio decreases in parallel with cold acclimation in mulberry (*Morus bombysis*) bark while its increase is associated with lower fluidity of the membrane (Picchioni *et al.*, 1994). In the current study, the total free sterols increased progressively towards to the end of the dormant phase in buds collected from both areas and for both seasons in agreement with the above studies. However, the reason for the higher level of free sterols observed in the Elgin buds when compared to the Koue Bokkeveld buds is unclear. This seems to indicate that the Koue Bokkeveld buds membrane was less fluid and permeable to solutes when compared to that in Elgin. It is possible that changes in the level of free sterols depend not only on the tissue, species, host environment and climatic conditions but also on other unknown factors. During the dormant phase, the total free phospholipids was higher in buds exposed to sufficient winter chill, especially in 2016, compared to buds exposed to deficient winter chill. The increase in the free sterols/ free phospholipid ratio in the Elgin buds could indicate membrane rigidity. However, the generally stable level of the ratio in the Koue Bokkeveld buds or the lab-chilled buds might be a sign of suitable environmental conditions for budbreak and metabolic activities. Wang *et al.* (1994) also reported a constant level of the ratio in apple buds two days after removing the terminal bud but the level thereafter decreased.

Growth resumption phase

Literature reports a decreasing trend in SFAs toward budbreak in apple (Wang and Faust, 1990a and 1990b) and blackberry (Izadyar and Wang, 1999) buds. It is thought that this declining trend may preserve the membrane fluidity and the physiological bilayer phase to enable growth resumption. Although this was not found in the present data for the overall %SFAs, the individual FAs (palmitic and stearic acid) did show a decreasing trend in some cases. Interestingly, it was only the colder region that showed this decreasing trend while in the warmer region the individual SFAs percentages either remained constant or increased during growth resumption. Generally, the percentage of the individual SFAs was also higher in the warmer region indicating a lower tendency to induce membrane fluidity.The MUFA, oleic acid, has also been reported to decrease prior to budbreak (Izadyar and Wang, 1999). This was not prominent in the present results during the growth resumption phase although in both years, the warmer region had significantly higher percentages of oleic acid compared to the colder region. The warmer winter site (Elgin) resulted in less desaturation; hence the higher SFA and oleic acid percentages during growth resumption.

A further (and sudden) desaturation of linoleic acid to linolenic acid prior to spring is well described under sufficient cold winter conditions (Wang and Faust, 1990b; Izadyar and Wang, 1999) and is thought to trigger metabolic activity and facilitate a transition from endodormancy to ecodormancy

(Beauvieux *et al*., 2018). Martz *et al.* (2006) stated that this increase in linolenic acid concentration occurred at the same time as an increase in plasma membrane H^+-ATPase and protein concentration in *Pinus resinosa* indicating a possible source for energy production during growth resumption. This phenomenon was very clear in the 2015 FA results and C18:3/C18:2 results from the colder region. Growth resumption was marked by an obvious increase in linolenic acid at the expense of linoleic acid. This polyunsaturation process producing linolenic acid was absent in the warmer region where the chill requirement was not met. However, it occurred when buds from this site were treated with hydrogen cyanamide and oil (Paper 4). This switch of linoleic to linolenic acid was, however, not recorded in the 2016 season as both regions maintained relatively stable percentages of these two FAs at the end of the trial. Although the 2016 season was a warmer season in both regions compared to 2015, the result was surprising. In the colder area, the main respiration pathways increased their activities to provide energy during the growth resumption phase and the absence of this increase in the Elgin buds was detected (Paper 1). A possible reason could be that the bud physiological activities were slower after warmer conditions and the switch in FA percentages was not yet evident/prominent when the trial was terminated. What was, however, confirmed in the 2016 results was the lack of high lipid desaturation during growth resumption in the region where the chill requirement was not met. In the warmer region it appears that the linoleic acid percentages could not be converted into adequate linolenic acid to sustain budbreak and growth (Erez *et al.* 1997).

The unsaturation in the current study was supported by the (C18:3+C18:2)/ (C18:1), (C18:3)/ (C18:1+C18:2), (C18:3/ C18:2) ratios which increased during the growth resumption phase. This may be related to the increase in membrane fluidity and organization in the physiological bilayer phase toward budbreak. Other authors reported the increase in unsaturation in (C18:3/C18:2) ratio in dormant apple (Wang and Faust, 1990), peach (Erez *et al*., 1997), blackberry cultivars (Izadyar and Wang, 1999) and also with the application of growth regulators under insufficient chilling conditions (Wang and Faust, 1988) and was ascribed to dormancy termination. This stimulation of unsaturation under insufficient chilling is also commented on in Paper 4 of this study. The unsaturation ratios support the finding that FA unsaturation increases in the response to the decrease in temperature (Erez *et al*., 1997; Cook and McMaster, 2002). FAs unsaturation not only contribute to membrane structure and degree of stability but also influence its fluidity and permeability (Cook and McMaster, 2002; Upchurch, 2008).

In the current study, we found a higher %SFA in the Elgin buds and higher %UFA and UFA/ SFA ratios in the Koue Bokkeveld buds during the growth resumption phase. The %SFAs are enriched with FAs with no double bonds and are known to increase membrane rigidity (Murata and Wada,

1995; Taiz and Zeiger, 2010). Stearic acid was found to be higher in the Elgin buds when compared with Koue Bokkeveld buds. It therefore seems that the warm winter area fails to desaturate the FAs prior to budbreak. The higher %SFA in Elgin buds occurred concomitantly with the lower %UFA and therefore lower UFA/ SFA ratio. The adaptation of plants to warm temperatures or other stresses results in higher %SFA and lower %UFA (Chihib *et al*., 2005; Zhukov, 2015). Apart from the %SFA, Elgin buds had also higher %MUFA prior to budbreak than in Koue Bokkeveld buds. Normally, as mentioned earlier, membranes with higher %SFA and %MUFA are resistant and not easily oxidised; however, those with higher %PUFA are fluid, flexible, permeable and susceptible to the lipid peroxidation (Catalá, 2009; Taiz and Zeiger, 2010; Jooste *et al*., 2014). We found lower %PUFA in Elgin buds compared to the colder region towards budbreak. PUFAs are known to be easily oxidised and membranes with a low MUFA/ PUFA ratio are more susceptible to lipid peroxidation, such as Koue Bokkeveld buds, than those enriched with MUFA (Taiz and Zeiger, 2010; Jooste *et al*., 2014). It seems that the higher MUFA/ PUFA ratio observed in the warmer region during the growth resumption phase might indicate a lower susceptibility to oxidative stress and changes in biochemical reactions prior to budbreak since membranes of Elgin buds are presumed to be less fluid and permeable. Beauvieux *et al.* (2018) indicated that membranes that are susceptible to stresses favour the release of buds from dormancy such as the Koue Bokkeveld buds.

Generally, the total free sterols increase is associated with the hydrolysis of steryl esters stock towards budbreak under cold conditions (Wang and Faust, 1990) and during budbreak and bud growth in apple buds induced with thidiazuron (TDZ) (Wang and Faust, 1988). Other studies reported that a high concentration of free phospholipid and a decline of the free sterols to free phospholipids ratio are linked with budbreak and occurred in parallel with the desaturation of fatty acids in the presence of sufficient winter chill or growth regulators such as TDZ and nitro-guanidine in apple (Wang and Faust 1988, 1989, 1990a and 1990b, Wang *et al*., 1994) and blackberry (Izadyar *et al*., 1999). The general high level of free sterols towards growth resumption found in these results, supports the above mentioned studies. Similarly, a higher concentration of total phospholipids is associated with dormancy release and budbreak (Wang and Faust, 1990a and 1990b). The free sterols to free phospholipids ratio remained at a consistently low level in the Koue Bokkeveld buds while increasing in Elgin towards to the end of the winter.

5. Conclusion

The results presented in this paper on seasonal changes of lipid composition in dormant apple buds appear to be comparable to the general pattern of lipid metabolism under winter field

conditions. Colder and warmer winter conditions affected the polar lipid levels and the most noticeable differences were observed at the end of the winter period and during growth resumption. The current findings generally showed that mild winter temperatures caused a lower degree of desaturation of FAs from SFAs to MUFAs and MUFAs to PUFA and especially from linoleic to linolenic acid at growth temperatures. This resulted in higher %SFA and %MUFA and lower %UFA and %PUFA. Milder winter conditions appears to increase the free sterols concentration and decrease the phospholipids concentration towards to the end of winter seasons. Such conditions may have decreased the fluidity and permeability of the membranes and in turn negatively affected growth resumption. This study thus shows that the lipid composition was affected by the warmer winter conditions, which reduced the polyunsaturation process that negatively affects metabolic activities during budbreak and bud growth. The lack of fluidity and permeability in the membranes from the warmer area could possibly obstruct or slow down the oxygen uptake and electron transport. This might decrease energy production as the mechanics of the oxidative phosphorylation reactions of respiration resides within the bi-layer of the mitochondria. Paper 1 confirmed this as both the activity of the TCA and CYT pathways failed to increase during growth resumption in the warm winter areas. It would, therefore, be interesting to repeat the study in parallel with extended studies on membrane permeability and fluidity and oxidative stress involvement using commercial orchards with traditional cold (northern hemisphere sites), moderate (South African conditions) and warm winter (South African conditions and North African conditions) to test the extent of the theory. This information would be helpful in understanding the mechanism and contribution of membrane lipids in dormant apple trees grown in areas with climatically marginal production capabilities.

6. Reference

Ames, B.N. (1966). Assay of inorganic phosphate, total phosphate and phosphateses. *Methods in Enzymology*. **8**, 115–118.

ARC-Infruitec, 1997. *South African deciduous fruit cultivars catalogue*. 2nd ed. ARC Infruitec-Nietvoorbij, Stellenbosch, SA. (as per Tharaga, 2014).

Beauvieux, R., Wenden, B. and Dirlewanger, E. (2018). Bud Dormancy in Perennial Fruit Tree Species: A Pivotal Role for Oxidative Cues. *Frontiers in Plant Science*. **9**, 657.

Catalá, A. (2009). Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids*. **157**, 1–11.

Chihib, N.E., Tierny, Y., Mary, P. and Hornez, J-P. (2005). Adaptational changes in cellular fatty acid branching and unsaturation of *Aeromonas* species as a response to growth temperature and salinity. *International Journal of Food Microbiology*. **102**, 113–119.

Christie, W.W. (1986). Separation of lipid classes by High Performance Liquid Chromatography with the 'Mass Detector'. *Journal of Chromatography*. **361**, 396–399.

Cook, H.W. and McMaster, C.R. (2002). Fatty acid desaturation and chain elongation in eukaryotes. *In D.E. Vance and J.E. Vance (Eds.) Biochemisty of Lipids, Lipoproleins and Membrane (4th Edn.)*

Cook, N.C. and Jacobs, G. (1999). Sub-optimal winter chilling impedes development of acrotony in apple shoots. *HortScience*. **34**, 1213-1216.

Cook, N.C. and Jacobs, G. (2000). Progression of apple (*Malus* x *domestica* Borkh.) bud dormancy in two mild winter climates. *Journal of Horticultural Science*. **75,** 233–236.

Cook, N.C., Calitz, F.J., .Allderman, L.A., Steyn, W.J. and Louw, E.D. (2017). Diverse patterns in dormancy progression of apple buds under variable winter conditions. *Scientia Horticulturae.* **226**, 307–315.

Cooke, J.E.K., Eriksson, M.E. and Junttila, O. (2012). The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. *Plant Cell Environment*. **35**, 1707–1728.

Courchaine, A.J., Miller, W.H. and Stein, D.B. (1959). Rapid semimicro procedure for estimating free and total cholesterol. *Clinical Chemitry*. **5**, 609–614.

Dennis, F. (2003). Problems in standardizing methods for evaluating the chilling requirements for the breaking of dormancy in buds of woody plants. *HortScience.* **28** (3), 347–350.

Duck-Chong, C.G. (1979). A rapid sensitive method for determining phospholipid phosphorous involving digestion with magnesium nitrate. *Lipids*. **14** (5), 492–497.

Dufourc, E.J. (2008). The role of phytosterols in plant adaptation to temperature. *Plant Signal Behavior*. **3**, 133–134.

Erez, A., Wang, S.Y. and Faust M. (1997). Lipids in peach buds during dormancy, a possible involvement in dormancy control. *Advances in horticultural Science*. **11**, 128–132.

Faust, M. and Wang, S.Y. (1993). Biochemical events associated with resumption of growth in temperate zone fruit trees. *Acta Horticulturae*. **329**, 257–264.

Folsch, J., Lees, M. and Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*. **226** (1), 497–509.

Furt, F., Simon-Plas, F. and Mongrand, S. (2011). Lipids of the plant plasma membrane. *The plant plasma membrane, Plant Cell Monographs*, **19.**

Gibson, P.G., Reighard, G.L., Powell, G.L. and Jenkins, T.C. (2004). Graft transmissible agents affect membrane fatty acid saturation during dormancy release in Peach. *Journal of the American Society for Horticultural Science*. **129**, 649–652.

Grunwald, C. (1971). Effects of free sterols, steryl ester and steryl glycoside on membrane permeability. *Plant Physiology*. **48**, 653–655.

Grunwald, C. (1978). Shading influence on the sterol balance of *Nicotiana tabacum* L. *Plant Physiology*. **61**, 76–79.

Halgryn, P.J., Theron K.I. and Cook, N.C. (2001). Genotypic response to chilling period of apple buds from two Western Cape localities. *South African Journal of Plant and Soil*. **18**, 21–27.

Izadyar, A.B. and Wang, S.W. (1999). Changes of lipid components during dormancy in 'Hull Thornless' and 'Triple Crown' blackbery cultivars. *Scientia Horticulturae*. **82**, 243–254.

Jooste, M., Rohwer, E.A., Kidd, M. and Huysamer, M. (2014). Comparison of antioxidant levels and cell membrane composition during fruit development in two plum cultivars (*Prunus salicina* Lindl.) differing in chilling resistance. *Scientia Horticulturae*. **180**, 176–189.

Kaluzny, M.A., Duncan, L.A., Merritt, M.V. and Epps, D.E. (1985). Rapid separation of lipid classes in high yield and purity using bonded phase columns. *Journal of Lipid Research*. **26**, 135–140.

Kodama, H., Horiguchi, C., Nishiuchi, T., Nishimura, M. and Iba K. (1995). Fatty acid desaturation during chilling acclimation is one of the factors involved in conferring low-temperature tolerance to young tobacco leaves. *Plant Physiology*. **107**, 1177–1185.

Laffargue, A., de Kochko, A. and Dussert, S. (2007). Development of solid-phase extraction and methylation procedures to analyze free fatty acids in lipid-rich seeds. *Plant Physiology Biochemistry*. **45**, 250–257.

Lang, G.A., Early, J.D., Martin, G.C. and Darnell, R.L. (1987). Endo-, para- and ecodormancy: physiological terminology and classification for dormancy research. *HortScience*. **22**, 371–377.

Liao, R., Tanabe, K., Tamura, F. and Itai, A. (1996). Seasonal variations in fatty acid composition and cold hardiness of Japanese pear in response to air temperature and growing stages. *Environmental Control in Biology*. **34**, 303–311.

Liu, D., Norman, H.A., Stutte, G.W. and Faust, M. (1991). Lipase activity during endodormancy in leaf buds of apple (*Malus domestica* Borkh.). *Journal of the American Society for Horticultural Science*. **116**(4), 689–692.

Martz, F., Sutinen, M.L., Kivineemi, S. and Palta, J.P. (2006). Changes in freezing tolerance, plasma membrane H+-ATPase activity and fatty acid composition in *Pinus resinosa* needles during cold acclimation and de-acclimation. *Tree Physiology*. **26**, 783–790.

Midgley, S.J.E. and Lötze, E. (2011). Climate change in the Western Cape of South Africa: trends, projections and implications for chill unit accumulation. *Acta Horticulturae*. **903**, 1127–1134.

Mittler, R., Finka, A. and Goloubinoff, P. (2012). How do plants feel the heat? *Trends in Biochemical Sciences*. **37**, 118–125.

Monteiro, J.P., Oliveira, J.P. and Jurado, A.S. (2013). Mitochondrial membrane lipid remodeling in pathophysiology: A new target for diet and therapeutic interventions. *Progress in Lipid Research*. **52**, 513–528.

Munro, K.D., Hodges, D.M., DeLong, J.M., Forney CF and Kristie, D.N. (2004). Low temperature effects on ubiquinone content, respiration rates and lipid peroxidation levels of etiolated seedlings of two differentially chilling-sensitive species. *Physiologia Plantarum*. **121** (3), 488–497.

Murata, N. and Wada, H. (1995). Acyl-lipid desaturases and their importance in the tolerance and acclimatisation to cold of cyanobacteria. *Biochemical Journal*. **308**, 1–8.

Nishida, I. and Murata, N. (1996). Chilling sensitivity in plants and cyanobacteria: The crucial contribution of membrane lipids. *Annual Review of Plant Physiology and Plant Molecular Biology*. **47**, 541–568.

Ohlrogge, J., Browse, J. (1995). Lipid biosynthesis. *Plant Cell*. **7**, 957–970.

Palta, J.P., Whitaker, B.D. and Weiss, L.S. (1993). Plasma membrane lipids associated with genetic variability in freezing tolerance & cold acclimation of *Solanum* species. *Plant Physiology*.**103**, 793-803

Pietsch, A. and Lorenz, R.L. (1993). Rapid separation of the major phospholipid classes on a single amino propyl cartridge. *Lipids*. **28** (10), 945 – 947.

Picchioni, G.A., Watada, A.E., Roy, S., Whitaker, B.D., and Wergin, W.P. (1994). Membrane lipid metabolism, cell permeability, and ultrastructural changes in lightly processed carrots. *Journal of Food Science*. **59** (3), 597–601.

Piispanen, R. and Saranpää, P. (2004). Seasonal and within-stem variations of neutral lipids in silver birch (Betula pendula) wood. *Tree Physiology*. **24**, 991–999.

Quinn, P.J. (1981). The fluidity of cell membranes and its regulation. *Progress in Biophysics and Molecular Biology*. **38**, 1–104.

Richardson, E.A., Seeley, S.D. and Walker, D.R. (1974). A model for estimating the release of rest for Redhaven and Elberta peach trees. *HortScience.* **9**, 331–332.

Rohde, A. and Bhalerao, R.P. (2007). Plant dormancy in the perennial context. *Trends Plant Science.* **12**, 217–223.

Sakamoto, T. and Murata, N. (2002). Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. *Current Opinion in Microbiology*. **5**, 208–210.

Schaller, H. (2004). New aspects of sterol biosynthesis in growth and development of higher plants. *Plant Physiology and Biochemistry*. **42**, 465–476.

Simon, E.W. (1974). Phospholipids and plant membrane permeability. *New Phytologist*. **73**, 377–420.

Spiteller, G. (2003). The relationship between changes in the cell wall, lipid peroxidation, proliferation, senescence and cell death. *Physiology Plant*. **119**, 5–18.

Taiz, L. and Zeiger, E. (2010). Plant Physiology. 5nd ed. Sinauer Associates Inc., Sunderland, M.A.

Tharaga, P.C. (2014). Impacts of climate change on accumulated chill units at selected fruit production sites in South Africa. MSc Thesis, Bloemfontein University, South Africa.

Theocharis, A., Clément, C. and Barka E.A. (2012). Physiological and molecular changes in plants grown at low temperatures. *Planta*. **235**, 1091–1105.

Uemura, M. and Steponkus, P.L. (1994). A contrast of the plasma membrane lipid composition of oat and rye leaves in relation to freezing tolerance. *Plant Physiology*. **104**, 479–496.

Upchurch, R.G. (2008). Fatty acid unsaturation, mobilization and regulation in the response of plants to stress. *Biotechnology Lett*ers. **30**, 967–977.

Van der Schoot, C. and Rinne, P.L.H. (2011). Dormancy cycling at the shoot apical meristem: transitioning between self-organization and self-arrest. *Plant Science*. **180**, 120–131.

Van Meer, G., Voelker, D.R. and Feigenson, G.W. (2008). Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology*. **9**, 112–124.

Wang, S.Y. and Faust, M. (1988). Changes of fatty acids and sterols in apple buds during budbreak induced by a plant bioregulator, thidiazuron. *Physiologia Plantarum*. **72**,115–120.

Wang, S.Y. and Faust, M. (1989). Changes of membrane polar lipids associated with bud break in apple induced by nitroguanidines. *Journal of Plant Growth Regulation*. **8**, 153–161.

Wang, S.Y. and Faust, M. (1990a). Seasonal changes of membrane lipids in apple shoots. *Journal of the American Society for Horticultural Science*. **115**, 462–467.

Wang, S.Y. and Faust, M. (1990b). Changes in membrane lipids in apple buds during dormancy and budbreak. *Journal of the American Society for Horticultural Science*. **115**, 803–808.

Wang, S.Y., Faust, M. and Line M.J. (1994). Apical dominance in apple (*Malus domestica* Borkh): The possible role of indole-3-acetic acid (IAA). *Journal of the American Society for Horticultural Science*. **119**(6), 1215–1221.

Wang, L.X., Tang, J.H., Xiao, B., Yang, Y.J. and Liu, J. (2013). Variation of photosynthesis, fatty acid composition, ATPase and acid phosphatase activities and anatomical structure of two Tea (*Camellia sinensis* (L.) O. Kuntze) cultivars in response to fluoride. *The Scientific World Journal*. **109367**, 9.

Wu, J., Seliskar, D.M. and Gallagher, J.L. (2005). The response of plasma membrane lipid composition in callus of the halophyte *Spartina patens* (Poaceae) to salinity stress. *American Journal of Botany*. **92**(5), 852–858.

Xue, H.W., Chen, X. and Mei, Y. (2009). Function and regulation of phospholipid signalling in plants. *Biochemical Journal*. **421**(2), 145–156.

Yoshida, S. (1984). Chemical and biophysical changes in the plasma membrane during cold acclimation of mulberry bark cells (*Morus bombycis* Koidz. Cv Goroji). *Plant Physiology*. **76**, 257–265.

Yoshida, S. (1986). Reverse changes in plasma membrane properties upon deacclimation of mulberry trees (*Morus bombysis* Koidz.). *Plant Cell Physiology*. **27**, 83–89.

Zhang, C. and Tian, S. (2009). Crucial contribution of membrane lipids' unsaturation to acquisition of chilling-tolerance in peach fruit stored at 0°C. *Food Chemistry*. **115**, 405–411.

Zhukov, A. V. (2015). Palmitic acid and its role in the structure and functions of plant cell membranes. *Russian Journal of Plant Physiology*. **62**, 706–713.

FIGURES

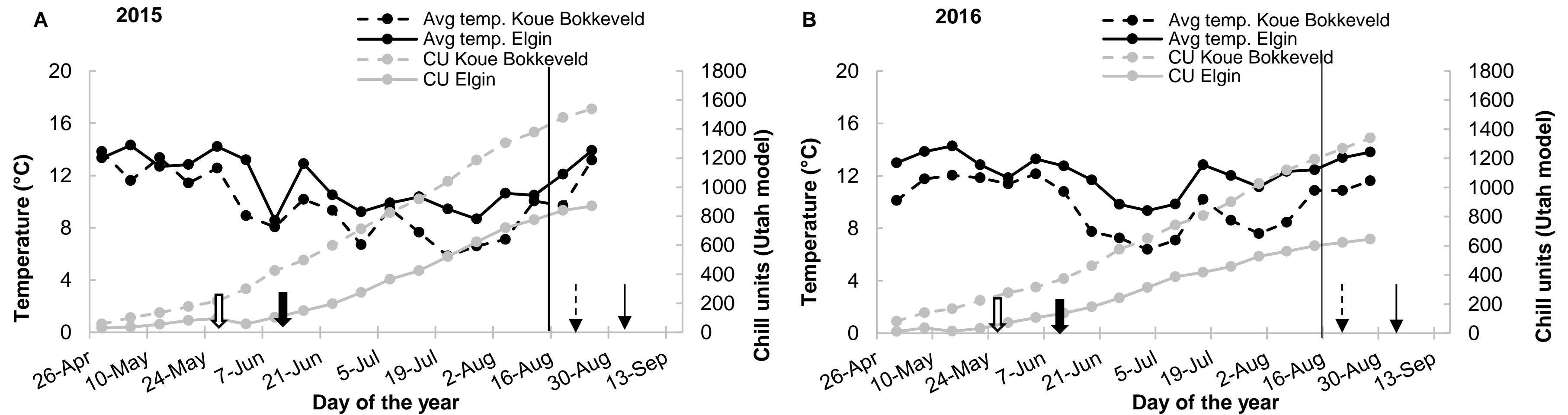


Fig. 1: Chill units (CU) (grey) and average daily temperature (black) for 2015 (A) and 2016 (B) in the Koue Bokkeveld (_ _ _) and Elgin (____). Arrows indicate leaf drop in the Koue Bokkeveld (white) and in Elgin (black). Line arrows show growth resumption in the Koue Bokkeveld (dotted) and Elgin (solid). The vertical line indicates the end of the dormant phase and the start of the growth resumption phase.

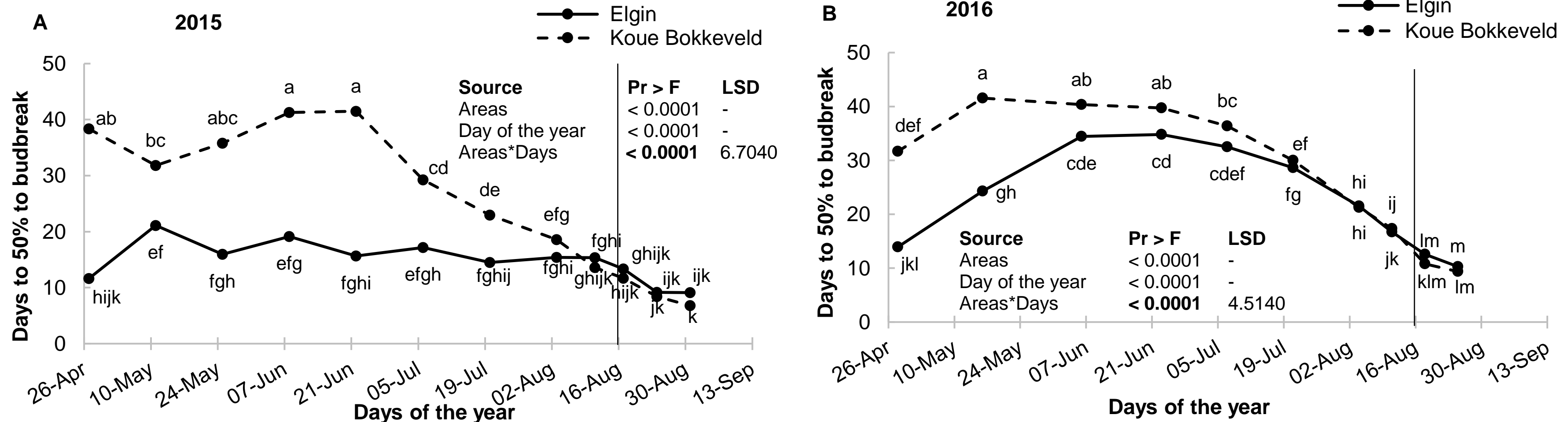


Fig. 2: Dormancy progression curves of 'Cripps Pink' buds sampled in the Koue Bokkeveld (_ _ _) and Elgin (____) during 2015 (A) and 2016 (B). Letters indicate significant differences at p=0.05. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase.

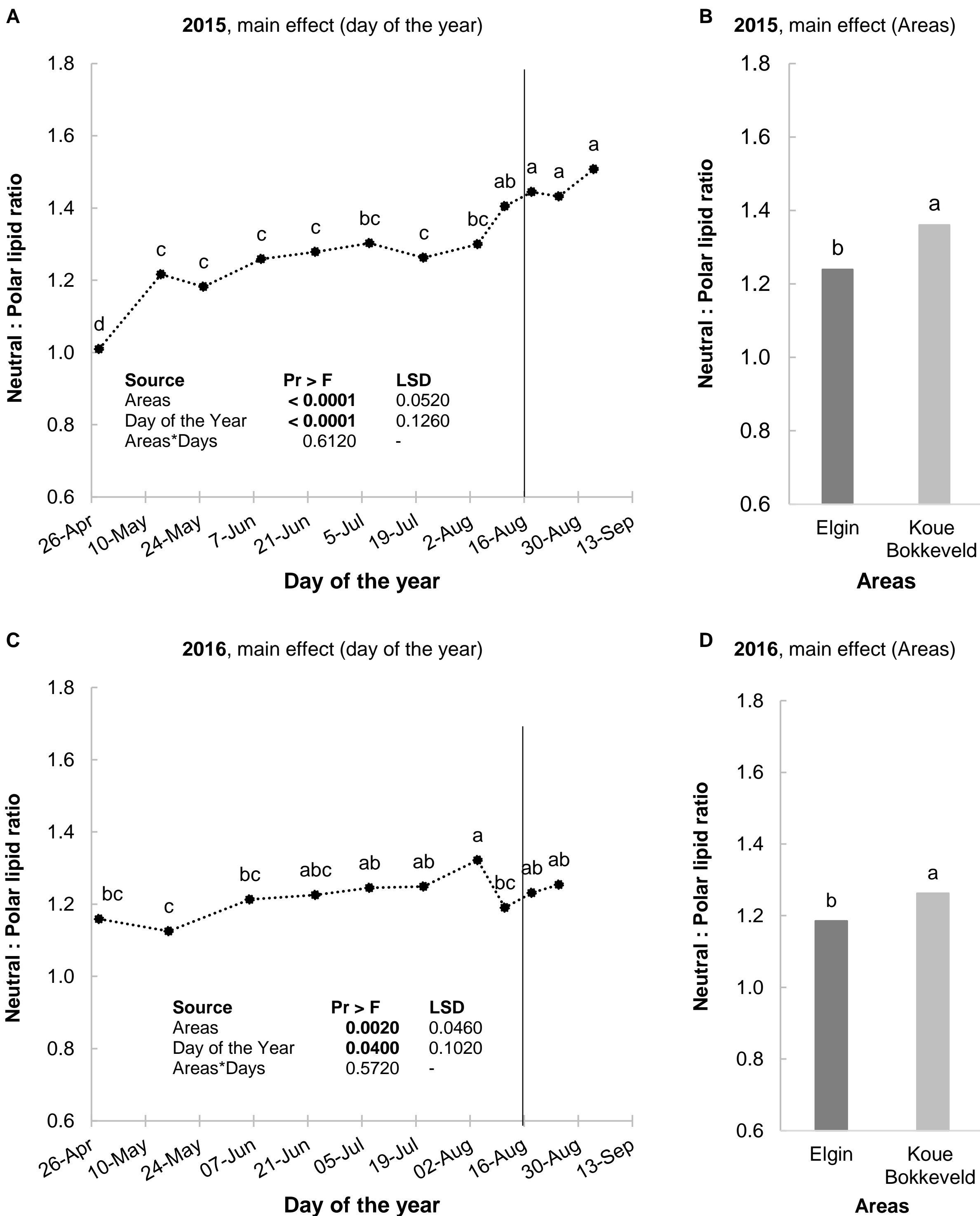


Fig. 3: Neutral: Polar lipid ratio of 'Cripps Pink' buds sampled in the Koue Bokkeveld and Elgin during 2015 (A, B) and 2016 (C, D). The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.

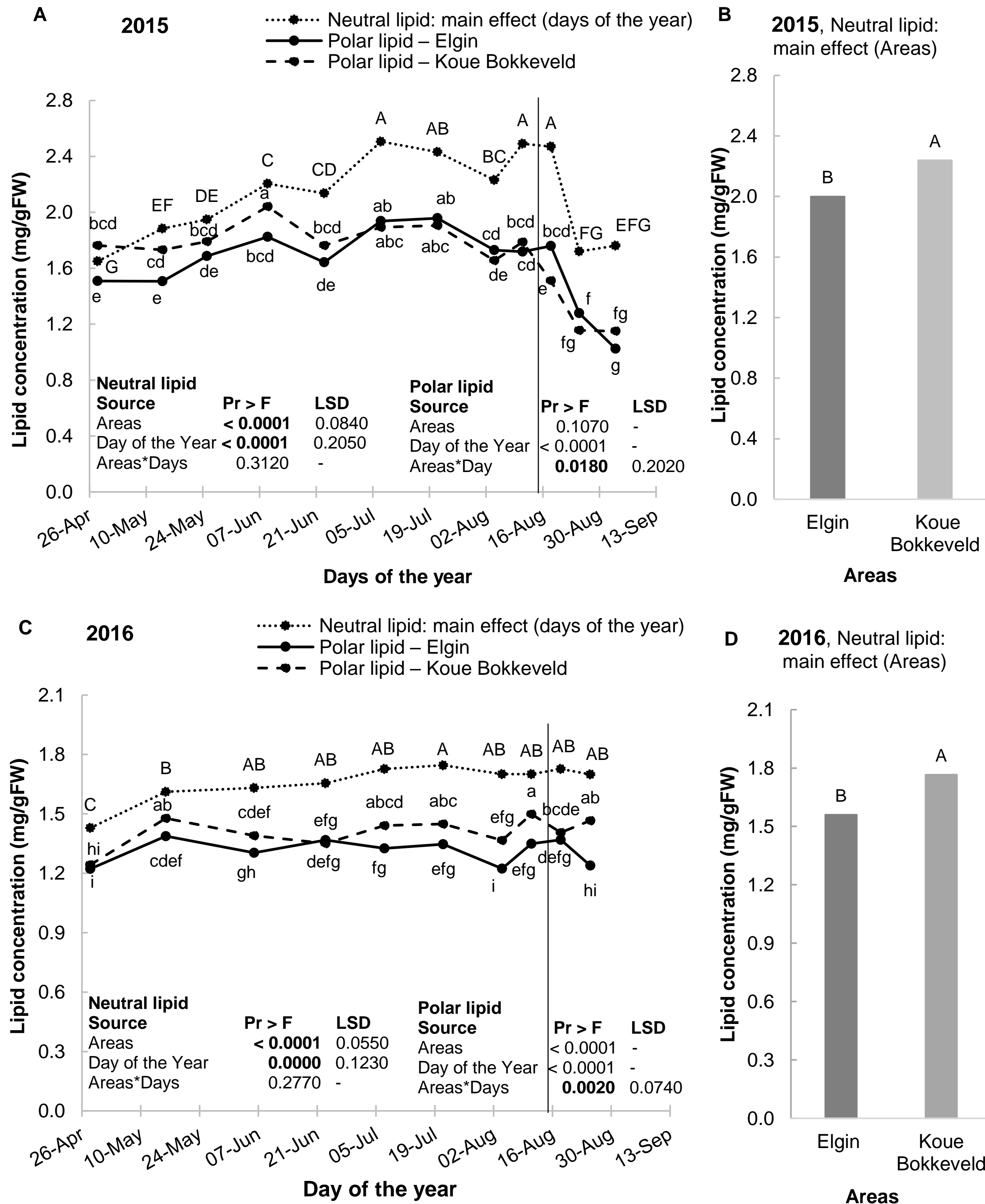
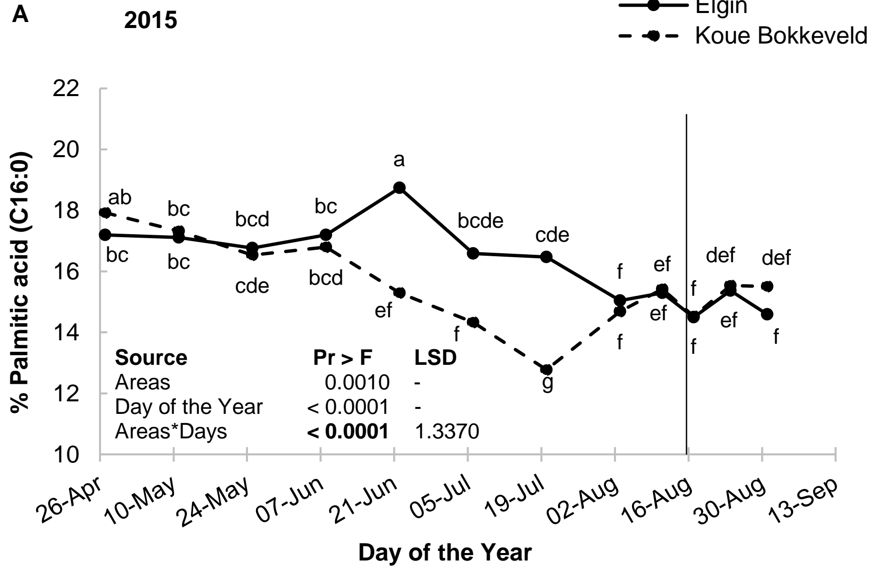
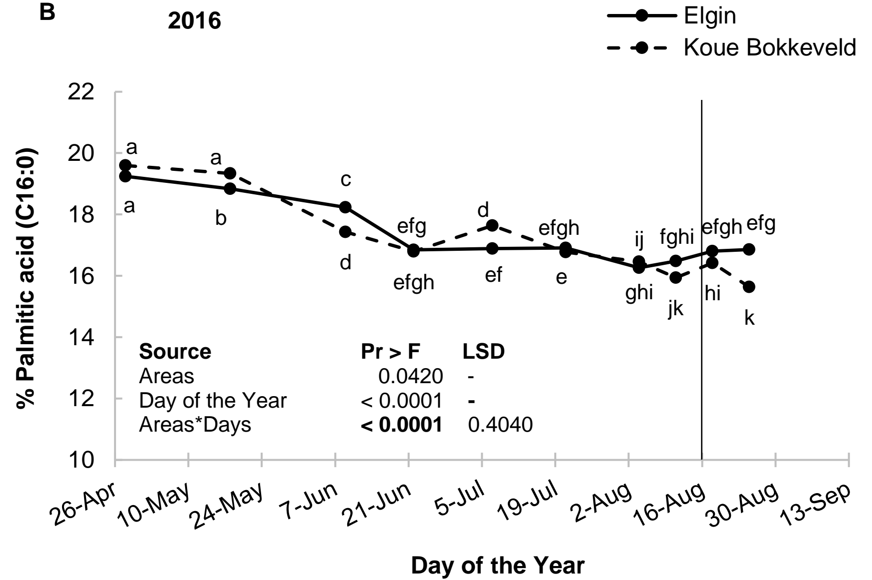
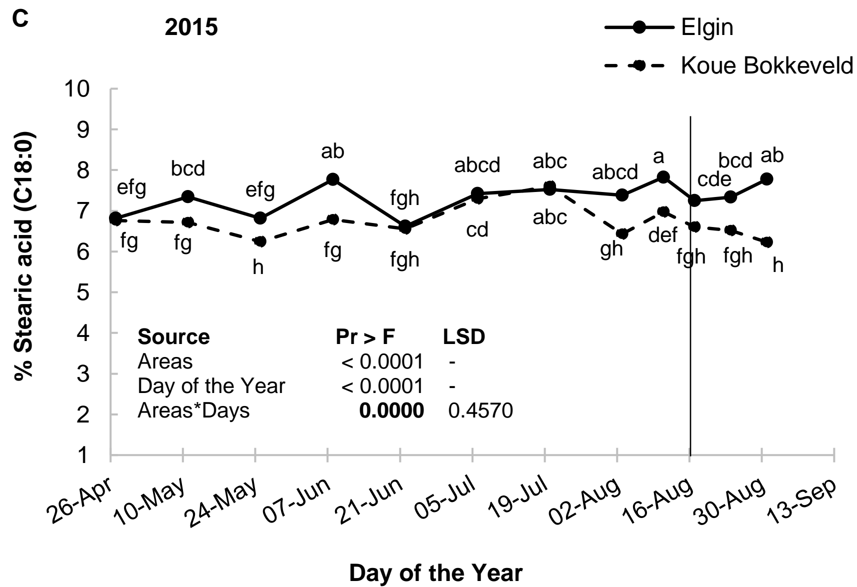


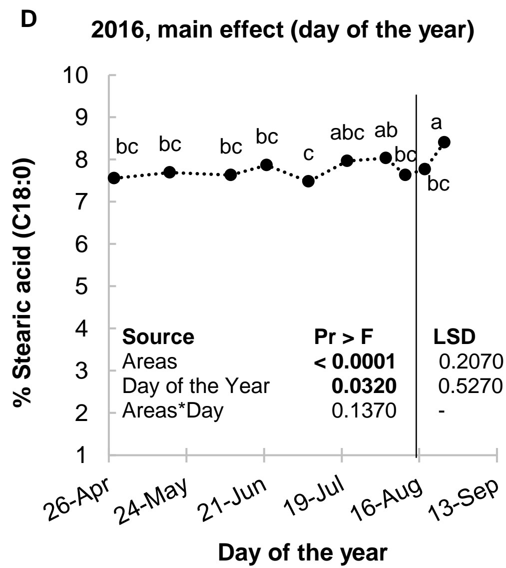
Fig. 4: Lipid concentration (mg/gFW) of the polar and neutral lipid fractions of 'Cripps Pink' buds sampled in the Koue Bokkeveld (_ _ _) and Elgin (____) during 2015 (A and B) and 2016 (C and D). The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences ($p < 0.05$) when interactions were not significant, the significant main effects are presented.

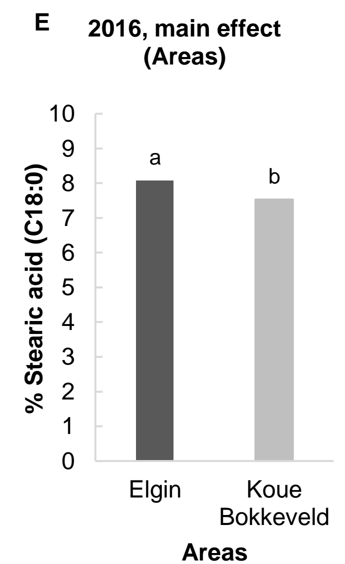
NOTE: In order to make graphs clear and readable, we present NL and PL ANOVAs in the same figure, NL with capital letters and PL with the small letters. Also note that the vertical axes for (2015) and (2016) differ.


A
2015
Elgin
Koue Bokkeveld
% Palmitic acid (C16:0)
Source Pr > F LSD
Areas 0.0010 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 1.3370
Day of the Year


B
2016
Elgin
Koue Bokkeveld
% Palmitic acid (C16:0)
Source Pr > F LSD
Areas 0.0420 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 0.4040
Day of the Year


C
2015
Elgin
Koue Bokkeveld
% Stearic acid (C18:0)
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year < 0.0001 -
Areas*Days 0.0000 0.4570
Day of the Year


D
2016, main effect (day of the year)
% Stearic acid (C18:0)
Source Pr > F LSD
Areas < 0.0001 0.2070
Day of the Year 0.0320 0.5270
Areas*Day 0.1370 -
Day of the year


E
2016, main effect (Areas)
% Stearic acid (C18:0)
Elgin
Koue Bokkeveld
Areas

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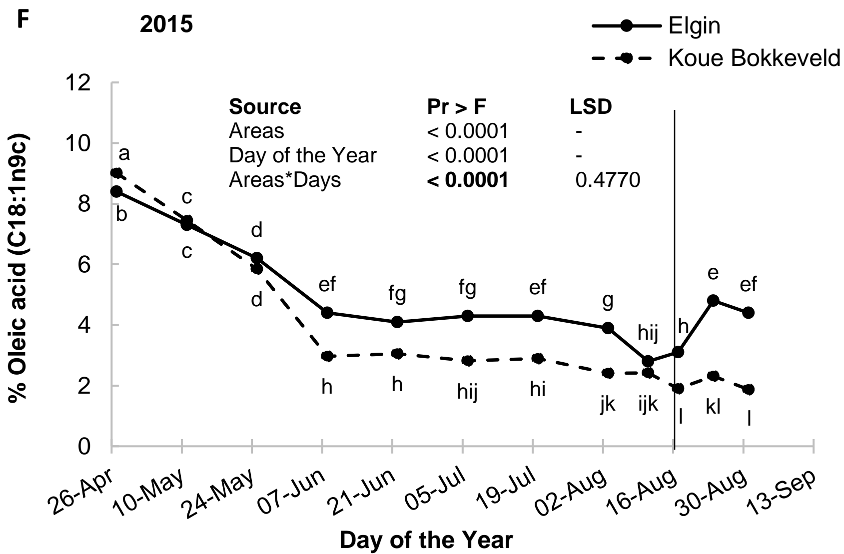
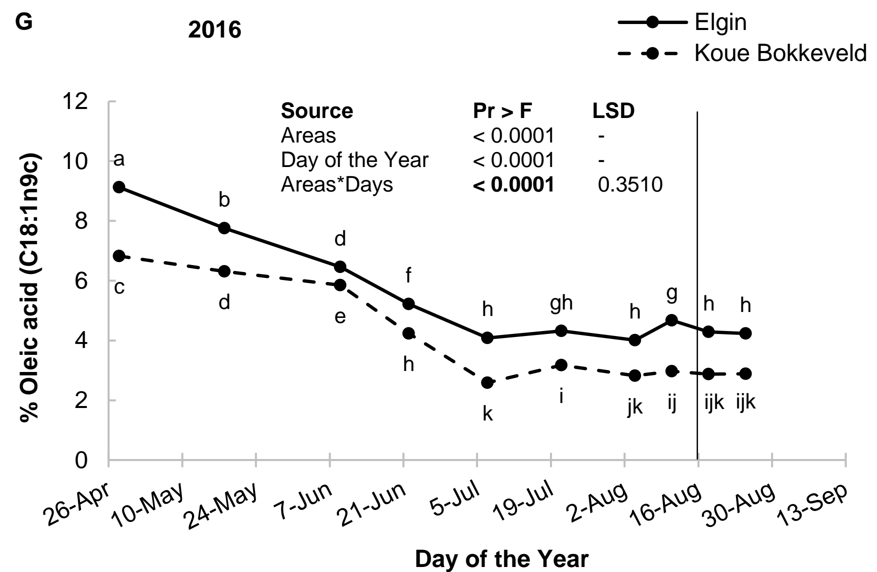
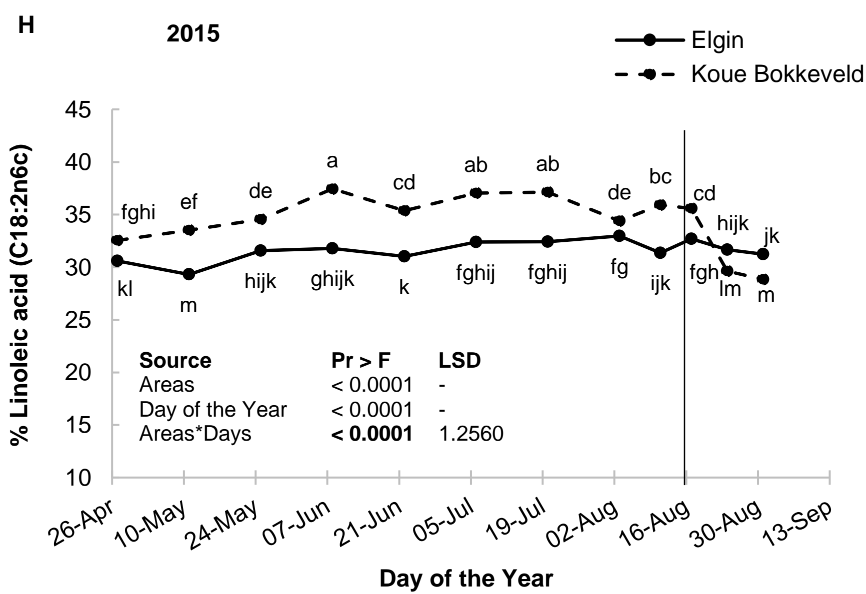
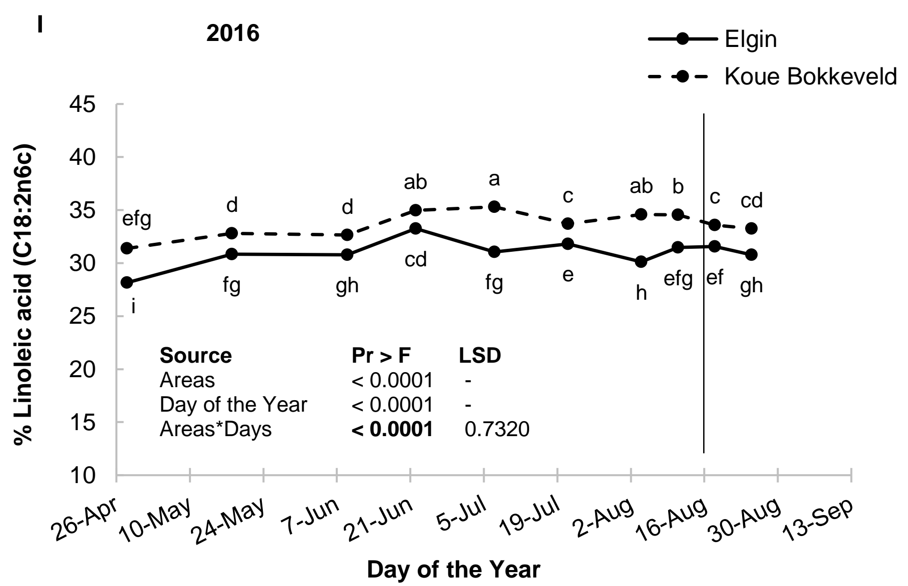








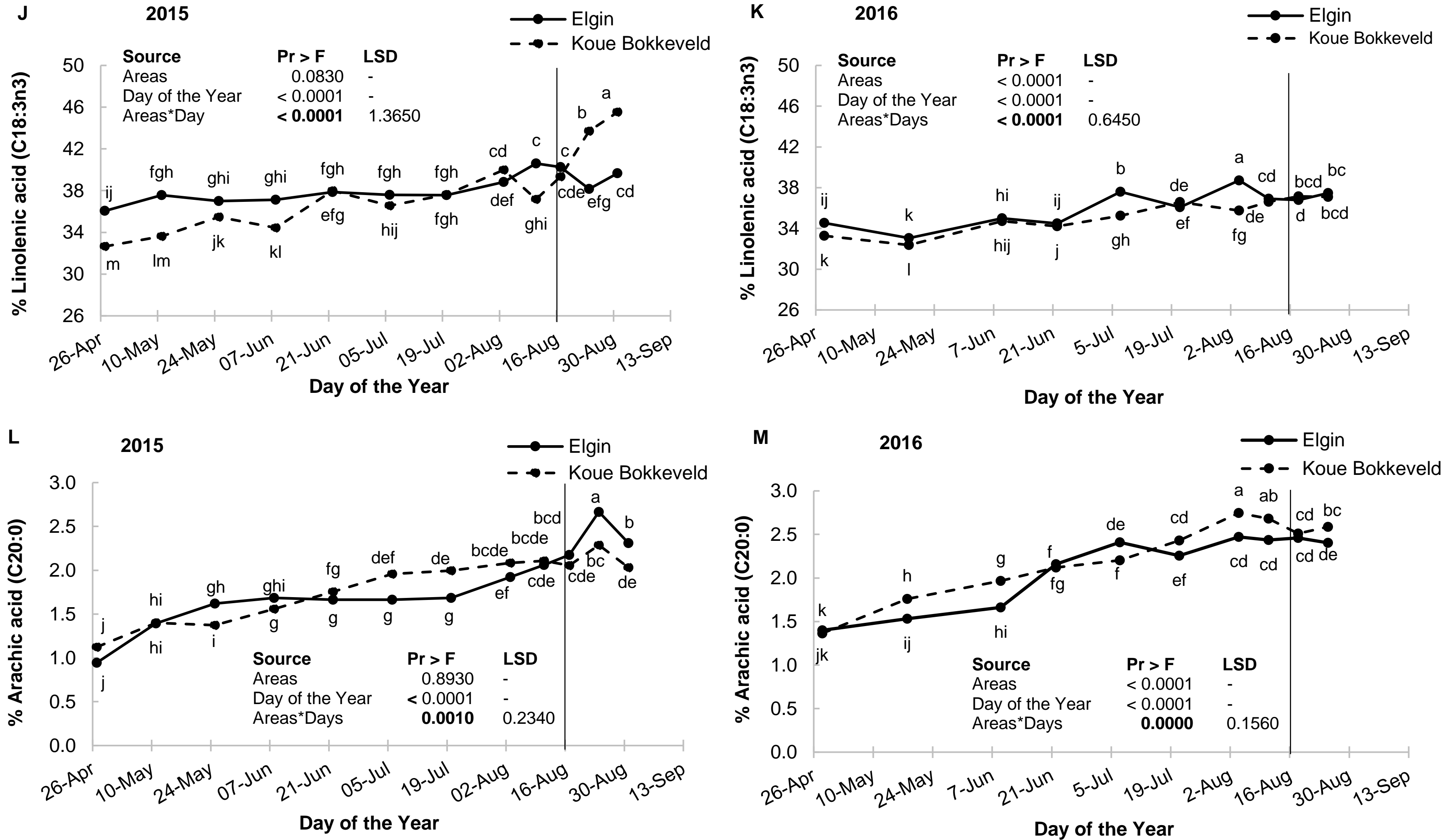


Fig. 5. Percentage palmitic acid (C16:0) (A, B), stearic acid (C18:0) (C, D, E), oleic acid (C18:1n9c) (F, G), linoleic acid (C18:2n6c) (H, I), linolenic acid (C18:3n3) (J, K) and arachic acid (C20:0) (L, M) in the total polar lipid fraction of 'Cripps Pink' buds sampled in Koue Bokkeveld (_ _ _) and Elgin (______) in 2015 and 2016. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.

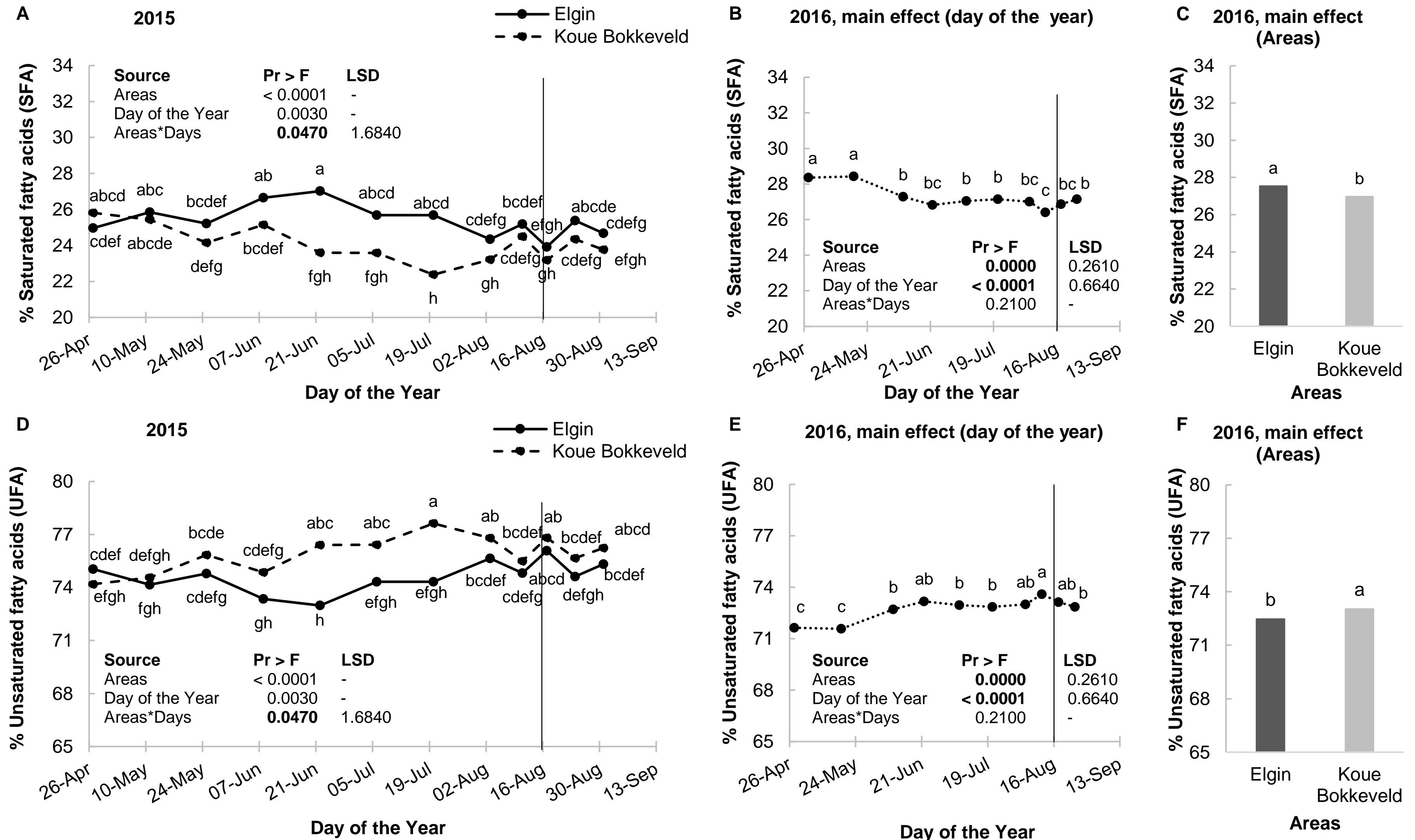


Fig. 6. Percentage saturated fatty acids (SFA) (A, B, C) and unsaturated fatty acids (UFA) (D, E, F) in the total polar lipid of 'Cripps Pink' buds collected from the Koue Bokkeveld (_ _ _) and Elgin (____) in 2015 and 2016. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.

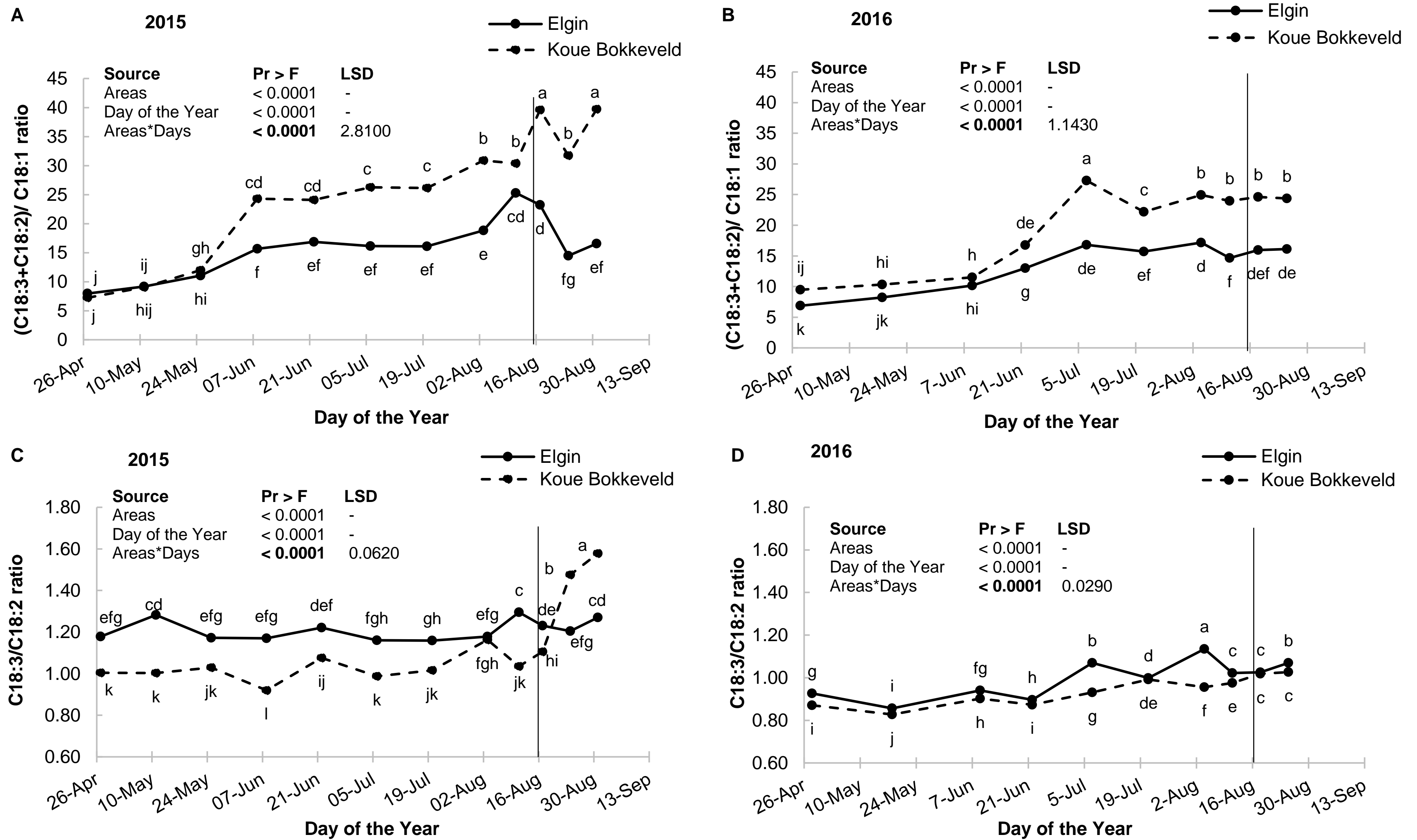
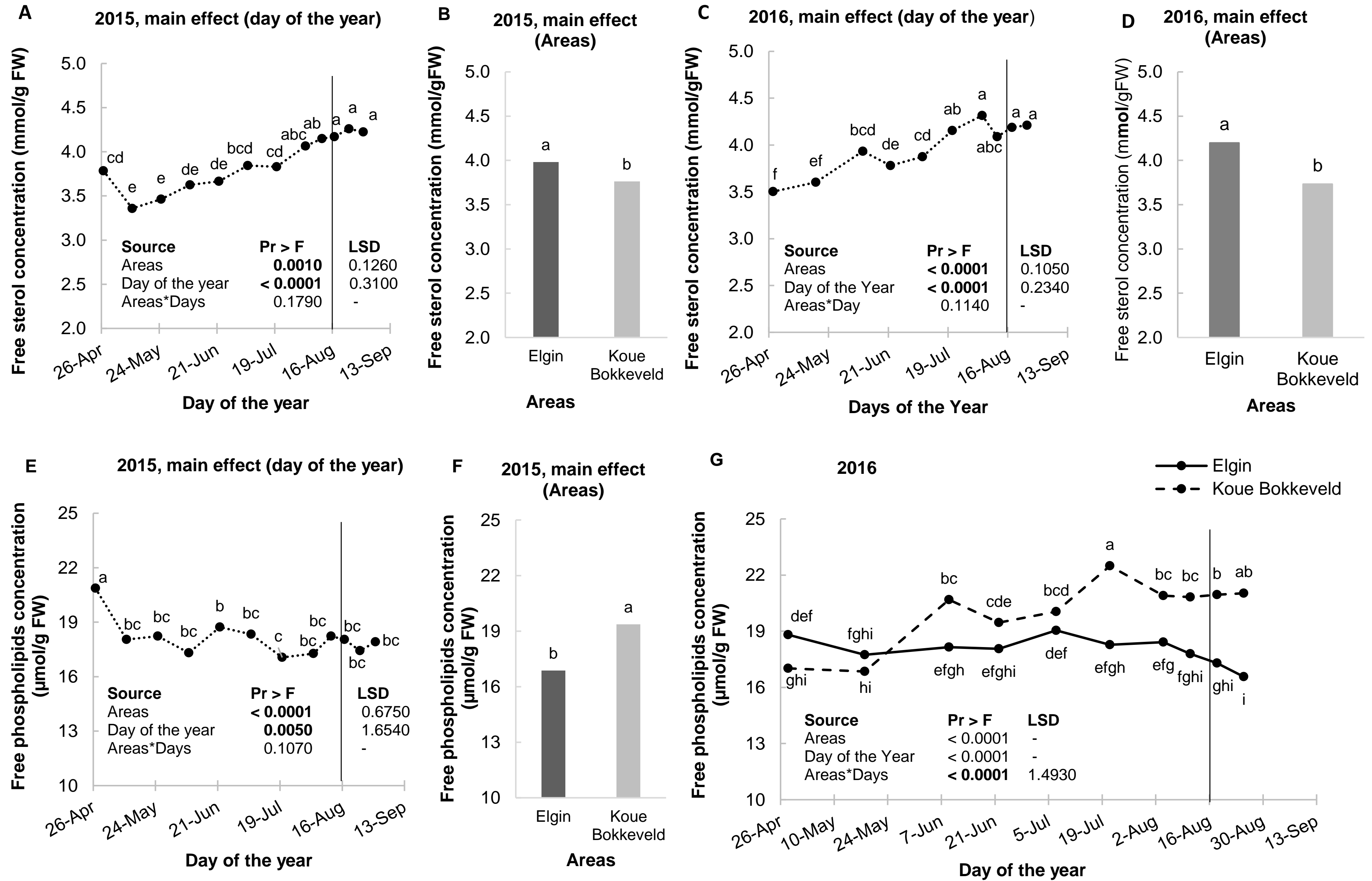


Fig. 7. The unsaturation of polar lipids (PL) in 'Cripps Pink' buds collected in 2015 and 2016 from the Koue Bokkeveld (_ _ _) and Elgin (_____) presented as the (C18:3+C18:2)/ C18:1 ratio (A, B) and the C18:3/ C18:2 ratio (E, F). The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$).


A
2015, main effect (day of the year)
Free sterol concentration (mmol/g FW)
Source Pr > F LSD
Areas 0.0010 0.1260
Day of the year < 0.0001 0.3100
Areas*Days 0.1790 -
Day of the year
B
2015, main effect (Areas)
Free sterol concentration (mmol/gFW)
Elgin
Koue Bokkeveld
Areas
C
2016, main effect (day of the year)
Free sterol concentration (mmol/gFW)
Source Pr > F LSD
Areas < 0.0001 0.1050
Day of the Year < 0.0001 0.2340
Areas*Day 0.1140 -
Days of the Year
D
2016, main effect (Areas)
Free sterol concentration (mmol/gFW)
Elgin
Koue Bokkeveld
Areas
E
2015, main effect (day of the year)
Free phospholipids concentration (µmol/g FW)
Source Pr > F LSD
Areas < 0.0001 0.6750
Day of the year 0.0050 1.6540
Areas*Days 0.1070 -
Day of the year
F
2015, main effect (Areas)
Free phospholipids concentration (µmol/g FW)
Elgin
Koue Bokkeveld
Areas
G
2016
Elgin
Koue Bokkeveld
Free phospholipids concentration (µmol/g FW)
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 1.4930
Day of the year

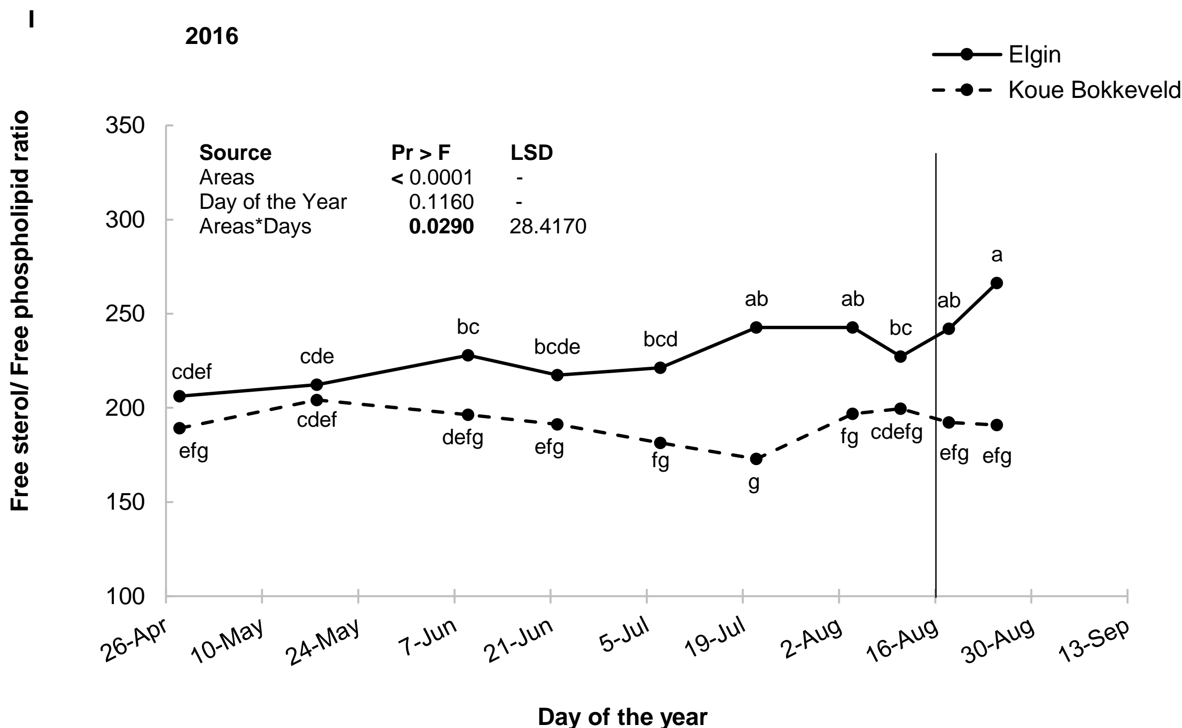


Fig. 8. Total free sterol concentration (A, B, C, D), total free phospholipids concentration (E, F, G) and their ratio (total free sterol/free phospholipids) (H, I) of 'Cripps Pink' buds sampled in the Koue Bokkeveld (_ _ _) and Elgin (____) during 2015 and 2016. The vertical line indicates the end of the dormant phase and the start of the growth resumption. Letters show significant differences ($p < 0.05$) and if the interaction was not significant, the mean effects are presented.

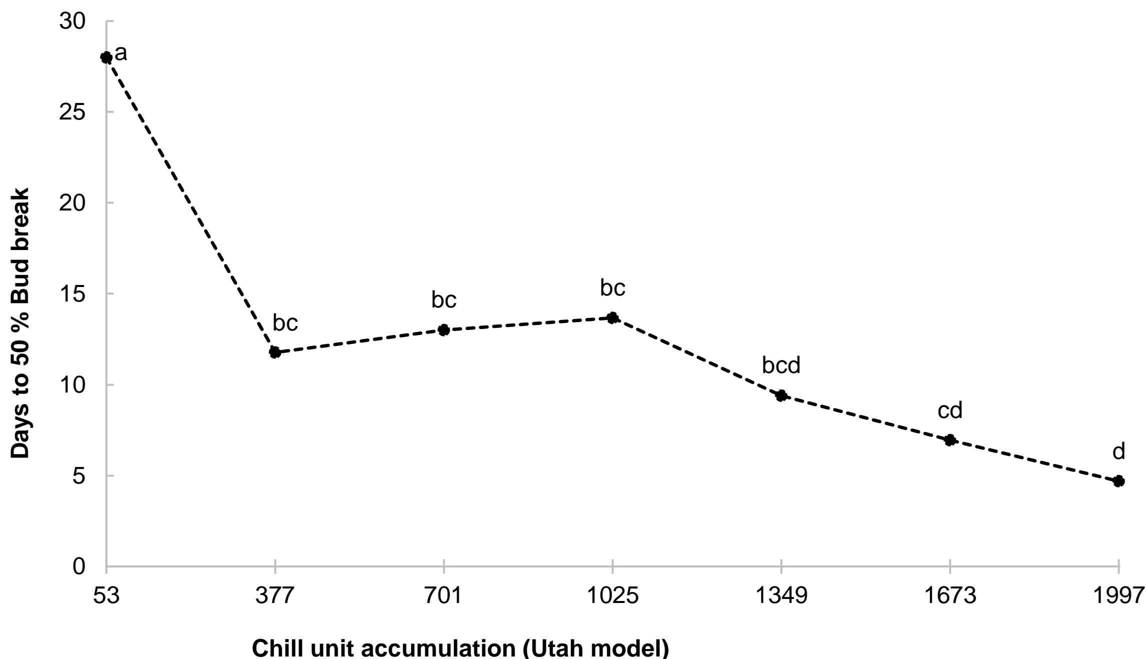


Fig. 9: Dormancy levels of 'Cripps Pink' lab-chilled buds that received progressively more chill units. Letters indicate significant differences between the means ($p < 0.05$).

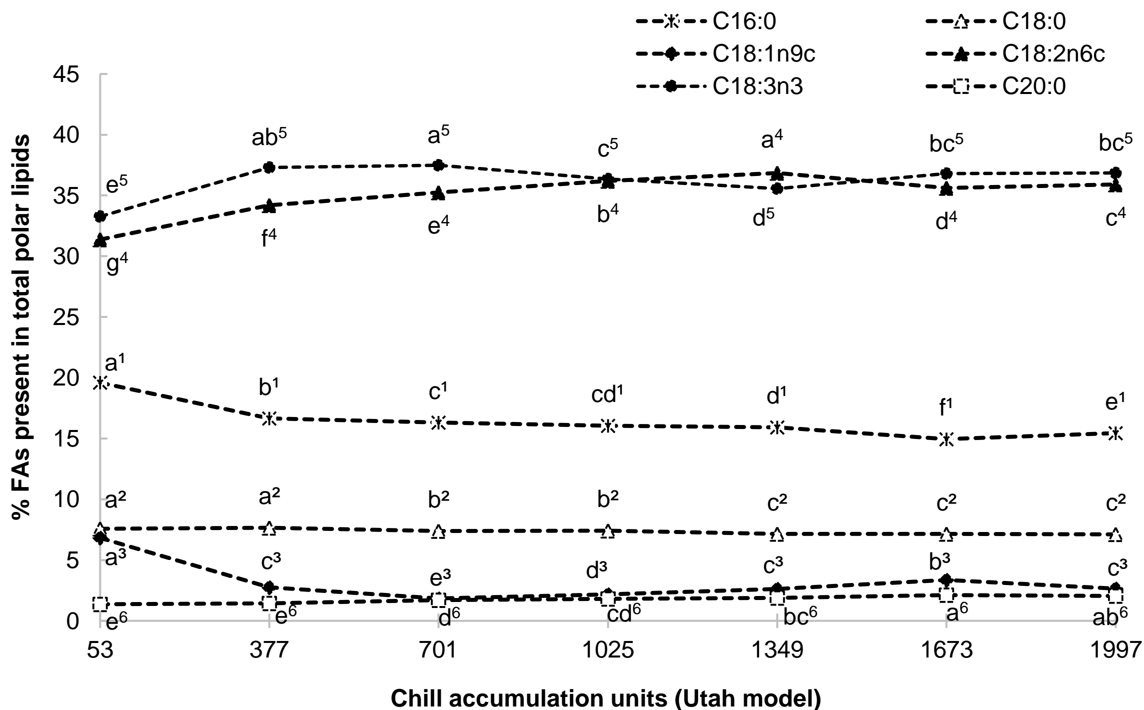


Fig. 10: Percentage palmitic acid (C16:0) (^{1}), stearic acid (C18:0) (^{2}), oleic acid (C18:1n9c) (^{3}), linoleic acid (C18:2n6c) (^{4}), linolenic acid (C18:3n3) (^{5}) and arachic acid (C20:0) (^{6}) in total PLs of 'Cripps Pink' lab-chilled buds that received progressively more chill units. Letters indicate statistical differences between the means ($p < 0.05$). Captions ($^{1, 2, 3, 4, 5, 6}$) indicate individual parameter evaluated alongside chilling accumulation.

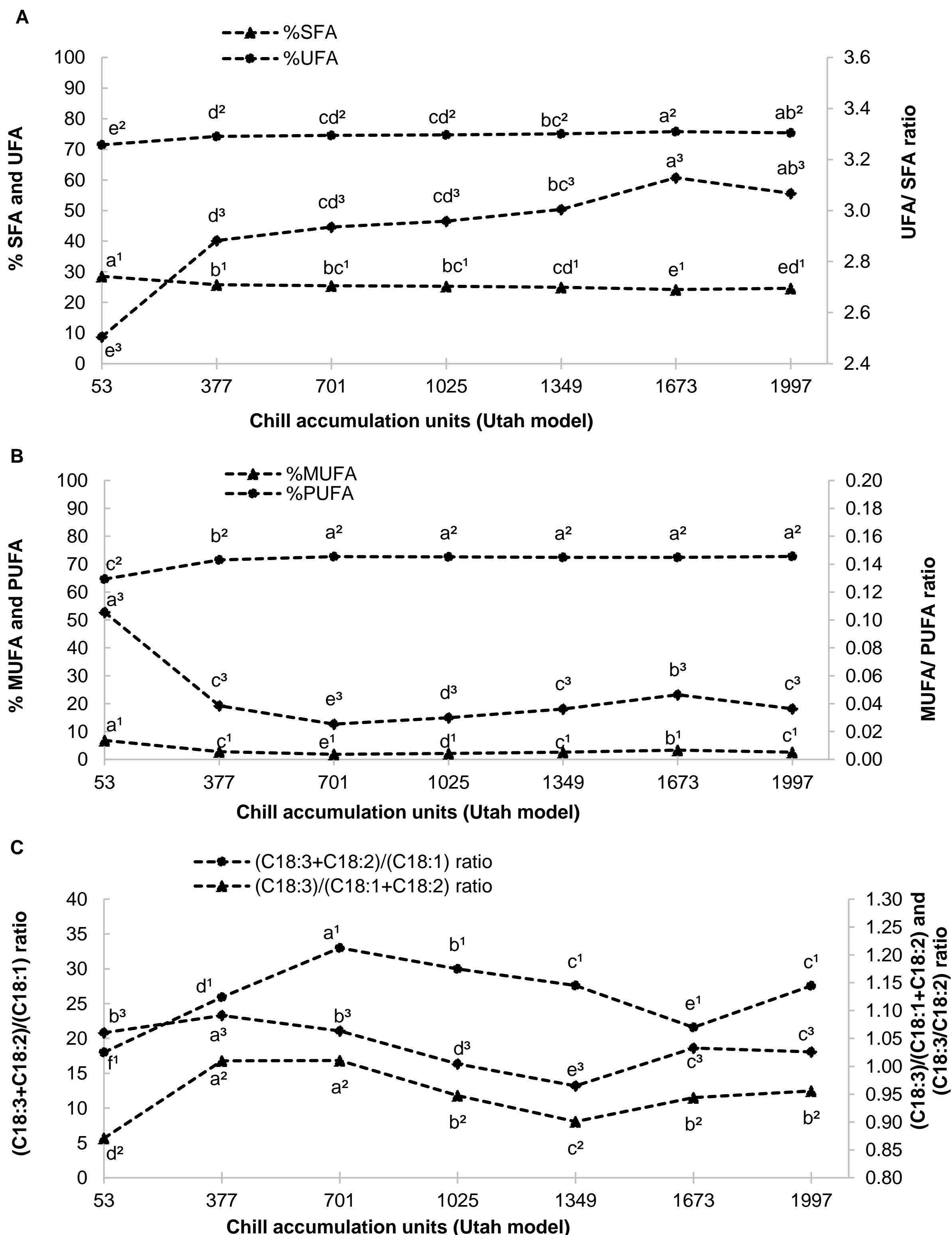


Fig.11. Percentage saturated (%SFA), unsaturated (%UFA) and unsaturated/saturated (UFA/ SFA) FA ratio (A), percentage monounsaturated (%MUFA), polyunsaturated (%PUFA) and MUFA/PUFA FA ratio (B), and ratios of C18:3+C18:2)/ (C18:1), (C18:3)/ (C18:1+C18:2) and (C18:3/ C18:2) (C) in the polar lipid fraction of 'Cripps Pink' lab-chilled buds that received progressively more chill units (Utah model). Letters indicate statistical differences between the means ($p < 0.05$). Captions ([1, 2, 3]) indicate individual parameter evaluated alongside chilling accumulation.

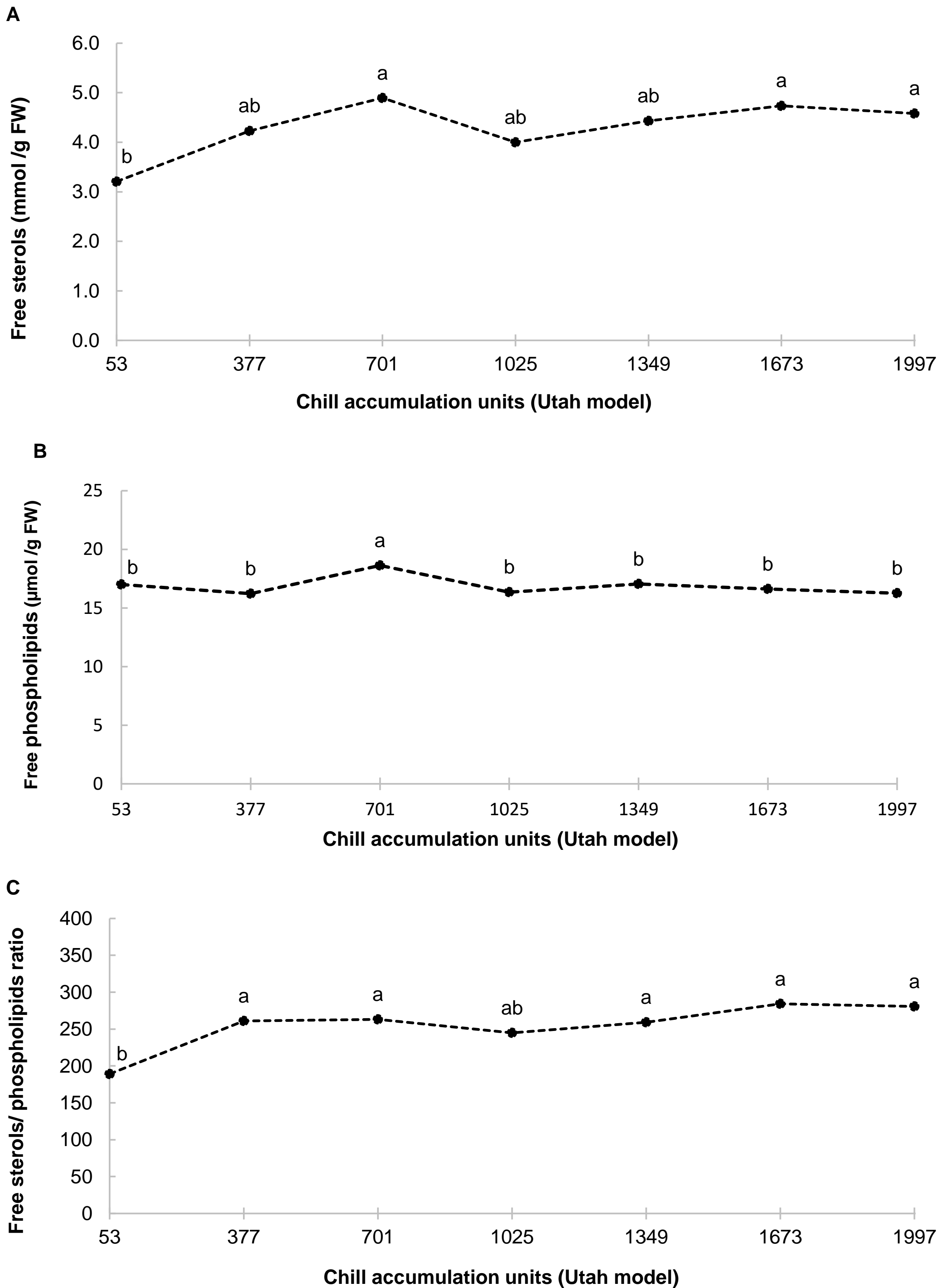


Fig. 12: Concentration of total free sterols (A), total free phospholipids (B) and ratio free sterols/ phospholipids (C) in lipids of 'Cripps Pink' lab-chilled buds that received progressively more chill units (Utah model). Letters indicate statistical differences between the means of buds ($p = 0.05$).

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PAPER 4

The effect of hydrogen cyanamide and oil on the lipids of 'Cripps Pink' terminal buds.

Abstract

In areas with mild winter conditions, inadequate chill accumulation can prolong dormancy in temperate fruit trees and compromise fruit production. To improve production in these areas, rest breaking agents are used to overcome dormancy. It has been shown that some rest breaking agents, e.g. thidiazuron (TDZ), affect the membrane lipid composition of buds thereby resulting in dormancy release. Yet, little is known about the effect of a commonly used rest breaking agent, i.e. hydrogen cyanamide (HC), on the composition of lipids. To address this knowledge gap, the current study investigated the effect of HC in combination with mineral oil (HCo) on the lipid composition of dormant apple buds exposed to insufficient winter chill under South African conditions. In 2015 and 2016, terminal buds from HCo treated and control 'Cripps Pink' apple trees were collected in commercial orchards (Elgin area, ±700 CU). Total lipids were extracted from the buds followed by the quantification of the fatty acid (FA) concentration of the polar and neutral lipid fractions using gas chromatography; free sterols and free phospholipids were measured spectrophotometrically. The FA composition of the polar fraction was affected by the HCo treatment close to budbreak and favoured the desaturation of C18 FAs to linolenic acid (C18:3), which increased the ratios of the C18 groupings, (C18:3+C18:2)/ (C18:1), (C18:3)/ (C18:1+C18:2) and (C18:3/ C18:2) in a similar way as sufficient winter chill. It is suggested that this unsaturation process induced membrane fluidity and permeability which stimulated growth resumption. In contrast, the increase in palmitic acid (C16:0) concentration and the low ratio of free sterols to phospholipids in the treated buds could be due to adverse stress conditions related to mild winter.

Keywords: *apple, budbreak, Dormex™, fatty acids, phospholipids, sterols*

1. Introduction

The apple is the most important deciduous fruit tree grown in South Africa. Previous studies have indicated that these temperate plants can perceive and process different environmental temperatures (Mittler *et al.,* 2012; Theocharis *et al.,* 2012) and need exposure to sufficient low temperatures during winter to exit their dormant phase and restart the growth cycle in spring (Saure, 1985). In areas lacking sufficient winter chill, deciduous fruit trees experience numerous abnormalities in their dormancy progression leading to changes in their growth and development, which may ultimately reduce yield (Cook and Jacobs, 1999; Petri and Leite, 2004). To sustain profitable yields, chemical rest breaking agents (RBAs) are used to compensate for the lack in winter chill and to overcome endodormancy (Faust *et al.,* 1997; Seif El-Yazal and Rady, 2013). A combination of two RBAs, hydrogen cyanamide and mineral oil (HCo), is most effective in promoting endodormancy release and advancing uniform bloom (Petri and Stuker, 1995; Schlemper *et al.,* 2010). HCo is effective in inducing budbreak in several crops such as grape (Rubio *et al.*, 2014), apple (Carvajal-Millán *et al.*, 2007; Amberger, 2013), peach and nectarine (Dozier *et al.*, 1990) and kiwi (McPherson *et al.*, 2001). Under South African growing conditions it has been proved to be a successful RBA in the pome and stone fruit industries (Costa *et al.,* 2004).

Although HCo is widely used, its mode of action is poorly understood. Hydrogen cyanamide (HC) application inhibits catalase activity, thereby increasing the reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), within a cell, which is linked to the oxidation of membrane lipids (Nir *et al.,* 1986; Pérez *et al.,* 2008; Ben Mohamed *et al.,* 2012; Amberger, 2013). In plant cells, polar lipids form the main structural component of membranes (van Meer *et al.,* 2008) whereas neutral lipids (lipid reserves) are larger molecules and serve primarily as storage of reduced carbon (Ohlrogge and Browse, 1995; Durrett *et al.,* 2008; Solovchenko, 2013). The fatty acid chains of both polar and neutral lipids consist of 16 or 18 hydrocarbons that can contain one to three *cis* double bonds. The most important fatty acids (FAs) in plants are the saturated palmitic (C16:0) and stearic (C18:0) acid, the monounsaturated oleic acid (C18:1) as well as the polyunsaturated linoleic (C18:2) and linolenic (C18:3) acid (Ohlrogge and Browse, 1995). Very long chain fatty acids (VLCFAs, >C18) are mostly part of the neutral lipids in plants (Ohlrogge and Browse, 1995). In addition to polar and neutral lipids, plant cell membranes also contain sterols and phospholipids that are involved in the biological regulation of the membrane structure, as well as in controlling permeability and fluidity of the membrane. Lipids also play a key role in metabolic processes during growth and development of plants (Hennessey, 1992; Hartmann, 1998; Dufourc, 2008). Despite the similarity in the synthesis of neutral and polar lipids, the lipid

composition can vary significantly across plant species (Uemura and Peter, 1994), plant organs (Quinn *et al.,* 1978; Wu *et al.,* 2005), and with environmental conditions (Palta *et al.,* 1993; Welti *et al.,* 2002). For example, the polar lipids in leaves of the common green bean (*Phaseolus vulgaris*) account for 59.5% of the total lipid content while the neutral lipids constitute 40.5% (Wilson and Crawford, 1974).

Unsaturated FAs play an important role in membrane structure and function. Most of the studies on lipids of temperate fruit trees focused on the membrane lipid composition during seasonal changes or that of buds exiting dormancy after receiving sufficient winter chill (Wang and Faust, 1990a and 1990b; Izadyar and Wang, 1999). Such studies show that the composition and desaturation of the membrane lipids change during dormancy progression and affects the membrane fluidity and permeability. Generally, during chill accumulation under natural/artificial low temperatures or chemical RBA treatment lipid desaturation is induced. Under such conditions Saturated fatty acids (SFAs, with no double bonds, e.g. palmitic acid C16:0 or stearic acid C18:0) are desaturated into monounsaturated FA (MUFAs, with one double bond, e.g. oleic acid C18:1) to preserve the membrane fluidity and maintain cellular viability (Murata and Wada, 1995, Wang and Faust, 1988, 1990; Meï *et al*., 2015). These FAs are then desaturated into polyunsaturated FAs (PUFAs, with two *cis*-double bonds, e.g. linoleic acid C18:2 or with three *cis*-double bonds, e.g. linolenic acid C18:3) to further increase in fluidity (Ohlrogge and Browse, 1995; Murata and Wada, 1995). The polyunsaturation of linoleic acid to linolenic acid occurs at growth temperatures once the chilling requirement is fulfilled or insufficient chill has been compensated for (Wang and Faust, 1988, 1990) resulting in an increase in the ratio of unsaturated to saturated fatty acids during budbreak and growth resumption (Wang and Faust, 1988; Bregoli *et al*., 2006). Furthermore, prior to budbreak, free sterol levels decrease while phospholipid levels increase in the membrane structure (thus, a decrease in free sterol: phospholipid ratio) also contributing to a more fluid membrane (Wang and Faust, 1988, 1990 and 1993; Izadyar and Wang, 1999). Generally, under low temperatures, plant membranes acclimatise by increasing cryostability. This process is crucial as it results in the preservation of the membrane function and cell compartmentation (Alberdi & Corcuera, 1991; Faust *et al*., 1997). The modification of membrane fluidity and permeability may result in an increased tolerance to contraction and expansion of the membrane during induced stresses (Uemura and Steponkus, 1994; Uemura *et al*., 2006) preventing membrane ruptures and cell content leakage. Decreased porosity and the smaller size of pores, brought about by structural and compositional changes, result in a slower rate of cellular water loss and ice formation (Nilsen and Orcutt, 1996; Uemura *et al*., 2006), thus decreasing dehydration due to freezing injury. Decreased porosity also increases the effectiveness of the cell membrane as an ice barrier preventing membrane rupture by ice crystals.

However, the effect of HCo on lipid composition during dormancy release in apple has not been studied to date. It is not known whether HCo application affects lipid composition in a similar way as natural winter chill and how this compares to buds that did not receive adequate chill. It is then important to understand how HCo modifies the cell structure to allow movement through the membranes in the areas with a lack of chilling during spring conditions. Therefore, the current study aims to investigate possible changes in the lipid, sterol and phospholipid concentration and saturation in apple buds that received an HCo rest-breaking treatment following a winter period of insufficient chill.

2. Materials and Methods

2.1. Experimental site, treatment and sample collection

This study was conducted on mature 'Cripps Pink' apple trees, which has a medium chill requirement (450 - 800 positive chill units; ARC-Infruitec, 1997 as per Tharaga, 2014), cultivated on a commercial farm located in the warm winter region (± 700 CU, Utah model) of Elgin (34ºS, 19°E; 305 m a.s.l.) in the Western Cape, South Africa. For the duration of the trial a logger (Tiny Tag, Gemini Data Loggers, Chichester, UK) was used to record hourly temperature in the orchard. Treatment (Day 0) and sample collection were similar to that presented in Paper 2 of this dissertation. One day prior to treatment (Day -1), baseline samples were taken and control shoots were randomly selected, tagged and covered with transparent LDPE bags. The orchard was treated with a blend of 0.5% Dormex™ (hydrogen cyanamide) (Nexus, Paarl, South Africa) and 4% mineral oil (Nexus, Paarl, South Africa) on 3 September and 31 August for the 2015 and 2016 seasons respectively. The plastic bags were removed from the control shoots within 24h post treatment. During both seasons, 105 one-year-old shoots (±25 cm) were randomly collected from both the treated and control shoots every 2-3 days until the orchard had reached 70 % 'green-tip' stage. At such time the control terminal buds (untreated) showed no sign of budbreak. The terminal buds from the treated and the control shoots were excised and pooled into three replicates (n = 35 buds per replicate) each. These replicates were frozen in liquid nitrogen and stored at – 80 °C until further processing.

2.2. Lipid composition

To determine the lipid composition, each replicate was milled with a ceramic mortar and pestle in liquid nitrogen. Thereafter, the total lipids were extracted from 0.5 g of each replicate using a modified method of Jooste (2014) as described in Paper 3 of this dissertation. During the lipid extraction process, 200 µL of 2 mg mL^{-1} n-Heptadecanoic acid (Merck, Johannesburg, South

Africa) was added to each sample as an internal standard. At the end of the extraction, the concentrated lipid extract was dried by vacuum aspiration and dissolved in a solution of 2 mL chloroform (Merck) mixed with 0.2 mg butylated hydroxytoluene (BHT) (w/v) and kept at –80 ºC until further analyses.

2.2.1. Neutral lipids, free fatty acids and polar lipids

To determine the amount of FAs present in the buds, 300 µL of the total lipid extract was applied to 6 mL (500 mg) NH_2 cartridges (Chromabond columns, Düren, Germany) and allowed to pass through the column. To fraction the lipid extract into three different lipid classes, i.e. neutral lipids (NLs), free fatty acids (FFAs) and polar lipids (PLs), the method described in Paper 3 was used. To elute the NLs from the column, 4 mL of a chloroform: isopropanol (Merck, Johannesburg, South Africa) (2:1, v/v) solution was run through the column four times. Thereafter, FFAs were eluted from the column by applying 4 mL of a diethyl ether: acetic acid (Merck) (98:2, v/v) solution four times to the column. Lastly, PLs were eluted from the column by applying 4 mL of HPLC-grade methanol (Sigma-Aldrich, Johannesburg, South Africa), again four times to each column. The solvents, for each of the lipid classes, were evaporated from the samples through vacuum aspiration at medium temperature (40 ºC). Subsequently, the samples were methylated with 0.4 mL of a 10% (w/v) boron trifluoride (BF_3) (Sigma-Aldrich) in a methanol solution, by heating each sample at 100 °C on heating block for 5 minutes and cooling the samples to room temperature in a water bath. The fatty acid methyl esters (FAMEs) were then extracted by adding 2 mL of HPLC-grade hexane (Sigma-Aldrich) and 1 mL deionized H_2O to each sample, followed by centrifugation for 5 min @ 3000 rpm at 20 °C.

The concentration of methylated NLs, FFAs and PLs present in the samples of each different lipid class was determined immediately by transferring 1 mL of the top hexane layer of each sample to a gas chromatography (GC) vial (Chemetrix; Johannesburg, South Africa). Thereafter, a 2 µL aliquot was subjected to GC analysis using a GC instrument (Model 7890A, Agilent Inc., Wilmington, USA) fitted with a flame ionisation detector (FID) and automatic sampler (Model 7683B, Agilent Inc., Wilmington, USA). To achieve this, the oven temperature of the GC was set to increase from 150 °C (isothermal for 1 min) to 170 ºC at 1.2 ºC min^{-1}, from 170 ºC to 180 ºC at 5 ºC min^{-1} and from 180 ºC to 250 ºC (isothermal for 6 min) at 20 ºC min^{-1}. To identify the FAMEs in the samples, the retention times of the unknown FAMEs where compared to that of the FAMEs in a Supelco 37 component FAME Mix (C4 - C24) (Supelco Inc., Bellefonte, PA, USA). Quantification of the unknown FAMEs was achieved by setting up a standard curve of the Supelco 37 component FAME Mix and subjecting the samples to a similar GC analysis as described

above and calculating the ratio between the obtained values and the internal standard (unmethylated n-Heptadecanoic acid), contained in the free fatty acid fraction.

2.2.2. Total free sterols

To quantify the total free sterols present in the buds the modified sterol quantification method was used as described in Paper 3. In brief, a 100 µL aliquot of each total lipid extract was evaporated to dryness in a stream of nitrogen in a fume hood. Subsequently, each extract was dissolved in 6 mL glacial acetic acid (Merck,) then mixed with 4 mL of ferric chloride colour reagent mixture prepared by dissolving 2.5 g $FeCl_3$ $6H_2O$ in concentrated phosphoric acid (w/v). The resulting mixtures were vortexed and transferred into plastic cuvettes (Lasec, Cape Town, South Africa) after 10 min. The amount of total free sterols present in each sample was subsequently determined by measuring the absorbance at 550 nm using a UV/VIS spectrophotometer (Varian, 50 Bio-UV-Visible Spectrophotometer, Mulgrave, Australia). The total sterol concentration present in each sample was calculated from a stigmasterol standard curve and was expressed in mmol g^{-1} FW.

2.2.3. Total free phospholipids

The concentration of the total free phospholipids present in the buds were quantified using the methods of Ames (1966) and Duck-Chong (1979) modified by Jooste *et al.* (2014), which was further modified as described in Paper 3 of this dissertation. To achieve this, 30 µL of a 10% (w/v) $Mg(NO_3)_2.6H_2O$ (Sigma- Aldrich Johannesburg, South Africa) in 95% (w/v) methanol solution was added to 25 µL of each total lipid extract as well as to tubes designated to the construction of a standard curve. All tubes were dried in a heating block for 5 minutes at 95 °C. The resulting precipitate was heated with a Bunsen burner in a fume hood until the brown fumes disappeared. The pyrophosphate reaction which was formed during burning was subsequently hydrolysed to phosphate by adding 300 µL of 0.5 M HCl (Merck, Johannesburg, South Africa) to each tube. The samples were then heated in a heating block at 100 °C for 15 min and immediately cooled by immersion in a water bath at room temperature. Subsequently, 700 µL of a 1:6 (v/v) mixture of 10% ascorbic acid (Sigma-Aldrich) and 0.42% ammonium molybdate tetrahydrate (Laboratory Supplies, South Africa) in 1 N H_2SO_4 (Merck) were added to each sample and to the standard curve designated tubes. The samples and standards were then incubated at 45 ºC for 20 min on a heating block. Thereafter, a 1 mL aliquot of each sample was added to a plastic cuvette (1 cm path length) and the absorbance was read at 820 nm using a 50 Bio-UV-Visible spectrophotometer (Varian, 50 Bio-UV-Visible Spectrophotometer, Mulgrave, Australia). The quantification of the total free phospholipids in each sample was determined using the absorbance

of the sodium phosphate standard curve measured at 820 nm. The total phospholipid concentration was expressed in µmol g^{-1} FW.

2.3. Calculations and statistical analyses

As described in Paper 3, the percentage of individual FAs in the NL and PL fractions were calculated and the different FA groupings and ratios are expressed to make it comparable to similar studies from the literature (Wang and Faust, 1988, 1990; Ruiz *et al.*, 2004; Jooste, 2012). The total percentage of saturated fatty acids (%SFA) was obtained by adding up all the individual percentages of saturated FAs present in the total fraction (PL or NL). Saturated fatty acid chains contain only single bonds (e.g. palmitic acid (16:0), stearic acid (C18:0)) while the unsaturated chains have one or more *cis* double bonds (e.g. oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3)). Similarly, the total percentage unsaturated fatty acids (%UFA) was determined by adding up all the individual percentages of the unsaturated FAs present in the total fraction (PL or NL). From this the UFA: SFA ratio for both the PL and NL fractions was calculated. The percentage monounsaturated fatty acids (%MUFA) were obtained by adding the percentages of all the FAs that had only one double bond (e.g. oleic acid (C18:1n9c)), while the percentage polyunsaturated fatty acids (%PUFA) was calculated by adding up the percentages of all the FAs that had two or more double bonds (e.g. linoleic acid (C18:2n6c), linolenic acid (C18:3n3)) and from this the MUFA: PUFA ratio was calculated for the PL and NL fractions. Lastly, the unsaturation ratios of the C18 FAs (C18:3+C18:2)/ (C18:1), (C18:3)/(C18:1+C18:2) and (C18:3/C18:2) in the PL and NL fractions, were calculated similarly to Wang and Faust (1988 and 1990b). Only results from the polar lipid fraction will be discussed in this chapter as they include membrane lipids that are the matrix for metabolic events, selective permeable barriers to macromolecules and solutes, regulate fluidity and act as transducing signals (Furt *et al.*, 2011). Mitochondrial membranes also house the respiration mechanics. Results from the neutral lipid fraction (main part of the stored lipid pool) are included in Appendix B as additional information.

To describe the effect of the treatment throughout the trial period a two-way ANOVA (including possible interactions with the treatment and the days pre and post the treatment as independent variables) was performed across the sample collection points within each season at a significance level of 5%. Where a significant interaction (Treatments*Days) was detected ($p < 0.05$), differences among the means were compared using a *post hoc* Fischer's Least Significant Difference test. If the interaction was found to be non-significant ($p > 0.05$) the main effects, i.e. Treatments and Days, are presented independently. All statistical analyses were performed using the XLSTAT package (Version 19.01.40777, XLSTAT Institute, Berkeley, USA).

3. Results

3.1. Phenological stages and weather conditions pre and post treatment.

In both years the HCo application was administered at the time of budswell (3 September and 31 August for the 2015 and 2016 seasons, respectively) and the buds were monitored until 70% of the orchard (treated trees) was at the green-tip stage. In 2015, the green-tip stage was reached 15 days after the application of HCo and in 2016 after 21 days. The daily maximum temperatures for the trial period are reported in Fig. 1. From this, it is clear that daily maximum temperatures directly after the application of the RBA were very different between the two years. In 2015, the two days following the application had relatively high temperatures (29.7 and 29.3 °C) with dry conditions followed by milder conditions (16.1 °C) on Day 4, compared to the 2016 season where the maximum temperature dropped to 16.1 and 13.1 °C on Days 1 and 2 after application with rainy orchard conditions that recovered to 25.6 °C on Day 4. In 2015 there was a warm spell with temperatures reaching 26.6ºC and 34.6 °C on Day 9 and Day 10, respectively. In 2015 the control shoots were bagged 24h prior to the HCo application (Day -1) and in 2016 it was done 48h (Day -2) prior to HCo application. During the winter period of 2015 a total of 854 CU (Utah) were accumulated versus 788 CU in 2016 (calculated from 1 May to 31 August in both years).

3.2. Lipid content and FA composition

In both seasons, the ratio of neural lipid to polar lipid (NL:PL) was between 0.91-1.44 % (Fig. 2A and B). In 2015, only the main effect, days post treatment, had a significant effect on the NL:PL ratio ($p < 0.0001$) (Fig. 2A). Although no difference was found between control and treated buds, the average neutral lipid concentration was significantly higher than the corresponding polar lipid concentration, with only the last sampling point (70% green-tip stage in the treated orchard) showing no significant difference. The ratio in both fractions increased on the first day of the trial and then progressively declined until the end of trial (Fig. 2A). Similar to the 2015 season, no significant interaction was found between the main effects during 2016; only the days post application had a significant effect on the ratio ($p < 0.0001$) (Fig. 2B). In 2016 the samples also showed higher levels of neutral lipids compared to polar lipids except at the last sampling date. The ratio remained stable for the first seven days post treatment after which the level also decreased until the end of the trial (Fig. 2B) similar to the 2015 trend.

Fig. 3 shows the concentration of the polar and the neutral lipids across the trial period for both years. Across seasons, no significant interaction ($p > 0.05$) was found between the main effects in the neutral lipids, and only the main effect, days post treatment, was found to be significant

($p < 0.0001$). However, a significant interaction between treatment and the days post treatment was detected for polar lipids in both years ($p = 0.0080$ in 2015 and $p = 0.0090$ in 2016). In 2015, the concentration of the neutral lipids of both the control and the treated buds remained constant from the onset of the trial and slowly decreased up to Day 11 after which the rate rapidly decreased until the end of the trial (Fig. 3A). The polar lipids were also affected by the treatment. Polar lipid concentrations in treated buds declined until Day 3 and remained stable until Day 7 before a progressive increase occurred until the end of the trial. The concentration of the polar lipids in the control buds remained relatively constant throughout the trial (Fig. 3A). In 2016, a similar pattern to 2015 was observed for the neutral lipids, the concentration of both the control and the treated buds remained constant and only declined after Day 16 (Fig. 3B). The most obvious trend in the neutral lipid fraction is the sudden decrease in lipid concentration of both the treated and the control buds at the last measurement of the trial. This phenomenon occurred in both years and was absent in the polar fractions. The polar lipid concentrations for both the treated and the control buds of 2016 remained relatively stable and did not differ significantly throughout the trial (except for Day 0 and Day 12) (Fig. 3B).

In the polar lipid fraction, a total of six FAs were detected in the treated and control buds, i.e. palmitic acid (16:0), stearic acid (C18:0), oleic acid (C18:1n9c), linoleic acid (C18:2n6c), linolenic acid (C18:3n3) and arachic acid (C20:0) (Fig. 4). Amongst these, linolenic acid, linoleic acid and palmitic acid were predominant for both 2015 and 2016 (Fig 4). These same six FAs were also detected in the NL fraction with the addition of three VLCFAs, i.e. heneicosanoic acid (C20:1), eicosatrienoic acid (C20:3n3) and lignoceric acid (C24:0). Although some variations were observed in the NL concentrations, little changes were detected in the individual FAs for both control and treated buds. The effects of HCo on the NL FAs and the saturation of FAs are presented in the Appendix B (Fig. 5 to Fig. 8). The rest of the chapter will focus on the effects of the treatment on the individual FA from the PL fraction.

3.3. Effect of HCo on FA composition of polar lipid fraction

3.3.1. Changes in individual FAs

3.3.1.1. Palmitic acid (C16:0)

Across samples and treatments, palmitic acid made up between 14 – 22% of the FA in the polar fraction. A significant interaction between treatment and days post application ($p < 0.0001$ in both years) was detected for palmitic acid (C16:0) in the buds sampled in 2015 (Fig. 4A) and 2016 (Fig. 4B). The baseline samples of the two years showed that in 2015 the palmitic acid percentage

in the buds was 4% lower compared to 2016. At the end of the trial, a higher percentage of palmitic acid was observed in the treated buds compared to the control buds, especially in 2015. In 2015, both the control and the treated samples increased their initial levels significantly by Day 1, but then remained relatively stable for the next 10 days after which it increased significantly in treated buds to end 19% higher than the control buds at the end of the trial on Day 16 (Fig. 4A).

In the following year (2016), buds showed little variation. Except for a significant increase in the treated buds on the day of treatment and on Day 2, the control and treated buds remained similar until Day 16 of the trial when the treated buds decreased significantly, only to increase again and end the trial 8% higher than the control (Fig. 4B).

3.3.1.2. Stearic acid (C18:0)

Stearic acid made up between 6 – 9 % of the polar fraction during the trial (Figs. 4C, D and E). The starting percentage of stearic acid was found to be lower during 2015 compared to 2016, but at 70% green-tip stage, the percentages of both seasons were similar. In 2015, no significant interaction ($p < 0.0001$) was found between the main effects, but both main effects were found to be significant (both $p < 0.0001$). The percentage FA in both the control and treated buds increased sharply on the first day of the trial and then declined steadily over the 14 day period of the trial to end upon a value similar to the onset of the experiment (Fig. 4C). On average throughout the season, the treated buds showed a significantly higher amount of stearic acid (7.5%) compared to the control buds (6.7%) (Fig. 4D).

Similar to the 2015 season, no significant interaction ($p > 0.05$) effect was detected in 2016 and of the two main effects, only time (days) had a significant effect on the percentage stearic acid ($p < 0.0001$) (Fig. 4E). The percentage stearic acid present in the buds (treated and control) remained relatively similar from the onset of the experiment until Day 14 post treatment, when it decreased significantly and then remained at this lower level until the end of the trial.

3.3.1.3. Oleic acid (C18:1n9c)

In both years, the oleic acid in the treated and control buds measured between 2 – 5 % of the total polar fraction (Fig. 4F, G and H). While no significant interaction was detected for the 2015 season, both main factors were significant ($p < 0.0001$). During this season, the percentage oleic acid present in the buds increased during the first three days after treatment, followed by a decline until the end of the trial (Fig. 4F). The average percentage oleic acid throughout the trial was significantly higher in the control buds (3.2%) than in the HCo treated buds (2.8%) (Fig. 4G).

In the 2016 season, a significant interaction was observed between the two main effects ($p < 0.0001$) (Fig. 4H). Although the treated buds had an increased level of oleic acid one day after treatment, the level decreased in both the control and treated buds on Day 2. Thereafter, levels gradually increased in the control and treated buds and declined again on Day 12 (control buds) and Day 16 (treated buds) after treatment. During 2016, the percentage oleic acid present in the treated buds was only significantly lower than the control buds at the last sampling day. This decline was more rapid in the treated buds compared to the control buds (Fig. 4H) with the treated buds having a level 39% lower than the control buds at the end of the trial. When comparing the two seasons, it seems that the 2015 season had lower initial levels of oleic acid but in both seasons the levels decreased showing significantly lower levels on the last days of the trial compared to the beginning.

3.3.1.4. Linoleic acid (C18:2n6c)

The linoleic acid levels for the two year period was between 26 – 34% of the total amount of lipids found in the polar fraction except for the treated buds in 2015 that decreased to levels just above 20% at the end of the trial. In both years, a significant interaction was observed between treatment and days post treatment ($p < 0.0001$) (Fig. 4I and J). During 2015, both the treated and the control buds showed a gradual decrease in linoleic acid percentage until Day 9 post treatment (Fig 2I). Subsequent to this, the levels in the control buds increased significantly while levels in the treated buds continued to decrease more sharply than before. At the end of the trial the treated buds had levels 30% lower than the control buds.

In contrast to this, the 2016 season showed similar and relatively constant levels of linoleic acid in both the control and treated buds until 19 days post treatment (Fig. 4J). Only the last sampling point show a significant difference with the percentage in the treated buds dipping (similar to 2015) to a level 13% lower than the control buds (Fig. 4J). Across seasons, the initial percentages of linoleic acid was slightly higher in 2015, but at the end of the trial a steeper decrease of was detected in the treated buds of 2015 compared to 2016.

3.3.1.5. Linolenic acid (C18:3n3)

Linolenic acid made up the biggest portion of the FA in the polar fraction and accounted for 35 – 50% of the pool during both seasons. The 2015 season started off with higher levels of linolenic acid (42%) compared to 2016 (36%) and in both years the buds were significantly affected by an interaction between treatment and days post treatment (2015: $p = 0.0000$ and 2016: $p < 0.0001$) (Fig. 4K and L). At the end of the trial the treated buds of both seasons showed higher

percentages of linolenic acid compared to control buds. However, the level of linolenic acid in the treated buds in the 2015 season was higher at the end of the trial compared to the 2016 season. During 2015, the linolenic acid level decreased in both the control and treated buds after treatment (Fig. 4K), followed by a gradual increase. After nine days post treatment the treated buds started to show a significant difference by increasing to a level 13% higher than the control buds by the end of the trial whilst levels in the control buds declined slightly. In 2016, a very similar pattern was seen, only here the increase was slower for both the treated and the control (until Day 19) after which the treated buds increased their level significantly to end 10% higher than the control samples at the end of the trial.

3.3.1.6. Arachic acid (C20:0)

Arachic acid was found to be the FA with the lowest contribution to the polar fraction with levels varying between 1 and 2% across the two seasons (Fig 3M, N and O). Both seasons showed a declining pattern of arachic acid, although the initial and final percentages in 2015 were lower compared to 2016.

In 2015, no significant interaction was observed but both the main effects tested significant ($p < 0.0001$) (Fig. 4M and N). Initially, a higher percentage of arachic acid was present in all the buds, but this declined gradually as the 70% green-tip stage of treated buds (Fig. 4M) was reached. In addition, the treated buds had on average a significantly higher (1.5%) level of arachic acid during the trial compared to the control buds (1.3%) (Fig. 4N). During the 2016 season, a significant interaction was observed between treatment and days post treatment ($p < 0.0001$) (Fig. 4O). Although the arachic acid was present at a higher percentage in the HCo treated buds for most of the trial period, both the treated and control buds showed levels that remained constant until Day 14 post treatment. Subsequently, the percentage of arachic acid declined in the treated buds until the end of the trial, while it only declined up to Day 16 post treatment and then remained constant in the control buds. At the end of the trial the control buds contained 25 % more arachic acid than HCo treated buds.

3.3.2. Effect of HCo on the saturation of the fatty acids

3.3.2.1. SFA and UFA in treated and control buds

In both years the trends in the SFA grouping (consisting of palmitic, stearic and arachic acid) were very similar to that of palmitic acid (Fig. 4A and B) as it was the dominant FA representing up to 65 % of the SFA grouping and is therefore presented in Appendix B (Fig 1A and B).

The trends of the UFA grouping (consisting of oleic, linoleic and linolenic acid) was mainly driven by changes in the linoleic (C18:2) and linolenic (C18:3) acids as they together constituted up to 96 % of the grouping. Because the UFA grouping is the counterpart to the SFA grouping in the polar lipid fraction, it will change in a trend that is the inverse of the SFA grouping with similar statistical results. The UFA results are presented in Appendix B (Fig. 1C and D).

To compare the effect of the treatment on the saturation levels of the FA we investigated the UFA:SFA ratio over time (Fig. 5). This ratio indicates how the saturation of the polar fraction changed over time in both the treated and the control samples. Because the UFA grouping is significantly larger than the SFA, the trends are almost identical to the respective %UFA graphs for both years presented in Appendix B (Fig. 1C and D). In 2015, a significant interaction ($p = 0.0010$) (Fig. 5A) was found between treatment and days post treatment and, similar to the %UFA trend (Appendix B (Fig 1C and D)), the UFA:SFA in the treated and control samples decreased until Day 3 and then increased in the control buds while remaining stable in the treated buds until Day 5. After this the UFA:SFA ratio in the treated samples decreased progressively and significantly to end lower compared to the control samples that maintained a constant level (Fig. 5A). For buds sampled in 2016, no significant interaction effect was observed, but all the samples (treated and control) were significantly affected by time ($p < 0.0001$) (Fig. 5B) showing a slow increase in the UFA:SFA towards the end of the trial. On average there was no difference between the UFA:SFA of the treated and control samples.

3.3.2.2. MUFA and PUFA in the treated and control buds

In the polar fraction the MUFA grouping includes only oleic acid (C18:1) and therefore the analysis of MUFA are identical to that of oleic acid presented in Fig. 4 F, G and H and not presented again. The PUFA grouping is represented by linoleic (C18:2) and linolenic (C18:3) acid and its trends (Appendix B, Fig. 2D and E) are very similar to that of the UFA grouping (Appendix B, Fig. 1C and D) that also includes oleic acid, but because the oleic acid is present in such low levels, it has very little effect on the UFA trend. The MUFA:PUFA results are presented in Appendix B, Fig. 2F, G and H as they also do not provide new trends but are indeed also very similar to the oleic acid trends already presented. The reason for this is that the MUFA (oleic acid) levels are very low compared to the PUFA levels and thus the MUFA: PUFA ratios are small numbers driven by oleic acid (Appendix B, Fig. 2F, G and H). Thus, to avoid duplication we are not including a description of the results of the MUFA and PUFA and MUFA: PUFA again. The only new information was that the MUFA: PUFA was between 0.35 and 0.71 during the two year study with lower levels

throughout 2015 compared to 2016. Downwards trends were present in all the samples in both seasons, especially in the treated buds of 2016.

3.3.2.3. Unsaturation ratios of the C18 FAs of treated and control buds

When groupings are made of the C18 FAs and their ratios it becomes interesting as the major contributing FAs are linolenic (C18:3) and linoleic (C18:2) acid. As the contribution of steric (C18:0) and oleic (C18:1) acid are relatively minor, the groupings that involve them are overridden by the much larger values of the major groups and consequently they do not alter the trends of the major groupings significantly. This led to some of the trends (not necessarily their actual values) of the C18 ratios being identical to some of the groupings we have already reported on, i.e. the (C18:3):(C18:1+C18:2) is similar to the (C18:3):(C18:2) trend (Appendix B, Fig. 3A and B versus Fig 5A and B) and (C18:3+C18:2):C18:1 has a similar trend to %MUFA (Appendix B, Fig. 3 C,D and E versus Appendix B, Fig. 2A B and C). To avoid duplication we present these graphs but do not comment again on their trends in this section.

The most interesting trends, however, are that of C18:3/C18:2 represented in Fig. 6 A and B. Here a significant interaction was detected between treatment and days post treatment ($p < 0.0001$) in 2015 and 2016 (Fig. 6A and B). In both years the ratios started at around 1.2 and increased to around 1.6, except for the treated samples of 2015 that increased to a ratio of around 2.2 in the final measurement. The ratios remained relatively similar between the treated and the control buds in 2015 and showed an increasing trend until Day 10 after which it started to differ significantly. The ratio of the control buds decreased until the end of the trial while the ratios of the treated buds increased drastically and ended up 39% higher than the control. In 2016, a similar trend was seen with the treated and control ratios remaining similar and increasing somewhat, but the sharp increase in the ratio of the treated buds was only seen from Day 19 and ended up 22% higher than the control buds that did not show any change over the same period.

3.4. Effect of HCo on the total free sterols and the phospholipids

The free sterol concentration presented in Fig. 7A and B show a wide range of between 2 – 7 mmol g^{-1} FW during the two seasons of the trial with 2015 having levels mostly significantly lower than in 2016. In both years the treated and control buds were significantly affected by an interaction between treatment and days post treatment ($p < 0.0001$). Apart from a decrease in the free sterol concentration in the treated buds on the first day post treatment in 2015, the concentration increased in both control and treated buds until the third day post treatment (Fig. 7A). It then decreased for 5 days until the end of the trial. During the 2016 season, the free sterol

concentration in control buds remained relatively constant from the onset of the experiment until Day 14 post treatment (Fig. 7B). A spike occurred in treated buds on the second day post treatment and a drop in sterol concentration occurred in control buds at nine days post treatment. Thereafter, the free sterol concentration in control and treated buds declined, but this reduction was more pronounced in treated buds. At the end of the trial the free sterol concentration was 27% lower in the treated buds compared to the control buds.

The free phospholipid results showed plenty of variability within and between the two seasons (Fig. 7 C and D). No significant interaction was detected between treatment and days post treatment for either of the two seasons. However, treatment had a significant effect on the average phospholipid concentration in buds sampled in 2015 (p = 0.038) (Fig. 7C) resulting in a higher average concentration of free phospholipids (18 µmol g^{-1} FW) compared to the control buds (17 µmol g^{-1} FW). During the 2016 season, no treatment effect was seen while concentrations of free phospholipids did differ over time ($p < 0.0001$) (Fig. 7D). In both the treated and the control buds, the concentration increased on the treatment day until Day 2 after which both declined slightly and then remained constant until Day 12. Thereafter free phospholipid concentrations increased significantly towards the end of the trial.

When the total free sterol/ free phospholipids ratio was investigated (Appendix B, Fig. 4A and B) the trends were very similar to that of the free sterol concentration of Fig 7A and B. As the free sterol values were much lower in comparison to the free phospholipid values they thus had little effect. A description of the trend is not repeated again here. In general, a huge variability was again seen across seasons with ratio values fluctuating between 150 and 450.

4. Discussion

4.1. Orchard conditions and the effect of HCo on the lipid fractions

For HCo to release dormancy effectively, warm weather has to occur immediately after the application of this RBA, since low temperatures might induce phytotoxicity and reduce floral budburst (Sheard *et al.*, 2009), while higher temperatures (> 24 ºC) activate metabolic activities (Erez, 1987). During the present study, it was observed that the two seasons differed with regard to the baseline values and orchard conditions following the HCo application. Since the first days following the treatment (Day 0 – Day 2) were warmer in 2015, the onset of the 70% green-tip stage was accelerated to 15 days after treatment. In contrast, the cooler post treatment temperatures (Day 0 – Day 2) of 2016 resulted in the trees reaching the 70% green-tip stage

seven days later (21 days post treatment). Because of this difference in the onset of green-tip stage the effect of HCo on the lipid composition of the buds was also observed later in 2016.

The size of the neutral and polar lipid fractions are considered to be important during budbreak as they indicate the ability to release energy from its stored form (NL) as well as the ability to form new membranes (PL) (Ohlrogge and Browse, 1995). NLs are responsible for the storage of energy (reduced carbons) (Taiz and Zeiger, 2010) and it has been shown that a decrease in NL levels leads to growth resumption (Athenstaedt and Daum, 2006). In the present study, the amount of NLs declined significantly during budbreak in both the treated and the control samples similarly to the reduction detected at the end of the winter period of 2015 and 2016 (see Paper 3). From this, it seems reasonable to assume that budbreak facilitates a high demand for NL FAs oxidation as growth is an energy consuming process. However, the effect of HCo on the NLs is not evident as the HCo treated and control buds showed a similar pattern in both years, thus, the control buds also seem to release stored energy although budbreak was delayed. The changes detected in the NL concentrations across seasons may be due to seasonal variation in temperature as indicated by Piispanen and Saranpää (2002 and 2004). The PL content is known to increase towards budbreak (Portrat *et al.,* 1995, Wang and Faust, 1990b, Erez *et al.*, 1997) as new cells are formed during growth resumption. This pattern was evident in the present data, especially during 2015 where the PL increase pairs with the decrease in NL. In 2016, this was less evident although the treated buds showed signs of budbreak earlier than the control buds. Reasons for the lack of an increase in the PL are unclear.

It is known that HCo acts immediately upon treatment and reaches a maximum activity within 48 hours before being broken down by the plant tissue (Amberger, 2013). In this study, it seems that the HCo effect on the lipid composition is not observed immediately, there is no initial stage (as presented in Paper 2), but the response was only measurable later. Interestingly, the first meaningful differences between the treated and control samples are seen at day 15 post treatment in 2015 and day 12 post treatment in 2016, suggesting that HCo takes longer to show an effect on FA levels compared to the respiration pathways. From this it is deducted that HCo has a more direct and immediate effect on the respiration mechanics of the cells first and the change in the FA levels is only evident later. Thus it seems as if the FAs composition possibly changed as the availability of ATP starts to increase via an increase in respiration. In turn, this increase in FA desaturation again simulates respiration as it is known that the activation energy of the succinate oxidase complex is generally high when membrane lipids are more fluid and declines when membrane lipids become rigid (Hannon and Raison, 1979). Similar results were observed in previous studies that focused on thidiazuron (TDZ), where it was demonstrated that

the greatest change in the composition of membrane lipids occurred towards budbreak in blackberry (Izadyar and Wang, 1999) and apple (Wang and Faust, 1988 and 1990) and can be linked to the amount of chill units already fulfilled at the time of application (Izadyar and Wang, 1999).

4.2. The effect of the HCo treatment on FAs

When the effect of HCo treatment on the FA composition of apple buds was assessed, the FA composition was found to change. The low levels of stearic acid (9.0 to 6.3 %) and oleic acid (4.9 to 2.4%) measured in this trial are not surprising, since it has been shown that the levels of these FAs are low in bud tissue during budbreak and bud growth (<10% of the total FAs) (Wang and Faust, 1990, Izadyar and Wang, 1999). In the present study, stearic acid, oleic acid and linoleic acid decreased in HCo treated buds, while linolenic acid increased towards the end of the trial. This corresponds to previous studies showing that the desaturation of FAs in higher plants under growth resumption conditions occurs in a step-like fashion *via* the desaturation of stearic acid (SFA, no double bound) into oleic acid (one double bond), then into linoleic acid (two double bonds) and linolenic acid (three double bonds) (Murata and Wada, 1995; Schmid and Ohlrogge, 2002; Durrett *et al.,* 2008). This kind of desaturation facilitates membranes becoming enriched in higher UFAs, such as linolenic acid (Taiz and Zeiger, 2010), which prevent tight packing of the FAs and thereby increasing the metabolic activity of the cell membranes (Wilson and Crawford, 1974). It seems that in the present study, with the simultaneous decrease of linoleic acid and increase of linolenic acid at 70% green tip, the HCo treatment possibly favoured the synthesis of linolenic acid *via* the conversion of linoleic acid and could be associated with budbreak and bud growth as found by Wang and Faust (1990) during dormancy release mediated by sufficient chilling. This is supported by the fact that FAs are synthesised, desaturated and/or elongated in order to maintain membrane fluidity (Taiz and Zeiger, 2010). Erez *et al.* (1997) also found that linoleic acid decreased, while linolenic acid increased in buds and attributed this conversion to sufficient winter chilling. Recently, this desaturation has also been linked to an increased growth rate of a plant cell culture (Meï *et al.*, 2015). To further support this, Gibson *et al.* (2004) showed that the degree of unsaturation generally increases prior to dormancy release however, once the lipid desaturation process is inhibited under stresses, the resumption of growth can be delayed. Thus, it seems that HCo might promote growth resumption in a similar way to sufficient chill, by enabling desaturation of FAs to higher PUFAs, especially C18:2 to C18:3 towards budbreak, which confer fluidity, flexibility and permeability to cellular membranes and facilitates growth resumption (Wang and Faust, 1988, 1990a and 1990b). The increase in linolenic acid was reported to enhance the fluidity of the plasma and chloroplast membranes (D'Angeli and

Altamura, 2016). Wang (2010) also indicated that the warming and cooling of tissues could increase the synthesis of FAs with two or three double bonds, meaning that the unsaturation process occurring in the buds may be influenced by many factors at once.

In addition to the relative increase of linolenic acid in the HCo treated apple buds, there was also a noticeable increase in the ratios of the C18 groupings, (C18:3+C18:2)/ (C18:1), (C18:3)/ (C18:1+C18:2) and (C18:3/ C18:2) prior to budbreak. Thus, a general increase in unsaturated C18 lipids occurred. This concurs with the results obtained by Wang and Faust (1990b) and it seems that the active growth or resumption of growth requires unsaturated fatty acids. It has been shown that an increase in these unsaturation ratios are associated with membrane fluidity, permeability, budbreak and bud growth in apple, peach and blackberry (Wang and Faust, 1988 and 1990; Erez *et al.,* 1997; Izadyar and Wang, 1999; Cook and McMaster, 2002). Furthermore, it was demonstrated that a high degree of unsaturation is closely correlated with plasma membrane ATPase specific activity (Martz *et al.*, 2006), an enzyme that is activated during dormancy release (Aue *et al.*, 1999). Likewise, peach buds induced with hydrogen cyanamide showed a continued increase in the ω3-desaturase transcript (Bregoli *et al.,* 2006), which codes for the enzyme that desaturates FAs that have double bonds on the A9 and A12 positions, for example C18:2 (positions 9 and 12), to form FAs that have three double bonds, for example C18:3 (positions 9,12, and 15) (Murata and Wada, 1995). The increase of this ω3-desaturase transcript was found to occur in parallel with increased membrane permeability and increased bud growth (Bregoli *et al.,* 2006). Based on all of these studies, it seems possible that treating apple buds with HCo in the present study induced FAs desaturation to PUFA, ultimately facilitated membrane fluidity and permeability which stimulated or at least facilitated growth resumption and budbreak towards the end of the trial.

In the present study, we also found a higher %SFA and a lower UFA: SFA ratio in HCo treated buds in 2015. Since the %SFA represents FAs that do not have double bonds, such as palmitic and stearic acid (Murata and Wada, 1995), which are known to increase membrane rigidity (Taiz and Zeiger, 2010), it could be possible that the increase in palmitic acid was a result of the stress initiated by HCo treatment and warm winter conditions of the area. Similarly palmitic acid was found to increase when peach vegetative buds were exposed to chilling accumulation during the daily cycling of low and high temperatures (Erez *et al.*, 1997). Mishra *et al.* (2006) related the increase in %SFA and concomitant decrease in %UFA and the UFA: SFA ratio in the expanding leaves of *Catharanthus roseus,* to leaf maturity. In contrast, others found that palmitic acid decreased in apple (Wang *et al.*, 1994) and blackberry (Izadyar and Wang, 1999) buds when treated with TDZ prior to dormancy release. It is possible, however, that the increase in palmitic

acid observed in the present study (both seasons) may be associated with adverse stress conditions related to mild winter conditions as it has been shown that palmitic acid can increase due to the stress adaptation of plants (Zhukov. 2015). This author reported an increase in palmitic acid in a series of plant species when exposed to stress, such as water deficits and increased salt concentrations. He stated that this increase in palmitic acid might be another alternative pathway of membrane lipids resisting stress in plants, apart from the general hypothesis of adaptation of cellular membrane fluidity. We suggest that as it is known that HCo creates oxidative stress in the buds, it is probable that in association with insufficient winter chill conditions, it could lead to an increase in the palmitic acid level resulting in increased %SFA levels.

An overall decline was detected in the %MUFA (oleic acid only) in HCo treated buds prior to dormancy release. Usually, membranes enriched with SFAs and MUFAs are resistant and not easily oxidised, while those enriched with PUFAs are fluid and susceptible to lipid peroxidation (Catalá, 2009; Jooste *et al.*, 2014). Although the %PUFA also decreased in HCo treated buds towards budbreak in 2015, it is important to consider that this %PUFA is calculated by taking both linoleic and linolenic acid into account and that linoleic acid decreased sharply in HCo buds towards budbreak. Thus, the decrease in %PUFA was most likely brought on by a decrease in the linoleic acid concentration. Yet, linolenic acid is the PUFA known to contribute the most towards membrane flexibility, fluidity and selective permeability, resulting in preserved plant viability, while cellular function is maintained (Schmid and Ohlrogge, 2002; Taiz and Zeiger, 2010). It is thus difficult to speculate whether the decline in %PUFA of the HCo treated buds affected budbreak in 2015. In addition to the %MUFA and %PUFA, the MUFA: PUFA ratio was lower in 2015 and declined in 2016 in the HCo buds. Generally, a membrane with a low MUFA: PUFA ratio is susceptible to oxidation under stress conditions (Ohlrogge and Browse, 1995; Schmid and Ohlrogge, 2002; Jooste *et al.*, 2014). It is also known that the use of HCo creates a temporary hypoxia which leads to various physiological changes including increased oxidative stress (Amberger, 2013; Beauvieux *et al.*, 2018) and in turn, if oxygen deficiency induces oxidative stress, membrane structure is modified (Beauvieux *et al.*, 2018). It is therefore speculated that in the present study the HCo treatment might have affected the membrane lipids by inducing lipid peroxidation.

4.3. The effect of the HCo treatment on the free sterols and phospholipids

Since changes in total free sterols and phospholipid concentration can modify membrane properties, integrity, fluidity and permeability in higher plants (Hartmann, 1988; Wang and Faust, 1988; Schaller, 2004), it becomes relevant when considering dormancy release and RBA

applications. It is known that free sterols decline with the increase in cold temperature and increase when the cold is reduced (Yoshida, 1986). Previous findings showed that free sterols increase in apple buds during dormancy release, budbreak and bud growth under TDZ treatment (Wang and Faust, 1988), after cold winter conditions (Wang and Faust, 1990b) and in the presence of nitro-guanidine (Wang and Faust, 1988 and 1989). Likewise, total free sterols were found to increase in decapitated shoots that were either induced or not induced with TDZ (Wang and Faust, 1994). Furthermore, Wang and Faust (1990b) suggested that the concentration of free sterols increased during budbreak, while sterols in an esterified form declined and that this was associated with metabolic processes in the cell membrane. Wang and Faust (1994) indicated also that the free sterols increase when lipid reserves in buds are hydrolysed and the membrane becomes permeable. In this study, we found that the free sterol concentration in both the HCo treated and the control buds decreased towards budbreak in both seasons. Although the current results generally stand in contrast to previous studies by showing fluctuating levels in the control and treated groups and a general decreasing trend implying that the membranes should be more rigid, the high degree of C18 FA unsaturation should keep the membranes more fluid and permeable (Meï *et al.*, 2015). This decrease may possibly be related to the relatively warm climate that the trial was performed in. The two seasons also showed different amounts of free sterols present at the time of HCo application and although there was a decreasing trend in both years, at the end of the trial the control and treatment groups did not behave the same. This variability makes the interpretation of the results difficult and inconclusive.

An increase in total phospholipid content is associated with dormancy progression and release (Wang and Faust, 1990b; Izadyar *et al.*, 1999). According to Wang and Faust (1989 and 1990b), all classes of galactolipids and phospholipids increase during winter but during growth resumption in spring the unsaturated FAs increase is only limited to galactolipids and two phospholipids classes (phosphatidylethanolamine and phosphatidylinositol). In a similar way, a rise in the phospholipid content of dormant peach buds exposed to sufficient cold (Erez *et al.,* 1997) and apple buds treated with TDZ (Wang and Faust, 1988) occurred during budbreak. Likewise, in buds of different blackberry cultivars, exposed to different chilling temperatures, the phospholipid concentration increased at the end of the forcing period and was found to be associated with dormancy release (Izadyar and Wang, 1999). In the current study, the total free phospholipid concentration was only affected by HCo treatment during the colder 2015 season. The higher free phospholipids concentration of HCo treated buds in 2015 agrees with the results obtained in the above mentioned studies. However in 2016, no difference was found between the treatment and the control but both groups showed an increase towards budbreak possibly due to the warmer conditions experienced in that season.

The free sterols/ free phospholipid ratio was thought to be an indication of the interaction between the free sterols and free phospholipids in membranes and an index of membrane fluidity and thus dormancy release (Quinn, 1981). In the past, a decrease in this ratio occurred in apple shoots during a cold winter period and was linked to increased membrane fluidity, in association with budbreak and growth resumption in the presence of the sufficient chill accumulation, growth regulators such as TDZ and nitro-guanidine (Wang and Faust 1988, 1989, 1990a and 1990b). In the present study this ratio was dominated by the sterol trend as it occurred in much higher concentrations compared to the free phospholipids. The decline in the free sterol: phospholipid ratio observed in this study contrast earlier findings; this ratio is known to increase with the decrease in cold hardiness towards spring periods (Yoshida, 1986; Wang and Faust, 1990a and 1990b). The decline in this ratio and free sterols could be due to the milder winter conditions of the experimental site. It is possible that abnormal endodormancy release, associated with insufficient winter chill, affects the membrane lipid composition by resulting in a change in phospholipid composition (Simon, 1974; Portrat *et al.,* 1995). However, when abnormal endodormancy release is counteracted with the HCo, the free sterol/ phospholipid ratio generally increases, which can activate the release of dormancy and stimulate budbreak (Wang and Faust, 1988).

5. Conclusions

The current results indicate that HCo treatment affected the FA composition of polar lipids towards budbreak. Although some differences in the baseline values of the two seasons were detected, most of the differences observed occurred when the treated orchard reached 70% green-tip stage. Overall, it seems that HCo treatment causes a desaturation of FAs to form the PUFA linolenic acid towards the 70% green-tip stage. This could possibly lead to increased membrane fluidity and permeability, as well as result in the mobilisation of storage lipids, thereby rendering more energy available for budburst and growth resumption. Budbreak occurred in the treated buds despite the increase in %SFA (due to increased palmitic acid levels) and concomitant decrease in %UFA, %MUFA and %PUFA in HCo treated buds (especially in the warmer 2015 season). Likewise, the degree of expected reduction in fluidity associated with a decrease in the free sterol/ phospholipid ratio did not seem to hamper budbreak possibly due to the polyunsaturation process to linolenic acid towards the 70% green-tip stage. It is likely that these parameters decreased due to the warm conditions of the experimental site, which might have indirectly affected the polyunsaturation process by inducing FA saturation *via* the production of palmitic acid. This study thus provides evidence that with the rest breaking treatment HCo induces desaturation that can activate metabolic activities, thereby leading to favourable conditions for

budbreak and bud growth. Future research should focus on repeating the experiment in an area that receives sufficient chilling, to determine if the environment contributes towards the saturation of membrane lipids when buds are treated with HCo. This experiment should include budbreak data of treated and control shoots to act as a phenotypical verification of the findings.

6. References

Alberdi, M., Corcuera, L.J. (1991). Cold acclimation in plants. *Phytochemistry*. **30**, 3177–3184.

Amberger, A. (2013). Cyanamide in plant metabolism. *International Journal Plant Physiology Biochemistry.* **5**, 1–10.

Ames, B.N. (1966). Assay of inorganic phosphate, total phosphate and phosphateses. *Methods in Enzymology*. **8**, 115–118.

ARC-Infruitec, 1997. *South African deciduous fruit cultivars catalogue*. 2nd ed. ARC Infruitec-Nietvoorbij, Stellenbosch, SA. (as per Tharaga, 2014).

Athenstaedt, K. and Daum G. (2006). The life cycle of neutral lipids: synthesis, storage and degradation. *Cellular and Molecular Life Sciences*. **63**, 1355–1369.

Aue, H.L., Lecomte, I., Gendraud, M. and Pétel, G. (1999). Change in plasma membrane ATPase activity during dormancy release of vegetative peach-tree buds. *Physiologia plantarum*. **106**, 41–46.

Ben Mohamed, H., Vadel, A. M., Geuns, J.M.C. and Khemira, H. (2012). Carbohydrate changes during dormancy release in superior seedless grapevine cuttings following hydrogen cyanamide treatment. *Scientia Horticulturae*. **140**, 19 –25.

Bregoli, A.M., Nardozza, S. andreotti, C, Magnani, R., Corelli-Grappadelli, L., Masia, A. and Costa G. (2006). Relationship between biometric and physiological parameters, application time and flower bud abscission induced by a dormancy breaking agent in peach. *Acta Horticulturae*. **713**, 191–196.

Carvajal-Millán, E., Gardea, A.A., Rascón-Chu, A., Orozco, J.A., Ponce de León, N., Márquez-Escalante, J.A., Romo Chacón, A. and Guerrero Centro, V.M. (2007). Respiratory response of apple buds treated with budbreaking agents. *Thermochimica Acta*. **457**, 109–112.

Catalá, A. (2009). Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids*. **157**, 1–11.

Cook, N.C. and Jacobs, G. (1999). Sub-optimal winter chilling impedes development of acrotony in apple shoots. *HortScience*, **34**. 1213–1216.

Cook, N.C. and Jacobs, G. (2000). Progression of apple (*Malus x domestica* Borkh.) bud dormancy in two mild winter climates. *Journal of Horticultural Science*. **75**, 233–236.

Cook, H.W. and McMaster, C.R. (2002). Fatty acid desaturation and chain elongation in eukaryotes. D.E. Vance and J.E. Vance (Eds.). *Biochemistry of lipid, lipoproteins and membrane (4th Edn.)*. pp: 24

Costa, C., Stassen, P.J.C. and Mudzunga, J. (2004). Chemical rest breaking agents for the South African pome and stone fruit industry. *Acta Horticulturae.* **636**, 295–302.

D'Angeli, S. and Altamura, M.M. (2016). Unsaturated lipids change in Olive tree drupe and seed during fruit development and in response to cold-stress and acclimation. *International Journal of Molecular*

Sciences. **17**, 1889.

Dozier, W.A/, Powell, A.A., Caylor, A.W., McDaniel, N.R., Carden, E.L and McGuire, J.A. (1990). Hydrogen cyanamide induces budbreak of peaches and nectarines following inadequate chilling. *HortScience* **25**:1573–1575.

Duck-Chong, C.G. (1979). A rapid sensitive method for determining phospholipid phosphorus involving digestion with magnesium nitrate. *Lipids*. **14**, 492–497.

Dufourc, E. J. (2008). Sterols and membrane dynamics. *Journal of Chemistry and Biology*. **1**, 63–77.

Durrett, T.P., Benning, C. and Ohlrogge, J. (2008). Plant triacylglycerols as feedstocks for the production of biofuels. *The Plant Journal*. **54**, 593–607.

Erez, A. (1987). Chemical control of bud break. *HortScience*, **22**, 1240-1243.

Erez, A., Wang, S.Y. and Faust, M. (1997). Lipids in peach buds during dormancy, a possible involvement in dormancy control. *Advances in Horticultural Science*. **11**, 128–132.

Faust, M., Erez, A., Rowland, L.J., Wang, S.Y. and Norman, H. A. (1997). Bud dormancy in perennial fruit trees: physiological basis for dormancy induction, maintenance, release. *HortScience*. **32**, 623–629.

Faust, M. and Wang, S.Y. (1993). Biochemical events associated with resumption of growth in temperate zone fruit trees. *Acta Horticulturae*. **329**, 257–264.

Furt, F., Simon-Plas, F., and Mongrand, S. (2011). Lipids of the plant plasma membrane. *Plant Cell Monographs* **19**, pp 29.

Gibson, P.G., Reighard, G.L., Powell, G.L. and Jenkins, T.C. (2004). Graft transmissible agents affect membrane fatty acid saturation during dormancy release in peach. *Journal of the American Society for Horticultural Science*. **129**, 649–652.

Hannon G.N. and Raison J.K. (1979). Seasonal changes in the structure and function of mitochondrial membranes of artichoke tubers: Acyl fatty acid composition and the effect of growth conditions. *Plant Physiology*. **64**, 754–756

Hartmann, M.A. (1998). Plant sterols and the membrane environment. *Trends Plant Science*. **3**, 170–175.

Hennessey, T.M. (1992). Effects of membrane plant sterols on excitable cell functions. *Comparative Biochemistry and Physiology*. **101**, 1–8.

Izadyar, A.B. and Wang, S.Y. (1999). Changes of lipid components during dormancy in ‘Hull Thornless’ and ‘Triple Crown Thornless’ blackberry cultivars. *Scientia Horticulturae*. **82**, 243–254.

Jooste, M. (2012). Membrane studies in Japanese plums (*Prunus salic cina* Lind l.). PhD dissertation, University of Stellenbosch, Pp.294.

Jooste, M., Rohwer, E.A., Kidd, M. and Huysamer, M. (2014). Comparison of antioxidant levels and cell membrane composition during fruit development in two plum cultivars (*Prunus salicina* Lindl.) differing in chilling resistance. *Scientia Horticulturae*. **180**, 176–189.

McPherson HG, Richardson AC, Snelgar WP and Currie MB (2001) Effects of hydrogen cyanamide on budbreak and flowering in kiwifruit (*Actinidia deliciosa* ‘Hayward’). *New Zealand J Crop and Hort Sci* 29:277–285.

Martz, F., Sutinen, M.L., Kiviniemi, S. and Palta, J.P. (2006). Changes in freezing tolerance, plasma membrane H+-ATPase activity and fatty acid composition in *Pinus resinosa* needles during cold acclimation and de-acclimation. *Tree Physiology*. **26**, 783–790.

Meï, C., Michaud, M., Cussac, M., Albrieux, C., Gros, V., Maréchal, E., Block, M.A., Jouhet, J. and Rébeillé, F. (2015). Levels of polyunsaturated fatty acids correlate with growth rate in plant cell cultures. *Scientific reports*. **5** (15207), 1–13.

Mittler, R., Finka, A. and Goloubinoff, P. (2012). How do plants feel the heat? *Trends in Biochemical Sciences*. **37** (3), 1–8.

Mishra, S., Tyagi, A., Singh, I.V. and Sangwan, R.S. (2006). Changes in lipid profile during growth and senescence of *Catharanthus roseus* leaf. *Brazil Journal Plant Physiology*. **18**, 447– 454.

Murata, N. and Wada, H. (1995). Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. *Biochemistry Journal*. **308**, 1–8.

Nilsen E.T. and Orcutt D.M. (1996). The physiology of plants under stress, 689. John Wiley & Sons, Inc. New York.

Nir, G., Shulman, Y., Fanberstein, L. and Lavee, S. (1986). Changes in the activity of catalase in relation to the dormancy of grapevine (*Vitis vinifera*) buds. *Plant Physiology*. **81**, 1140–1142.

Ohlrogge, J. and Browse, J. (1995). Lipid biosynthesis. *Plant Cell*. **7**, 957–970. 1996.

Palta, J.P., Whitaker, B.D. and Weiss, L.S. (1993). Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of *Solanum* species. *Plant Physiology*. **103**, 793–803.

Pérez, F.J., Vergara, S. and Rubio, S. (2008). H_2O_2 is involved in the dormancy-breaking effect of hydrogen cyanamide in grapevine buds. *Plant Growth Regulation*. **55**, 149–155.

Petri, J.L. and Stuker, H. (1995). Effect of mineral oil and hydrogen cyanamide concentrations on apple dormancy, cv. 'Gala'. *Acta Horticulturae.* **395**, 161-167.

Petri, J.L. and Leite, G.B. (2004). Consequences of insufficient winter chilling on apple tree budbreak. *Acta Horticulturae*. **662**, 53–60.

Piispanen, R. and Saranpää, P. (2002). Neutral lipids and phospholipids in Scots pine (*Pinus sylvestris*) sapwood and heartwood. *Tree Physiology*. **22**, 661–666.

Piispanen, R. and Saranpää, P. (2004). Seasonal and within-stem variations of neutral lipids in silver birch (*Betula pendula*) wood. *Tree Physiology*, **24**, 991–999.

Portrat, K., Mathieu, C., Motta, C. and Pétel, P. (1995). Changes in plasma membrane properties of peach tree buds and stands during dormancy. *Journal of plant physiology*. **147**, 346–350.

Quinn, P.J. and Williams, W.P. (1978). Plant lipids and their role in membrane function. *Progress in Biophysics and Molecular Biology*. **34**, 109–173.

Quinn, P.J. (1981). The fluidity of cell membranes and its regulation. *Progress in Biophysics and Molecular Biology*. **38**, 1–104.

Rubio, S., Donoso, A. and Pérez, F.J. (2014). The dormancy-breaking stimuli "chilling, hypoxia and cyanamide exposure" up-regulate the expression of α-amylase genes in grapevine buds. *Journal of Plant Physiology*. **171**, 373–381.

Ruiz, J., Antequera, T., Andres, A.I., Petron, M.J., Muriel, E. (2004). Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. *Analytica Chimica Acta*, **520**, 201–205.

Saure M. 1985. Dormancy release in deciduous fruit trees. *Horticultural Reviews*, **7**, 239-300.

Schaller, H. (2004). New aspects of sterol biosynthesis in growth and development of higher plants. *Plant Physiology and Biochemistry*. **42**, 465–476.

Schmid, K.M. and Ohlrogge, J.B. (2002). Lipid metabolism in plants. *Biochemistry of lipids, lipoprooteins and membranes (4th Edn.).* D.E. Vance and J.E. Vance *(Edn.).* pp: 34.

Seif El-Yazal, M.A. and Rady, M.M. (2013). Foliar-applied Dormex^{TM} or thiourea-enhanced proline and biogenic amine contents and hastened breaking bud dormancy in 'Ain Shemer' apple trees. *Trees*. **27**, 161–169.

Schlemper, C., Rufato, L., Kretzschmar, A.A., Mendes M. and Macedo T.A. (2010). Overcoming the endodormancy of apple trees in southern Brazil. *Acta Horticulturae.* **884**, 88.

Sheard, A.G., Johnson, S.D. and Cook N.C. (2009). Effect of timing and concentration of rest breaking agents on budburst in 'Bing' sweet cherry under conditions of inadequate winter chilling in South Africa, *South African Journal of Plant and Soil*. **26**, 73–79.

Simon, E. W. (I974). Phospholipids and plant membrane permeability. *New Phytologist*. **73**, 377–420.

Solovchenko, A. E. (2013). Physiological role of neutral lipid accumulation in eukaryotic microalgae under stress. *Plant Physiology*. **59**, 167–176.

Taiz, L. and Zeiger, E. (2010). Plant Physiology. 5nd ed. Sinauer Associates Inc., Sunderland, M.A. pp: 782.

Tharaga, P.C. (2014). Impacts of climate change on accumulated chill units at selected fruit production sites in South Africa. MSc Thesis, Bloemfontein University.

Theocharis, A., Clément, C. and Barka, E.A. (2012). Physiological and molecular changes in plants grown at low temperatures. *Planta*, **235,** 1091–1105.

Uemura, M. and Steponkus, P.L. (1994). A contrast of the plasma membrane lipid composition of oat and rye leaves in relation to freezing tolerance. *Plant Physiology*. **104**, 479–496.

Uemura, M., Tominaga, Y., Nakagawara, C., Shigematsu, S., Minami, A and Kawamura, Y. (2006). Responses of the plasma membrane to low temperatures. *Physiologia Plantarum*. **126**, 81–89.

Upchurch R.G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnology Letters*. **30**, 967–977.

van Meer, G., Voelker, D.R. and Feigenson, G.W. (2008). Membrane lipids: Where they are and how they behave. *Nature Reviews Molecular Cell Biology*. **9**, 112–124.

Wang, S.Y. and Faust, M. (1988). Changes of fatty acids and sterols in apple buds during budbreak induced by a plant bioregulator, thidiazuron. *Physiologia Plantarum*. **72**, 115–120.

Wang, S.Y. and Faust, M. (1989). Nitroguanidines induce budbreak and change sterol content in apple. *Journal of Plant Growth Regulation*. **8**, 143–151.

Wang, S.Y. and Faust, M. (1990a). Seasonal changes of membrane lipids in apple shoots. *Journal of the American Society for Horticultural Science*. **115**, 462–467.

Wang, S.Y. and Faust, M. (1990b). Changes in membrane lipids in apple buds during dormancy and budbreak. *Journal of the American Society for Horticultural Science*. **115**, 803–808.

Wang, S.Y. and Faust, M. (1993). Biochemical events associated with resumption of growth in temperate zone fruit trees. *Acta Horticulturae*. **329**, 257–264.

Wang, S.Y., Faust, M. and Line, M.J. (1994). Apical dominance in apple (*Malus domestica* Borkh): The possible role of indole-3-acetic acid (IAA). *Journal of the American Society for Horticultural Science*. **119**, 1215–1221.

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Wang, C.Y. (2010). Alleviation of chilling injury in tropical and subtropical fruits. *Acta Horticulturae*. **864**, 267– 273.

Welti, R., Li, W., Li, M., Sang, Y., Biesiada, H., Zhou, H., Rajashekar, C.B., Williams, T.D. and Wang, X. (2002). Profiling membrane lipids in plant stress responses. Role of phospholipase D alpha in freezing-induced lipid changes in *Arabidopsis*. *Journal of Biological Chemistry*. **277**, 31994–32002.

Wilson, J.M. and Crawford, R.M.M. (1974). The acclimatization of plants to chilling temperatures in relation to the fatty-acid composition of leaf polar lipids. *The New Phytologist*. **73**, 805-820.

Wu, J., Seliskar, D.M. and Gallagher, J.L. (2005). The response of plasma membrane lipid composition in callus of the halophyte *Spartina patens* (Poaceae) to salinity stress. *American Journal of Botany*. **92**, 852–858.

Yoshida, S. (1986). Reverse changes in plasma membrane properties upon deacclimation of mulberry trees (*Morus bombysis* Koidz.). *Plant Cell Physiology*. **27**, 83-89.

Zhukov, A. V. (2015). Palmitic acid and its role in the structure and functions of plant cell membranes. *Russian Journal of Plant Physiology*. **62**, 706–713.

FIGURES

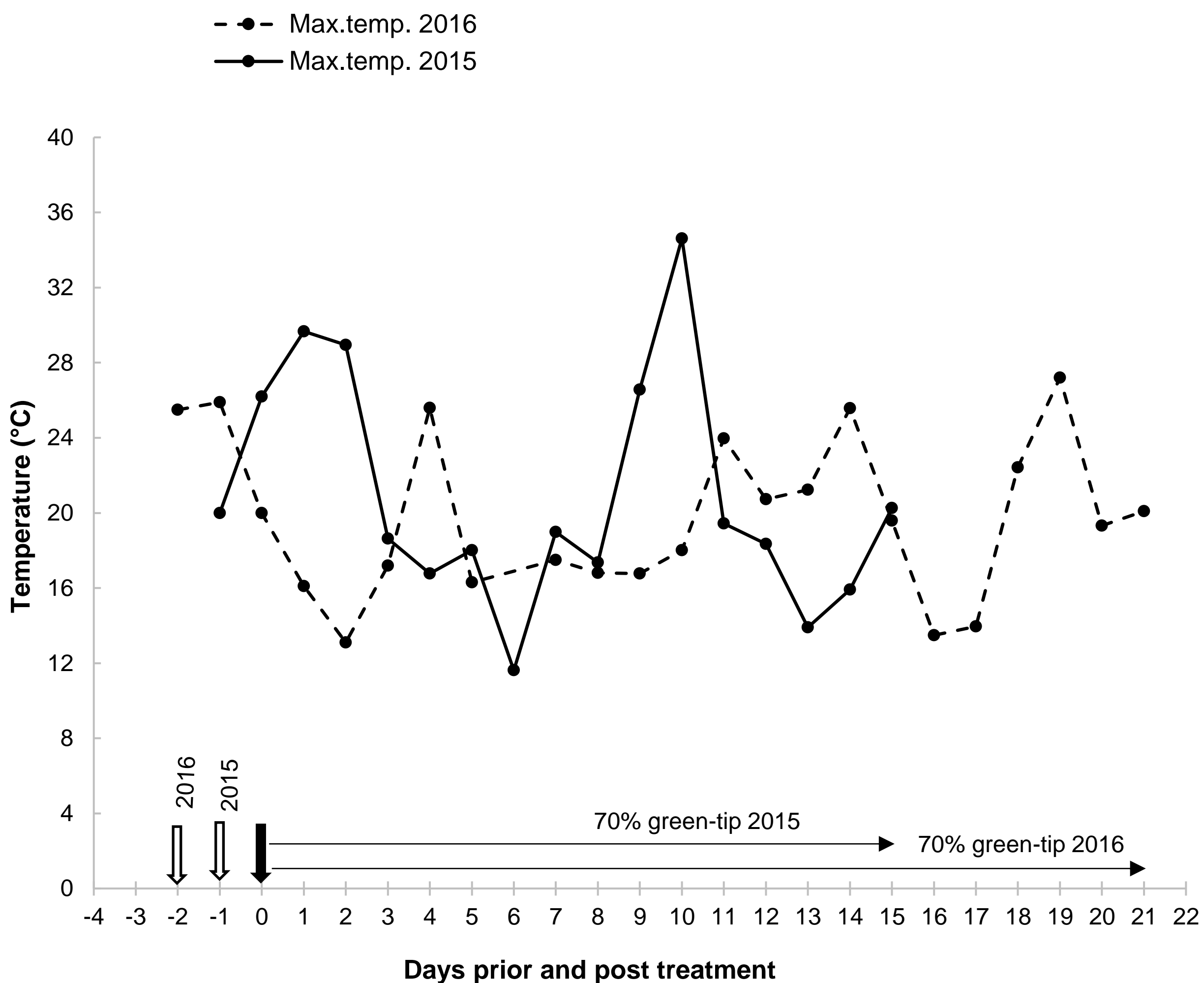
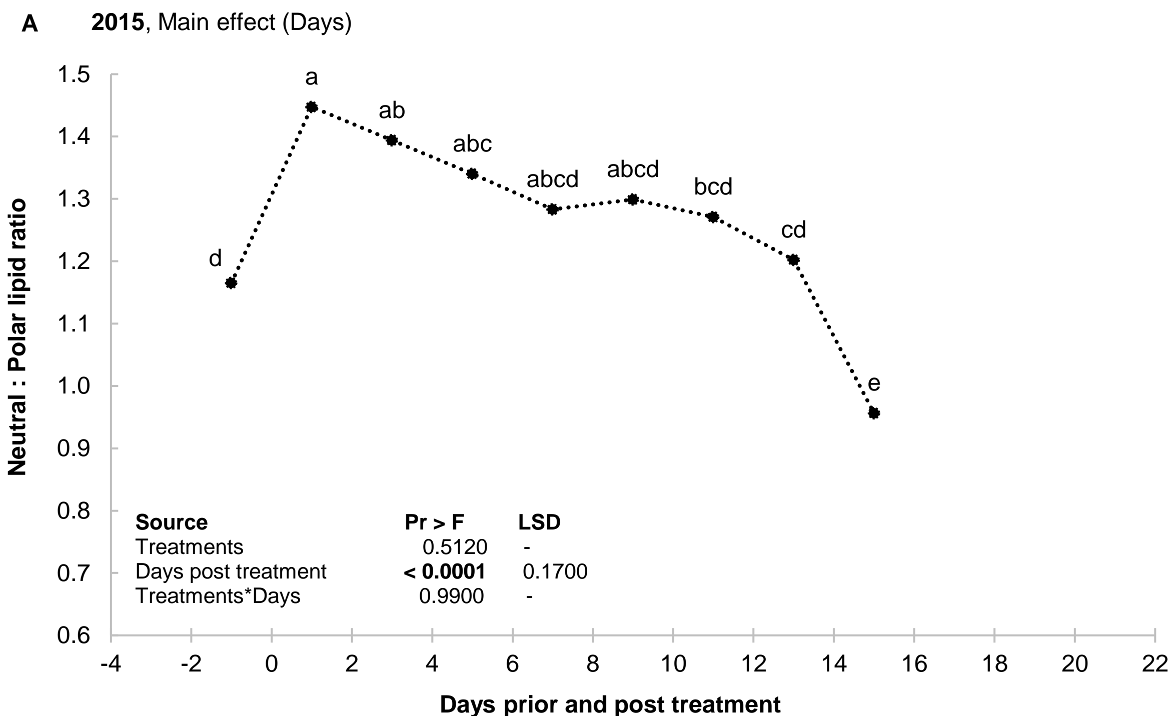


Figure.1: Daily maximum temperatures during 2015 (——) and 2016 (- - -). Negative values represent days prior to treatment. Day 0 is the day of treatment and positive values indicate days post treatment. Open arrows indicate covering of control shoots and the shaded arrow indicates the removal of the bags. Horizontal arrows show the time to 70% green-tip stage of the treated orchard.



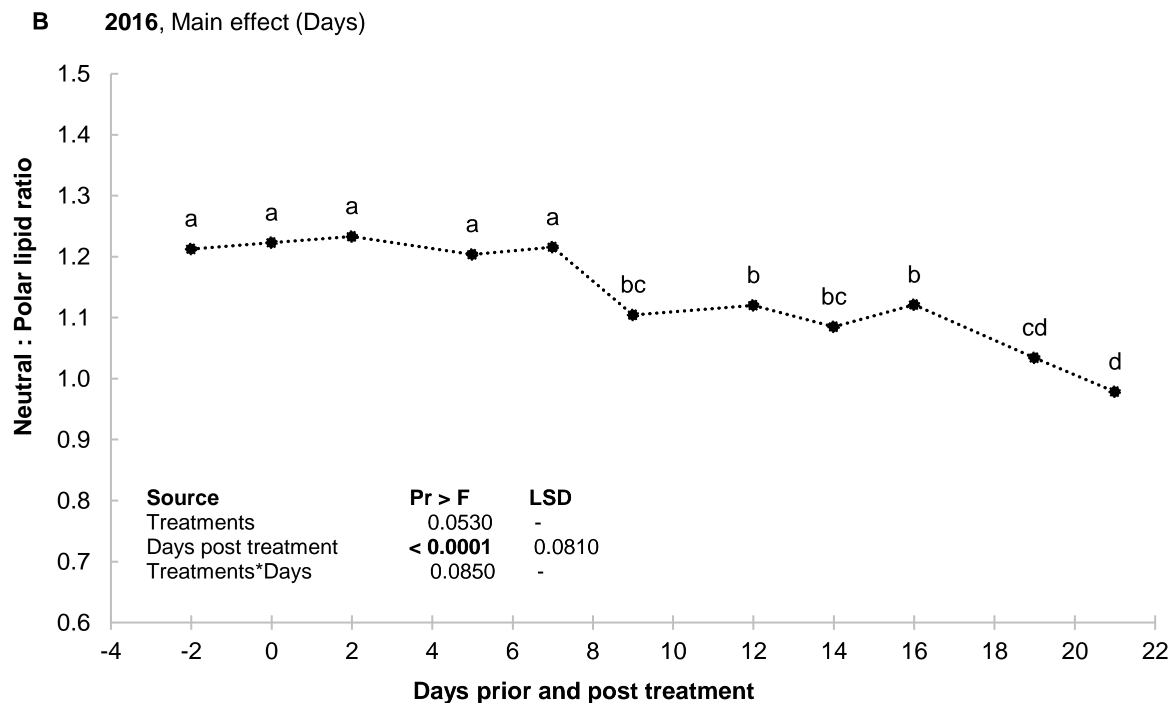


Figure 2: Neutral:Polar lipid ratio of the treated and control buds for 2015 (A) and 2016 (B). Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$. In both cases no interaction was detected and the significant main effects (days after treatment) is presented.

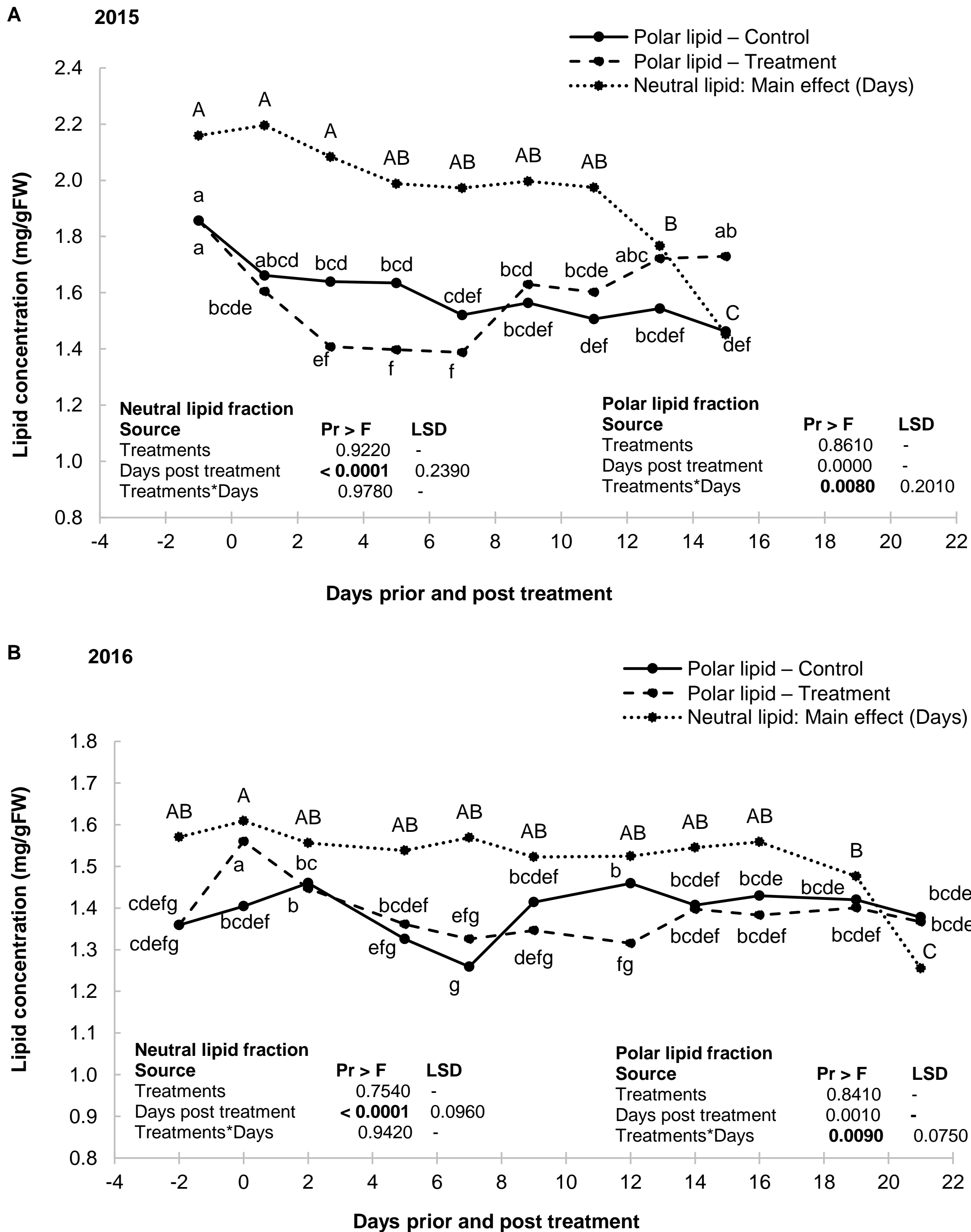
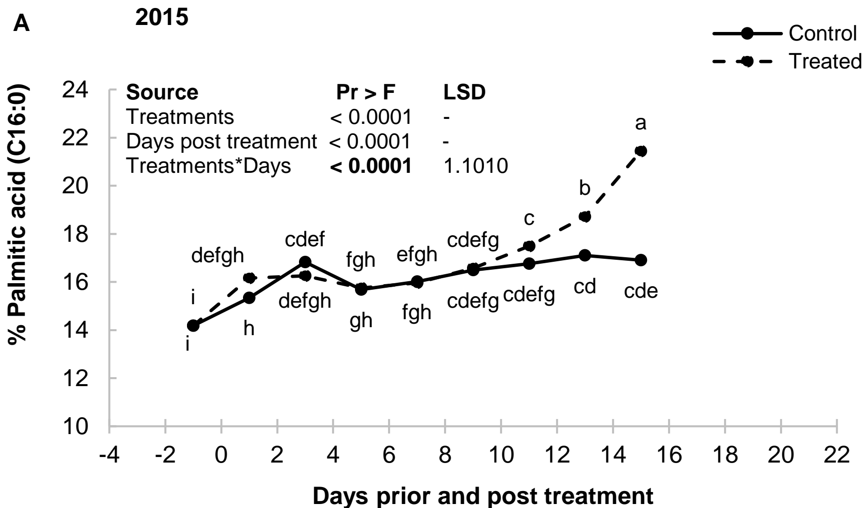
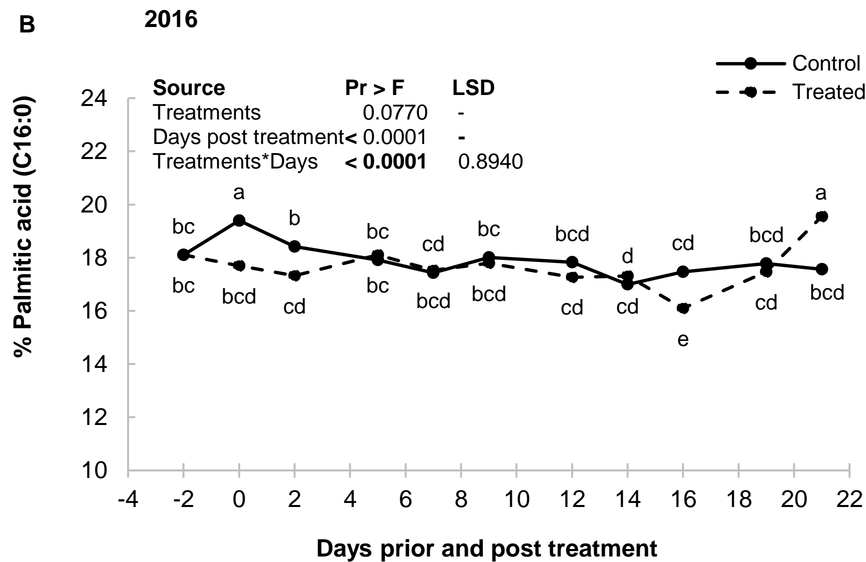
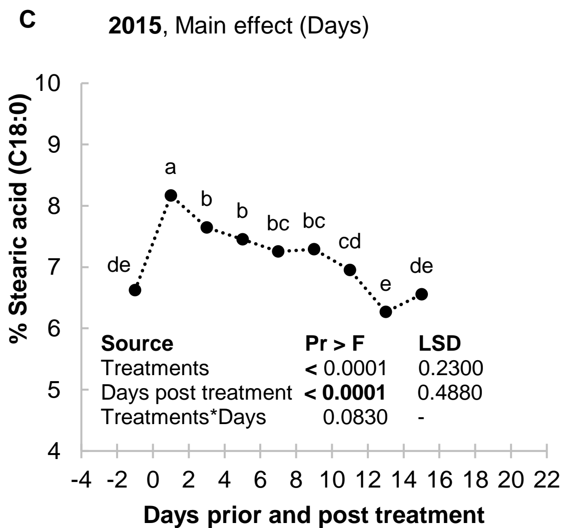


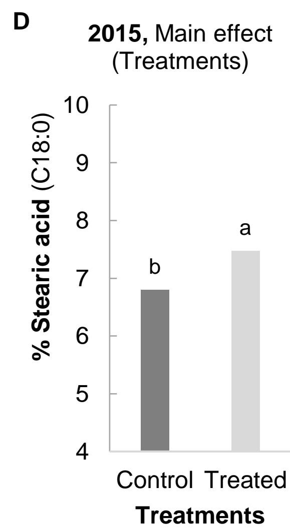
Figure 3: Lipid concentration (mg g^{-1} FW) of the polar and neutral lipid fractions of the treated and control buds for 2015 (A) and 2016 (B). Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$ and in cases where the interaction was not significant, the significant main effects are presented.

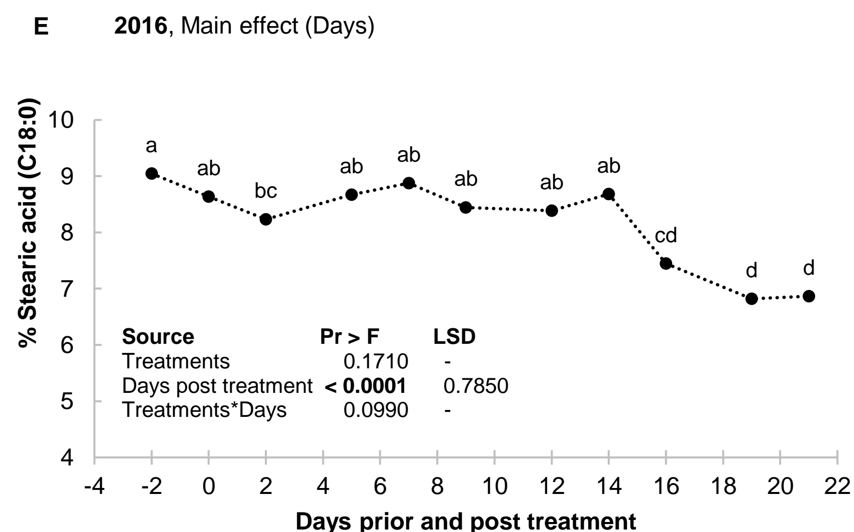
NOTE: To facilitate interpretation we present both ANOVAs in one figure by using capital letters for the neutral lipid results and lower case letters for the polar lipid results. Also note that the vertical axes for A (2015) and B (2016) differ.

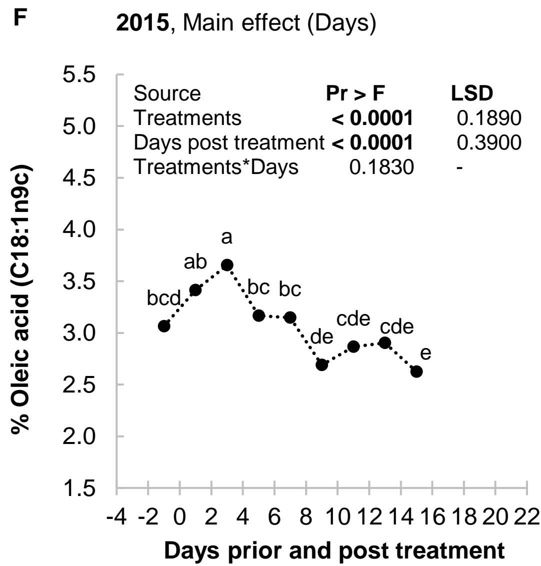

A
2015
Control
Treated
Source Pr > F LSD
Treatments < 0.0001 -
Days post treatment < 0.0001 -
Treatments*Days < 0.0001 1.1010
% Palmitic acid (C16:0)
Days prior and post treatment

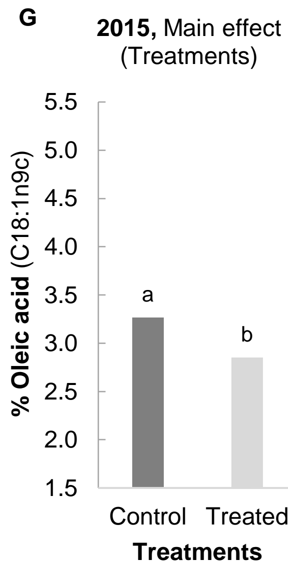

B
2016
Control
Treated
Source Pr > F LSD
Treatments 0.0770 -
Days post treatment < 0.0001 -
Treatments*Days < 0.0001 0.8940
% Palmitic acid (C16:0)
Days prior and post treatment

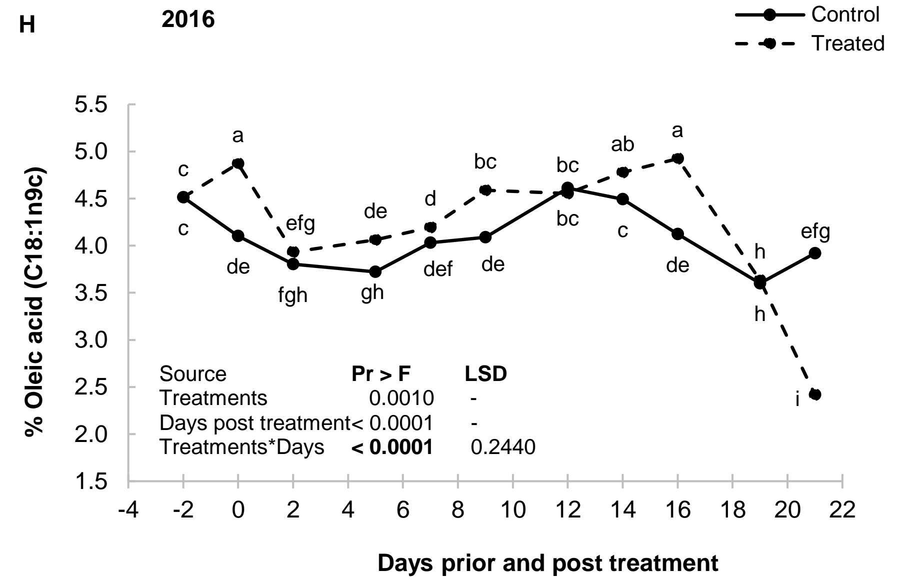

C
2015, Main effect (Days)
Source Pr > F LSD
Treatments < 0.0001 0.2300
Days post treatment < 0.0001 0.4880
Treatments*Days 0.0830 -
% Stearic acid (C18:0)
Days prior and post treatment

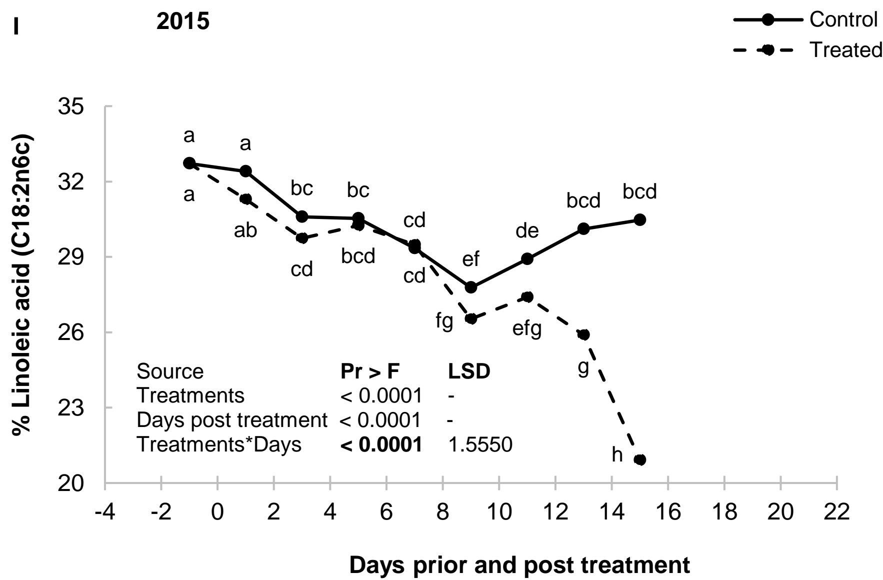

D
2015, Main effect (Treatments)
% Stearic acid (C18:0)
Control
Treated
Treatments

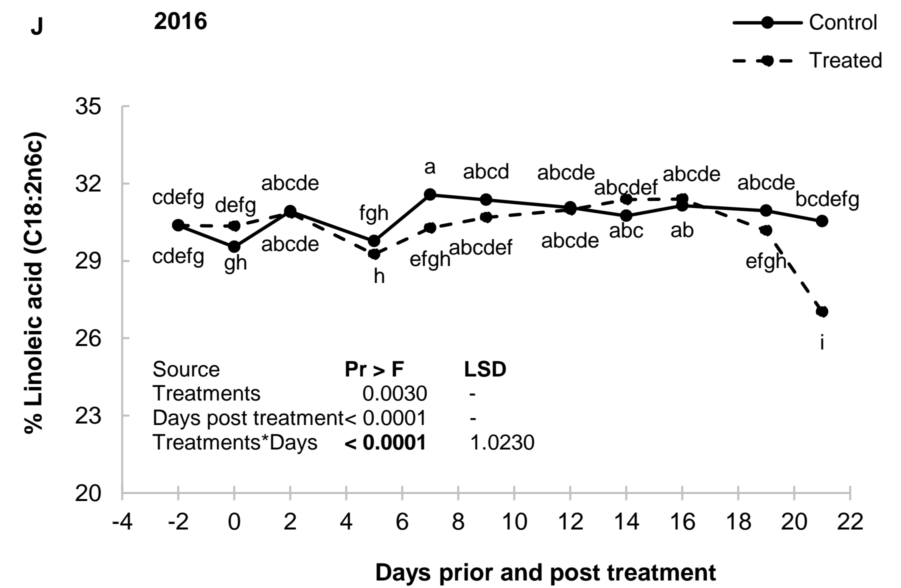

E
2016, Main effect (Days)
Source Pr > F LSD
Treatments 0.1710 -
Days post treatment < 0.0001 0.7850
Treatments*Days 0.0990 -
% Stearic acid (C18:0)
Days prior and post treatment


F
2015, Main effect (Days)
Source Pr > F LSD
Treatments < 0.0001 0.1890
Days post treatment < 0.0001 0.3900
Treatments*Days 0.1830 -
% Oleic acid (C18:1n9c)
Days prior and post treatment


G
2015, Main effect (Treatments)
% Oleic acid (C18:1n9c)
Control
Treated
Treatments


H
2016
Control
Treated
Source Pr > F LSD
Treatments 0.0010 -
Days post treatment < 0.0001 -
Treatments*Days < 0.0001 0.2440
% Oleic acid (C18:1n9c)
Days prior and post treatment


I
2015
Control
Treated
Source Pr > F LSD
Treatments < 0.0001 -
Days post treatment < 0.0001 -
Treatments*Days < 0.0001 1.5550
% Linoleic acid (C18:2n6c)
Days prior and post treatment


J
2016
Control
Treated
Source Pr > F LSD
Treatments 0.0030 -
Days post treatment < 0.0001 -
Treatments*Days < 0.0001 1.0230
% Linoleic acid (C18:2n6c)
Days prior and post treatment

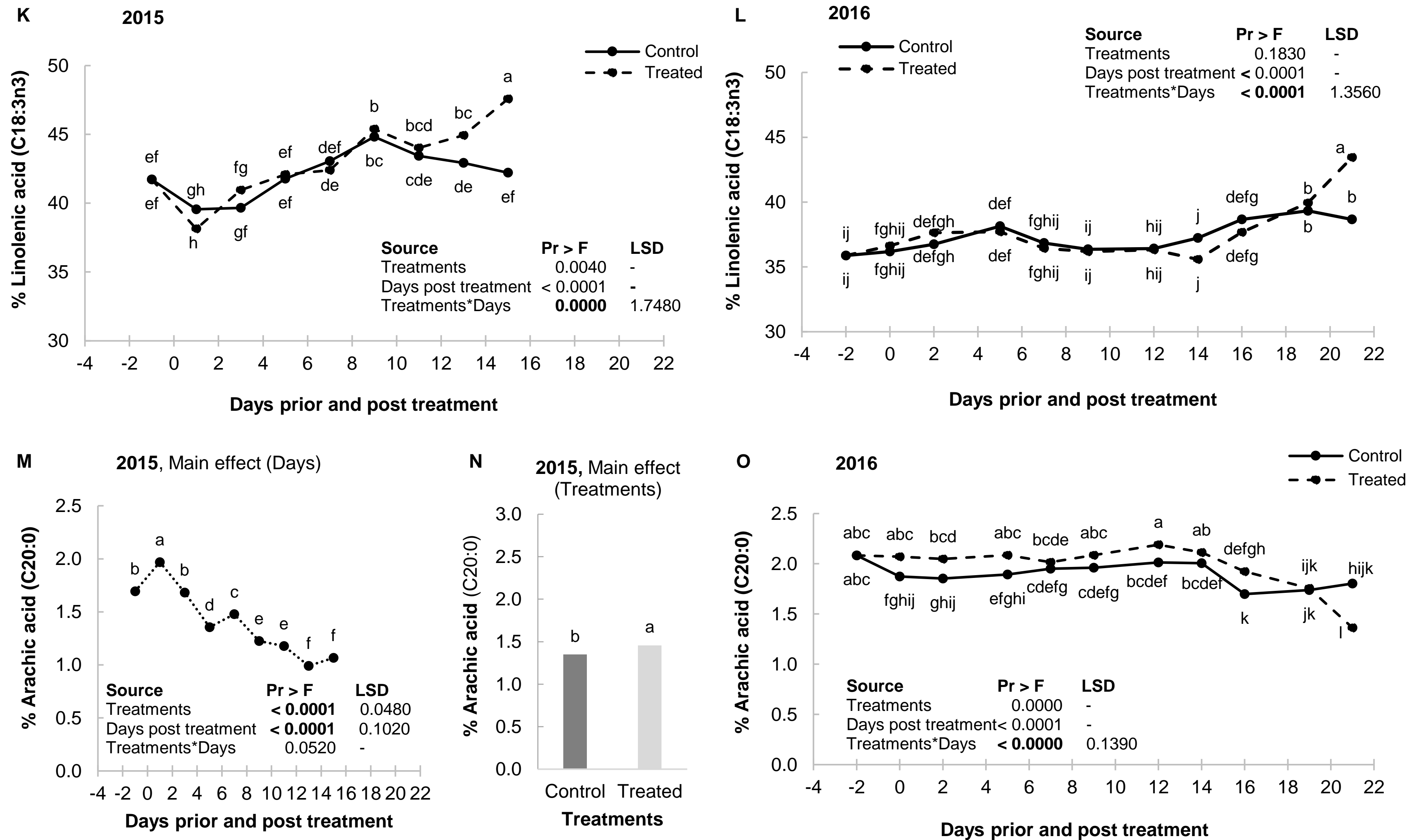


Figure 4: Percentage palmitic acid (C16:0) (A, B), stearic acid (C18:0) (C, D, E), oleic acid (C18:1n9c) (F, G, H), linoleic acid (C18:2n6c) (I, J), linolenic acid (C18:3n3) (K, L), and arachic acid (C20:0) (M, N, O) extracted from treated and control buds during 2015 and 2016. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at p < 0.05 and in cases where the interaction between the main effects (Treatments*Days) was not significant, the significant main effects are presented.

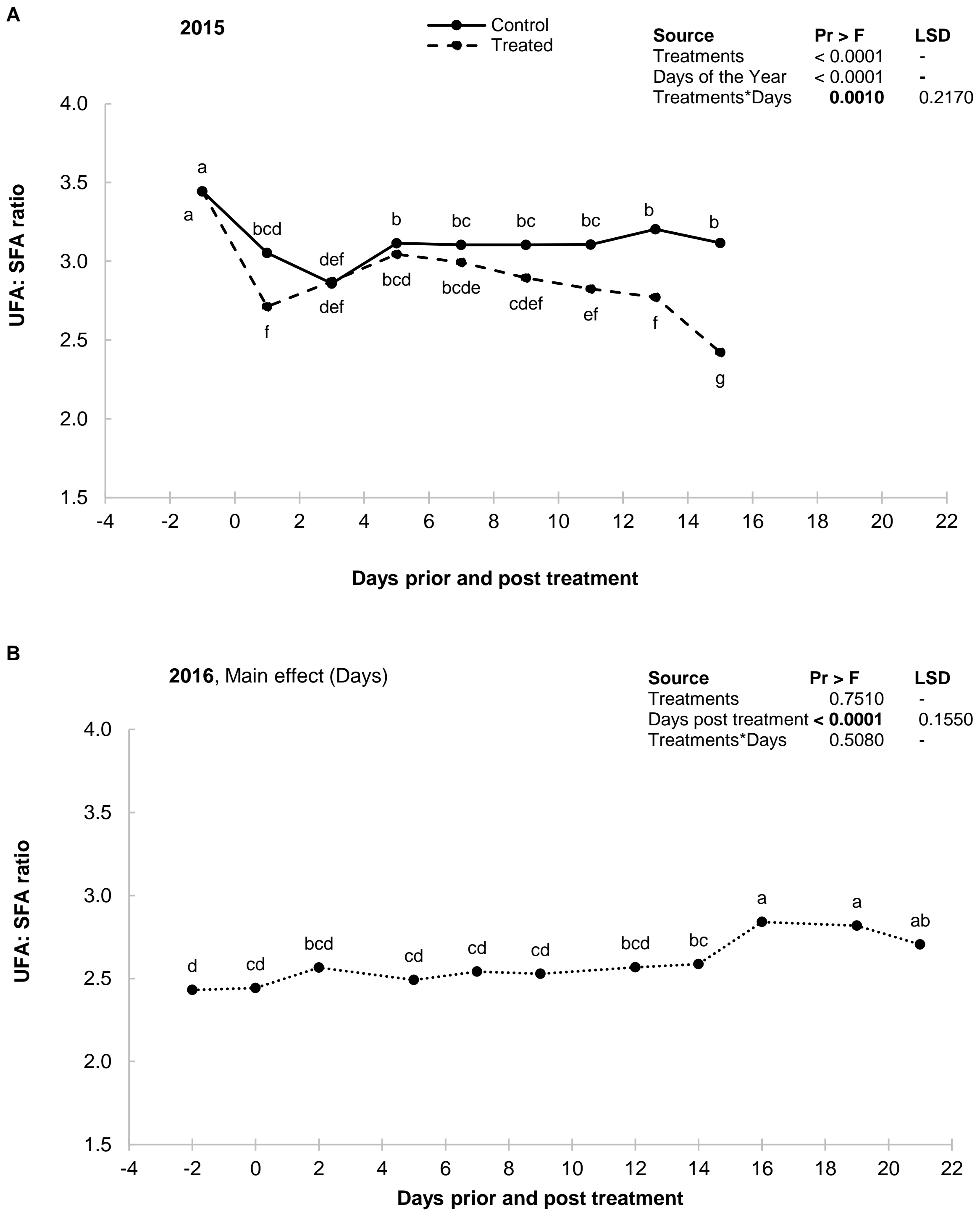


Figure 5: The unsaturated fatty acid (UFA) to saturated fatty acid (SFA) ratio (SFA/ UFA ratio) extracted from treated and control buds during 2015 (A) and 2016 (B). Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$ and in cases where the interaction between the main effects (Treatments*Days) was not significant, the significant main effects are presented.

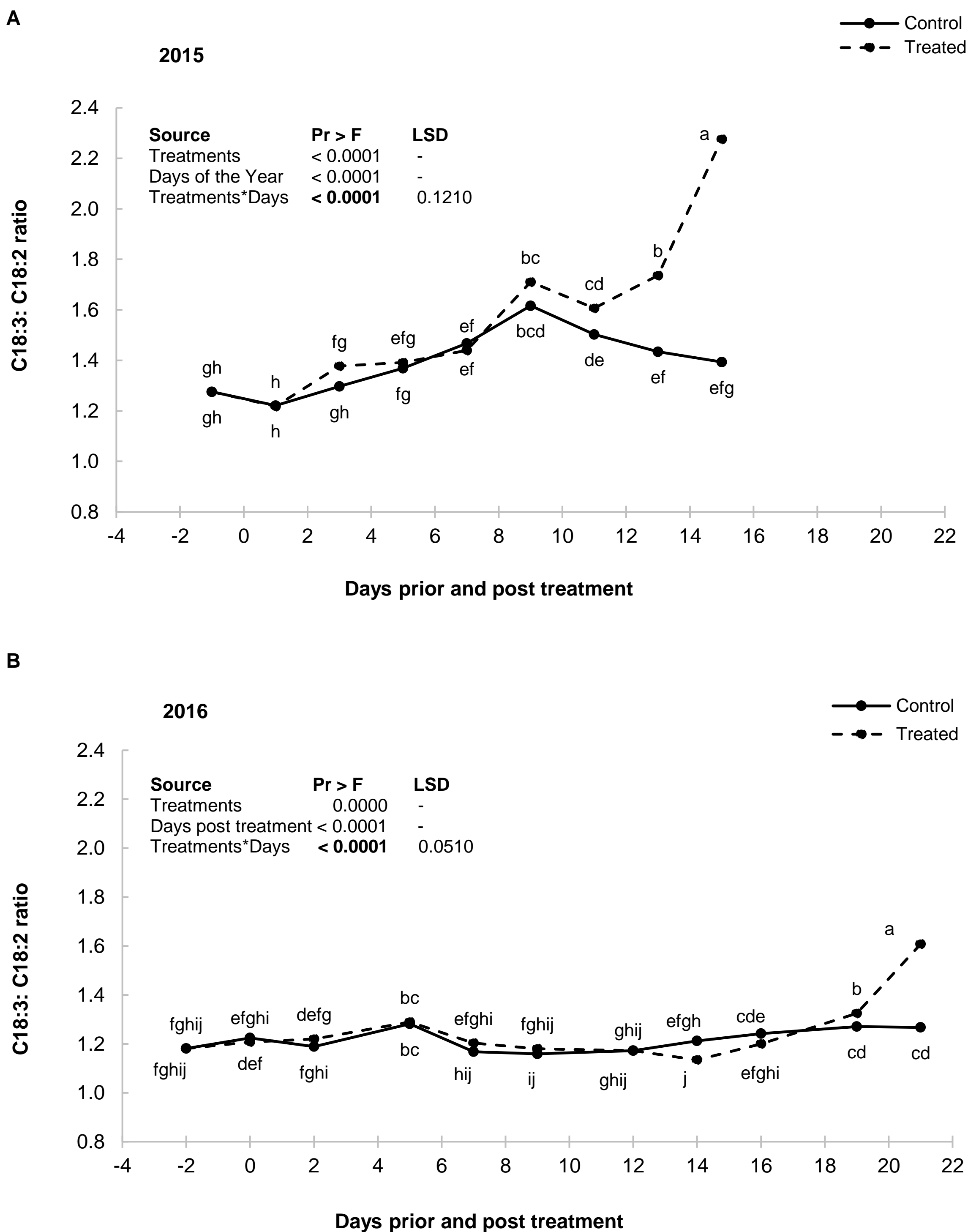


Figure 6: The unsaturation of the C18 FAs in treated and control buds during 2015 and 2016 presented as the C18:3/ C18:2 ratio (A, B). Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$.

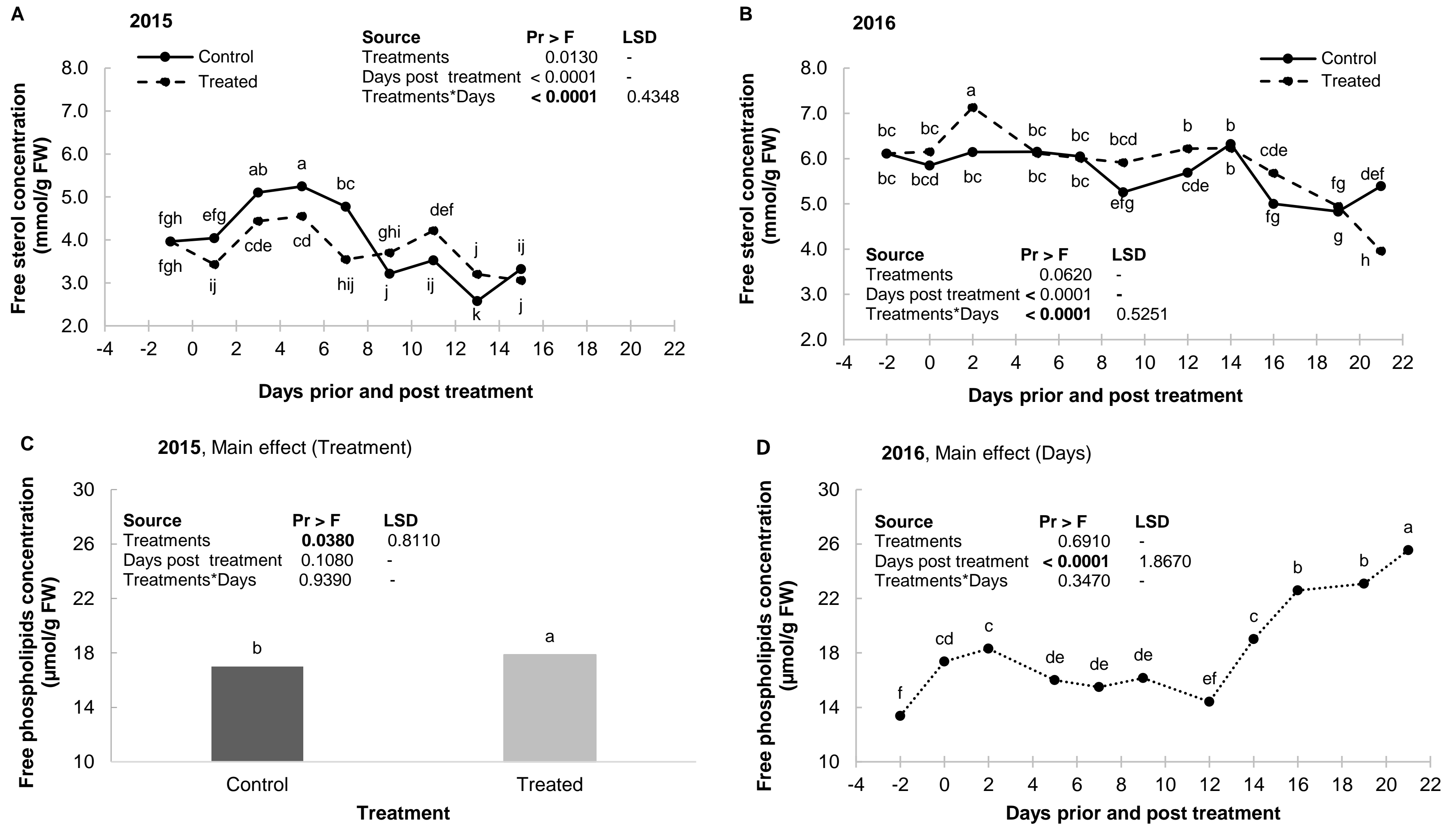


Figure 7: Free sterol (A, B) and free phospholipids concentration (C, D) in treated and control buds for 2015 and 2016. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$ and in cases where the interaction between the main effects (Treatments*Days) was not significant, the significant main effects are presented.

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GENERAL DISCUSSION AND CONCLUSIONS

Apple cultivars, such as 'Cripps Pink', grown in the Elgin/Grabouw area of the Western Cape in South Africa, are known to experience mild winter conditions that affect endodormancy release and delay budbreak in spring (Cook and Jacobs, 1999 and 2000; Cook *et al.*, 2017). Typically, the lower temperatures of winter reduce the metabolic activity in a bud and enhance entry into endodormancy. The same low temperatures also release the bud from endodormancy in order for synchronised budbreak to occur in the beginning of spring when warmer temperatures occur (Wang and Faust, 1990 a and b; Beauvieux *et al.*, 2018). Milder winter temperatures delay endodormancy induction and release as well as growth resumption activities in the bud (Cook and Jacobs, 2000; Pérez *et al.*, 2007). The present study expands on previous research done by Cook and Jacobs (2000) by comparing the dormancy progression of buds from trees grown in Elgin (warm winter region) and the Koue Bokkeveld (colder winter region) in terms of their respiration and lipid composition dynamics; two physiological aspects that are crucial for successful dormancy release and growth resumption. Below follows a discussion of the general trends observed in this study placed into the context of a dormant bud as it passes through autumn, winter and eventually early spring under different climatic conditions and the use of a rest-breaking agent. It aims to relate these findings to energy production and membrane characteristics. The general flow of the ideas are depicted in Figure 1.

Generally, in temperate fruit trees at high latitudes shoot growth cessation in mid-summer is caused by correlative inhibition originating from periodic root growth and/or the presence of leaves, and in the case of mature trees, the presence of fruit (Borchert, 1991). The most likely physiological basis for this inhibition is internal water deficit leading to competition amongst a large number of meristems (Borchert, 1991). Hormone imbalances, especially between cytokinin and absisic acid are instrumental in establishing and maintaining the gradual decrease and eventual cessation of the meristems. This inhibition of growth due to other plant structures are referred to as paradormancy and persists through autumn when cold hardening occurs due to low temperatures. In winter the inability to grow gradually develops into endodormancy where the source of the inhibition shifts to physiological factors within the bud itself. Once the chilling requirement has been satisfied, endodormancy is released and the buds progress into a state of ecodormancy whereby they are physiologically ready to commence growth, but fail to do so due

to the low environmental temperatures that are unfavourable for metabolic activity. This restriction is lifted when warmer spring temperatures arrive and result in synchronised budbreak that starts off the new growing season (Faust *et al.*, 1997; Cooke *et al.*, 2012). The present results on 'Cripps Pink' apple buds showed that dormancy progression and the depth of dormancy are altered under milder climatic conditions and lower chill accumulation. Under cooler winter conditions, endodormancy commenced earlier in autumn (April) before much chill accumulated and reached a maximum level of 42 days to 50% budbreak in both seasons compared to the slower entrance into endodormancy and 21 and 34 days to 50% budbreak in the milder winter area for the 2015 and 2016 seasons, respectively. These results are in agreement with findings from studies done by Cook and Jacobs (2000), Midgley and Lötze (2011) and by Cook *et al.* (2017) for the same locations.

Low temperatures in autumn and winter are known to reduce oxygen consumption and can limit ATP production and demand in plants with both these aspects recovering again following exposure to growth temperatures in spring (Atkin and Tjoelker, 2003; Kurimoto *et al.* 2004). This was evident from the current respiration results as the total respiration rate, tricarboxylic acid (TCA) and cytochrome C (CYT) pathways generally decreased during autumn in the bud tissue from both climatic regions. A decrease in the CYT pathway will typically result in a decline in the electron transport due to the reduction in oxygen consumption. This indirectly reduces the TCA cycle. The rate of the TCA cycle itself may also be sensitive to low temperatures. This reduction in the respiration pathway activity results in a decrease in energy (ATP) production during the dormant phase (Young, 1990; Gai *et al.*, 2013). In these results the pentose phosphate pathway (PPP) and alternative pathway (ALT) activities were also reduced in parallel with the increase in chill accumulation in both climatic regions, indicating that even under chill conditions that were unsatisfactory for budbreak, the lower winter temperatures were able to slow down the respiration mechanics. The lower winter temperature (in both areas) also favoured the sequential desaturation of fatty acids C16:0 to C18:0, C18:1 and C18:2 to allow increased membrane fluidity, which was accentuated in the colder area. For the two physiological aspects under investigation in this study, most differences between the areas were detected at the end of the winter period and during early spring when temperatures were more conducive for growth resumption. Buds that accumulated more chill rapidly exited dormancy and the rates of the main respiratory pathways increased. This was possibly due to sufficient chill accumulation during the dormant phase similar to results from Florez-Sarasa *et al.* (2007) who linked an increase in CYT and ATP with growth resumption. In the present results, when growth-conducing environmental

temperatures occurred after sufficient chill accumulation, it also resulted in a decrease in C18:2 FAs with a simultaneous increase in C18:3. It is thought that this led to increased membrane fluidity and permeability and probably increased oxygen transport, similar to results observed in blackberry (Izadyar and Wang, 1999) and in apple (Wang and Faust, 1990a and b). In the colder area, the end of the experiment also coincided with a decline pattern in the ratio of total free sterols to total phospholipids. All results from the colder area point towards the fulfilment of the chill requirement that resulted in an increase in the membrane fluidity as well as the energy producing respiration pathways. Since the oxidative phosphorylation component of respiration is situated within the lipid bi-layer of the mitochondria, it is plausible to assume that changes to the composition of the bi-layer might affect the respiration rate. It is thought that a more fluid membrane enhances oxygen uptake via the complex IV subunit and could be the limiting element regulating the cytochrome pathway when low temperatures are experienced (Matos *et al*, 2007). This favourable “membrane landscape” and the increased ATP production within the bud tissue facilitated synchronised budbreak even under relatively cool spring conditions.

Under mild winter conditions, the chill requirement is not fulfilled, buds remain dormant or show delayed and poor budbreak that negatively impact on fruit set, yield and quality (Petri and Leite, 2004). This insufficient chill accumulation obstructs the flow of the respiration pathways by keeping the buds in a state of high level PPP and low TCA activity (Tan *et al.*, 2010). It also alters lipid composition by disturbing the phospholipid packing and unsaturation process (Erez *et al.*, 1997). In general, the PPP and ALT pathways are known to be pathways to relieve stress (Tan *et al.*, 2010 and 2013) and are thought to be involved in dormancy maintenance (Florez-Sarasa *et al.* 2007). The current study supports the findings of Florez-Sarasa *et al.* (2007) as higher activities of PPP and ALT were observed in the milder area and buds from this area had a higher percentage saturated and mono-unsaturated FAs and a low percentage unsaturation during autumn and winter. During growth resumption under spring conditions, bud tissue exposed to insufficient winter chill failed to increase the activity of their TCA and CYT pathways, but instead increased their PPP and ALT pathway activity, perhaps to compensate for the low energy status at a time of high energy demand. This study also showed that under milder winter conditions the buds showed a reduction in the polyunsaturation process of their polar lipids compared to buds that experienced a colder winter and similarly an increase in the ratio of total free sterols to total phospholipids. The higher SFAs and MUFAs, the low UFAs and lower poly-unsaturation of C18:2 to C18:3 possibly made the membranes less fluid and permeable during growth resumption potentially rendering the bud unable to start growing thereby delaying budbreak. The lack of

change in the membrane fluidity could possibly hamper the functioning of the complex IV subunit and decrease the ATP production capacity. Interestingly, it is found that the oxygen uptake step of the ALT pathway is less affected by changes in membrane fluidity (Matos *et al*, 2007) and therefore the buds from the warmer area were able to continue using this pathway to prevent respirational uncoupling. The “membrane landscape” of the bud tissue from the warmer winter area was potentially less accommodating in supporting an increase in ATP production via respiration and this could result in buds that are unable/less able to grow, even under favourable environmental conditions.

When a mix of rest breaking products (hydrogen cyanamide and mineral oil, HCo) was applied to trees in the area with insufficient winter chill, it changed the physiology within the buds and resulted in a budbreak pattern more comparable to that of buds that received sufficient winter chill. HCo can thus be seen as a quick and useful option to partly compensate for the deficient chill accumulation in warm winter areas and ensure synchronised budbreak (El-Yazal and Rady, 2012). These results showed that the effectiveness of HCo increased when the application was followed by warm weather conditions that accelerated the onset of the green-tip stage (15 days in 2015) while cooler post-treatment conditions delayed growth resumption (21 days in 2016). Similar to results from Pérez *et al.* (2007) and Amberger (2013) for hydrogen cyanamide, we found that the immediate effect of the HCo application caused hypoxia in the apple bud tissue. Under such low oxygen conditions, the total respiration and the TCA and CYT pathways were interrupted while the activity of the PPP and ALT pathways increased to possibly compensate for the lack in energy production and to counter the high stress levels. Two to three days later a switch occurred in the treated buds and the main respiration pathways started to increase again while the PPP and ALT activity decreased revealing the long-term effect of the treatment. During this period, the main respiration pathways in the untreated buds remained low and the activity of PPP and ALT increased. This sudden change in the treated buds brought about a respiration pattern that was comparable to findings reported for the colder winter area. This immediate effect of hypoxia seen in the buds after HCo treatment was, however, not observed in the lipid composition. The HCo treatment effect on FAs was a decrease in linoleic acid while linolenic acid increased and only occurred closer to or at the green-tip stage. The decrease in linoleic acid contribution and simultaneous increase in the contribution of linolenic acid to the polar FA pool possibly leads to increased membrane fluidity and permeability as demonstrated by Wang and Faust (1988). In the current results, the green-tip phase of the treated buds also coincided with a decline in the ratio of total free sterols to total phospholipids. Similar results have been reported

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close to budbreak in studies where thidiazuron (TDZ) (synthetic cytokinin-like compound that is used as rest breaking application on apple) was applied in early spring or when sufficient winter chill occurred in blackberry (Izadyar and Wang, 1999) and in apple (Wang and Faust, 1988, 1990a and 1990b). In general, these physiological aspects provide evidence that treatment with HCo in the area with milder winter conditions changed the "membrane landscape" by increasing the polyunsaturated fatty acids and enhancing energy producing respiration pathways which lead to favourable conditions for bud growth. It also provides evidence that the rest breaking mechanism of the hydrogen cyanamide-oil combination uses a similar physiological mechanisms to that of sufficient winter chill to induce growth resumption of bud meristems. All the results may assist in the future development of products and procedures to improve apple cultivation in marginal production areas by improving productivity and profitability, especially in the Western Cape of South Africa

Limitations and recommendation for future research

The reported data on dormancy progression, respiration and lipid dynamics during winter and under HCo treatment in apple buds from marginal climates in South Africa creates a platform for future studies. Future studies could include trials that quantify oxidative stress under different environmental conditions as it seems to stand in close relationship to all the respiration pathways. Lipid studies can be extended and expanded to include investigations into fatty acids from the major individual glycolipids and phospholipids in both polar lipids and neutral lipids, as well as more detailed studies on sterol composition during the dormancy cycle to better understand and describe the changing "membrane landscape". Studies including the direct measurement of membrane fluidity and permeability will also confirm the functionality of the lipid changes observed in the study. Designing experiments to test the direct relationship between lipid composition, fluidity and CYT activity will also aid to clarify the interface between respiration and membrane characteristics. In the RBA chapters, no budbreak data is presented in terms of the whole orchard. As this is the visual response of the plant to the preceding winter and spring conditions, it would have been useful to illustrate how the physiological dynamics manifests in a phenotypical reaction at orchard level. Another limitation is that no data (respiration and lipid composition) were recorded in the untreated buds to see if they would eventually (beyond the 15 – 21 days) also change their physiology to increase their respiration after a longer period. The matter of "sufficient" chill should also be further investigated by perhaps including a study site from a Northern hemisphere orchard where more traditional winter conditions are experienced. We acknowledge that although the higher chill accumulation in the Koue Bokkeveld was used to serve as a "cold"

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contrast to the warmer/milder Elgin area, it is not similar to a region that receives a more pronounced autumn and a much colder winter. It is also indispensable that further studies should include more production areas/years with variable winter conditions and different RBA dosages to shed more light on the robustness of the results across production areas and seasons.

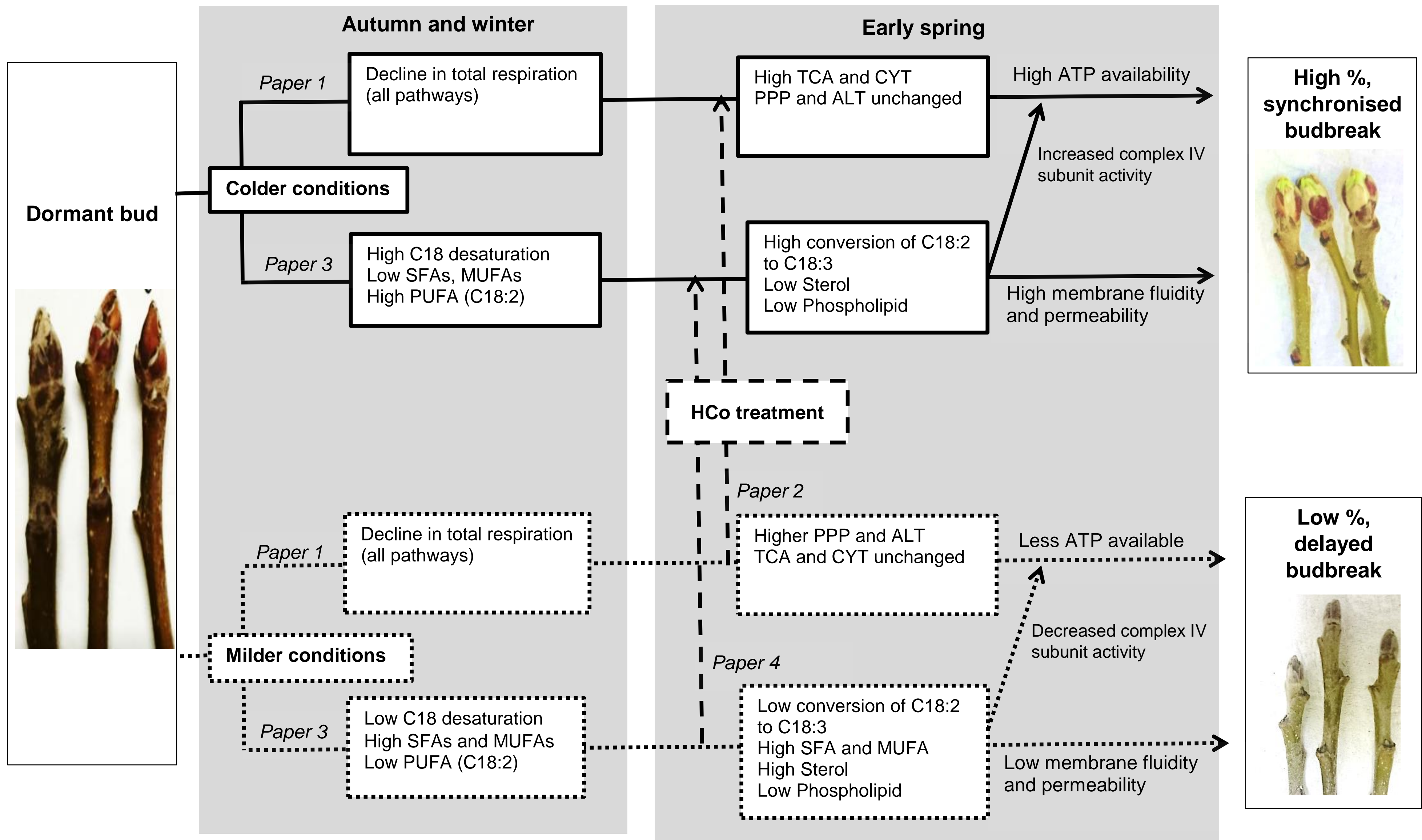

Figure. 1: The proposed physiological dynamics within a dormant apple bud during different environmental conditions and the use of hydrogen cyanamide and oil (HCo) as a rest-breaking agent.

References

Amberger, A. (2013). Cyanamide in plant metabolism. *International Journal of Plant Physiology and Biochemistry*. **5**, 1–10.

Atkin, O.K. and Tjoelker, M.G. (2003). Thermal acclimation and the dynamic response of plant respiration to temperature. *Trends in Plant Science*. **8**, 343–351.

Beauvieux, R., Wenden, B. and Dirlewanger, E. (2018). Bud dormancy in perennial fruit tree species: A pivotal role for oxidative cues. *Frontiers in Plant Science*. **9**, 657.

Borchert, R. 1991. Growth periodicity and dormancy, pp 221-245. In: A.S. Raghavendra (Ed). Physiology of trees. John Wiley & Sons, NY.

Cook, N.C. and Jacobs, G. (1999). Sub-optimal winter chilling impedes development of acrotony in apple shoots. *HortScience*. **34**, 1213-1216.

Cook, N.C. and Jacobs, G. (2000). Progression of apple (*Malus x domestica* Borkh.) bud dormancy in two mild winter climates. *Journal of Horticultural Science*. **75**, 233-236.

Cook, N.C., Calitz, F.J., Allderman, L.A., Steyn, W.J. and Louw, E.D. (2017). Diverse patterns in dormancy progression of apple buds under variable winter conditions. *Scientia Horticulturae.* **226**, 307–315.

Erez, A., Wang, S.Y. and Faust M. (1997). Lipids in peach buds during dormancy, a possible involvement in dormancy control. *Advances in Horticultural Science*. **11**, 128–132.

Florez-Sarasa, I.D., Bouma, T.J., Medrano, H., Azcon-Bieto, J. and Ribas-Carbo, M. (2007). Contribution of the cytochrome and alternative pathways to growth respiration and maintenance respiration in *Arabidopsis thaliana*. *Physiologia Plantarum*. **129**, 143–151.

Gardea, A.A., Moreno, Y.M., Azarenko, A.N., Lombard, P.B. and Daley, L.S. (1994). Changes in metabolic properties of grape buds during development. *Journal of the American Society for Horticultural Science*. **119**, 756–760.

Izadyar, A.B. and Wang, S.Y. (1999). Changes of lipid components during dormancy in 'Hull Thornless' and 'Triple Crown thornless' blackberry cultivars. *Scientia Horticulturae*. **82**, 243–254.

Kurimoto K., Millar A.H., Lambers, H., Day, D.A., Noguchi, K. (2004). Maintenance of growth rate at low temperature in rice and wheat cultivars with a high degree of respiratory homeostasis is associated with a high efficiency of respiratory ATP production. *Plant and Cell Physiology*. **45**, 1015–1022.

Matos, A.R., Hourton-Cabassa, C., Cicek, D., Reze, N., Arrabac, J.D., Zachowski, A. and Moreau, F. (2007). Alternative oxidase involvement in cold stress response of *Arabidopsis thaliana fad2* and FAD3+ cell suspensions altered in membrane lipid composition. *Plant Cell Physiology*. **48(6)**, 856–865.

Midgley, S.J.E. and Lötze, E. (2011). Climate change in the western cape of South Africa: trends, projections and implications for chill unit accumulation. *Acta Horticulturae*. **903**, 1127-1134.

Pérez, F.J., Rubio, S. and Ormeño-Nuñez, J. (2007). Is erratic bud-break in grapevines grown in warm winters areas related to disturbances in mitochondrial respiratory capacity and oxidative metabolism? *Functional Plant Biology*. **34**, 624–632.

Seif El-Yazal, M.A. and Rady, M.M. (2012). Changes in nitrogen and polyamines during breaking bud dormancy in Anna apple trees with foliar application of some compounds. *Science of Horticulture*. **136**, 75-80.

Tan, Y., Gao, D.S., Li, L., Chen, X.D. and Xu, A.H. (2010). Respiratory response of dormant nectarine floral buds on chilling deficiency. *Agricultural Sciences in China*. **9**, 1448–1454.

Tan, Y., Li, L., Leng, C., Li, D., Chen, X. and Gao, D. (2013). Respiratory response of dormant nectarine vegetative buds to high temperature stress. *Journal of Integrative Agriculture*. **12**, 80–86.

Wang, S.Y. and Faust, M. (1988). Changes of fatty acids and sterols in apple buds during budbreak induced by a

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plant bioregulator, Thidiazuron. *Physiologia Plantarum*. **72**, 115–120.

Wang, S.Y. and Faust, M. (1990a). Seasonal changes of membrane lipids in apple shoots. *Journal of the American Society for Horticultural Science*. **115**, 462–467.

Wang, S.Y. and Faust, M. (1990b). Changes in membrane lipids in apple buds during dormancy and budbreak. *Journal of the American Society for Horticultural Science*. **115**, 803–808.

Young, E. (1990). Changes in respiration rate and energy of activation after chilling and forcing dormant apple trees. *Journal of the American Society for Horticultural Science*. **115**, 809–814.

Wang, S.Y. and Faust, M. (1990a). Seasonal changes of membrane lipids in apple shoots. *Journal of the American Society for Horticultural Science*. **115**, 462–467.

Wang, S.Y. and Faust, M. (1990b). Changes in membrane lipids in apple buds during dormancy and budbreak. *Journal of the American Society for Horticultural Science*. **115**, 803–808.

Young, E. (1990). Changes in respiration rate and energy of activation after chilling and forcing dormant apple trees. *Journal of the American Society for Horticultural Science*. **115**, 809–814.

APPENDIX A

This appendix contains the results from the analysis of polar and neutral lipids seasons (2015 and 2016) refered to in Paper 3.

1. Fatty acids composition of the polar lipid fraction

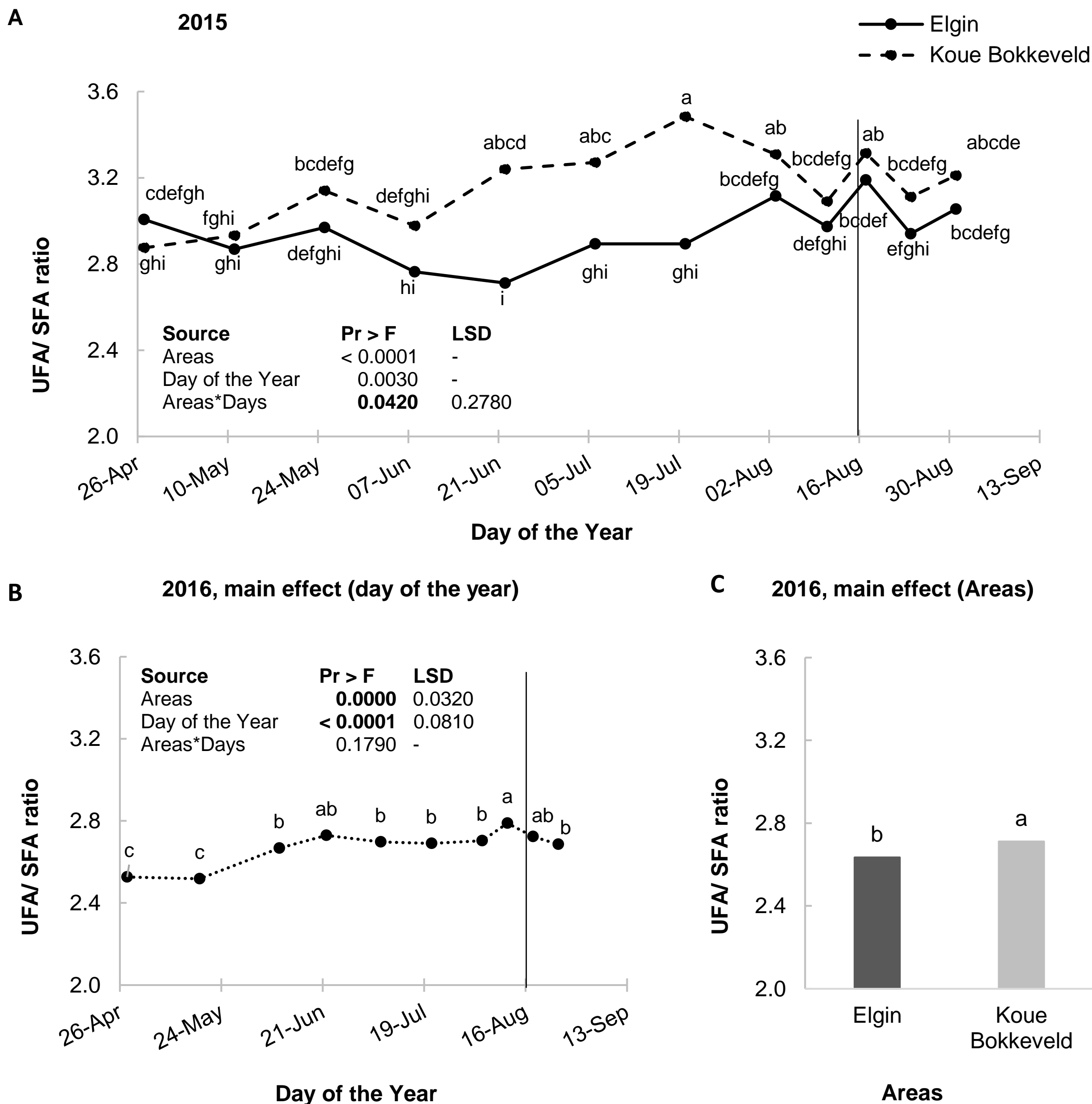
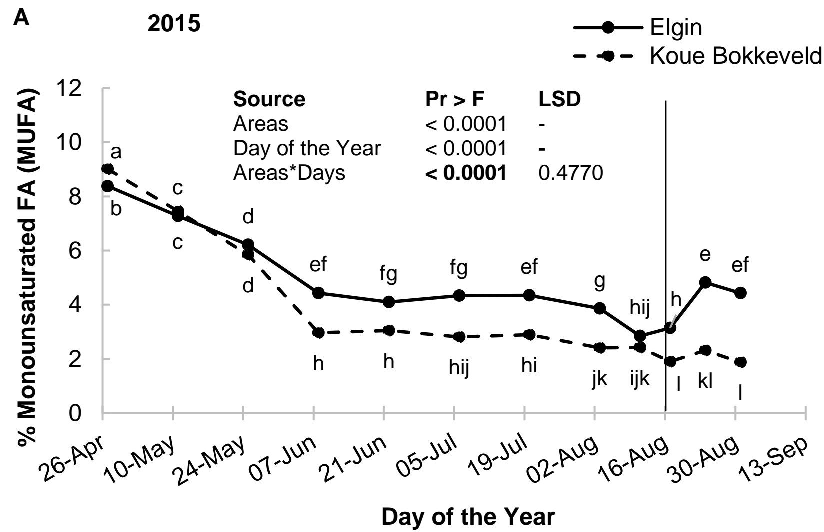
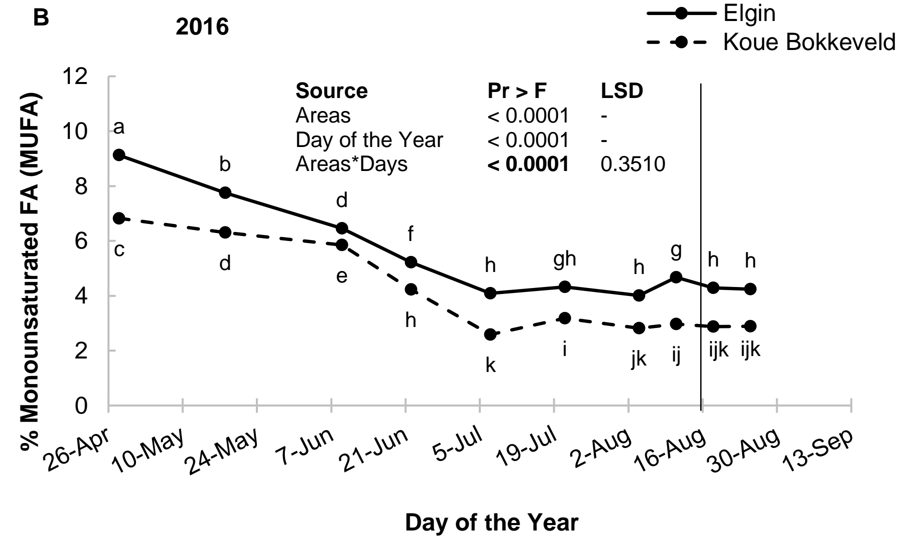
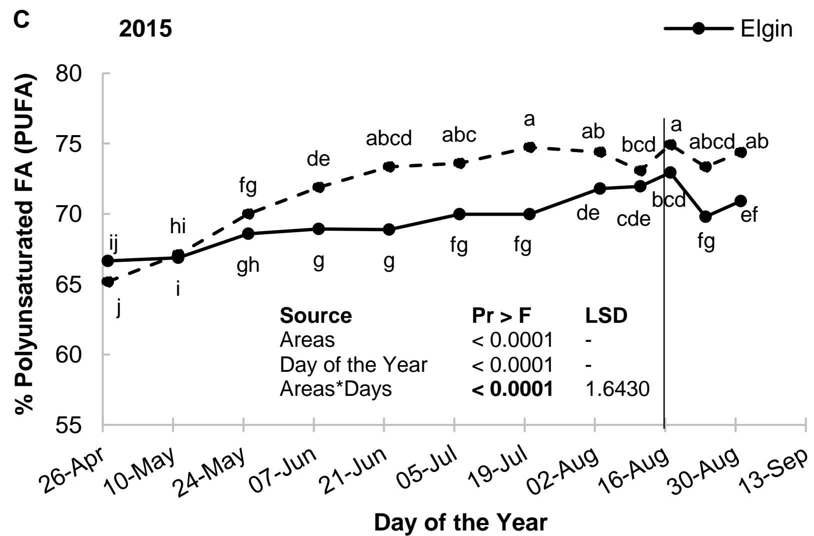
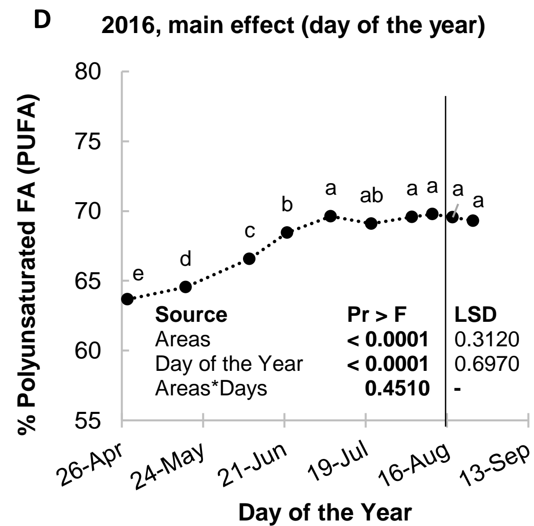


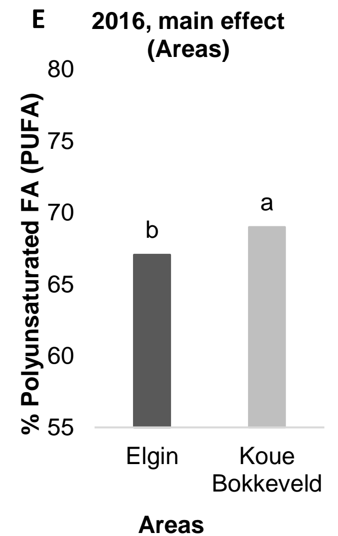
Fig. 1. The unsaturated (UFA)/ saturated (SFA) FA ratio (UFA/ SFA ratio) (A, B, C) present in the total polar lipid of ‘Cripps Pink’ buds collected from the Koue Bokkeveld (_ _ _) and Elgin (____) in 2015 and 2016. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the main factors are presented individually.


A
2015
Elgin
Koue Bokkeveld
% Monounsaturated FA (MUFA)
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 0.4770
Day of the Year
26-Apr 10-May 24-May 07-Jun 21-Jun 05-Jul 19-Jul 02-Aug 16-Aug 30-Aug 13-Sep


B
2016
Elgin
Koue Bokkeveld
% Monounsaturated FA (MUFA)
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 0.3510
Day of the Year
26-Apr 10-May 24-May 7-Jun 21-Jun 5-Jul 19-Jul 2-Aug 16-Aug 30-Aug 13-Sep


C
2015
Elgin
% Polyunsaturated FA (PUFA)
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 1.6430
Day of the Year
26-Apr 10-May 24-May 07-Jun 21-Jun 05-Jul 19-Jul 02-Aug 16-Aug 30-Aug 13-Sep


D
2016, main effect (day of the year)
% Polyunsaturated FA (PUFA)
Source Pr > F LSD
Areas < 0.0001 0.3120
Day of the Year < 0.0001 0.6970
Areas*Days 0.4510 -
Day of the Year
26-Apr 24-May 21-Jun 19-Jul 16-Aug 13-Sep


E
2016, main effect (Areas)
% Polyunsaturated FA (PUFA)
Elgin
Koue Bokkeveld
Areas

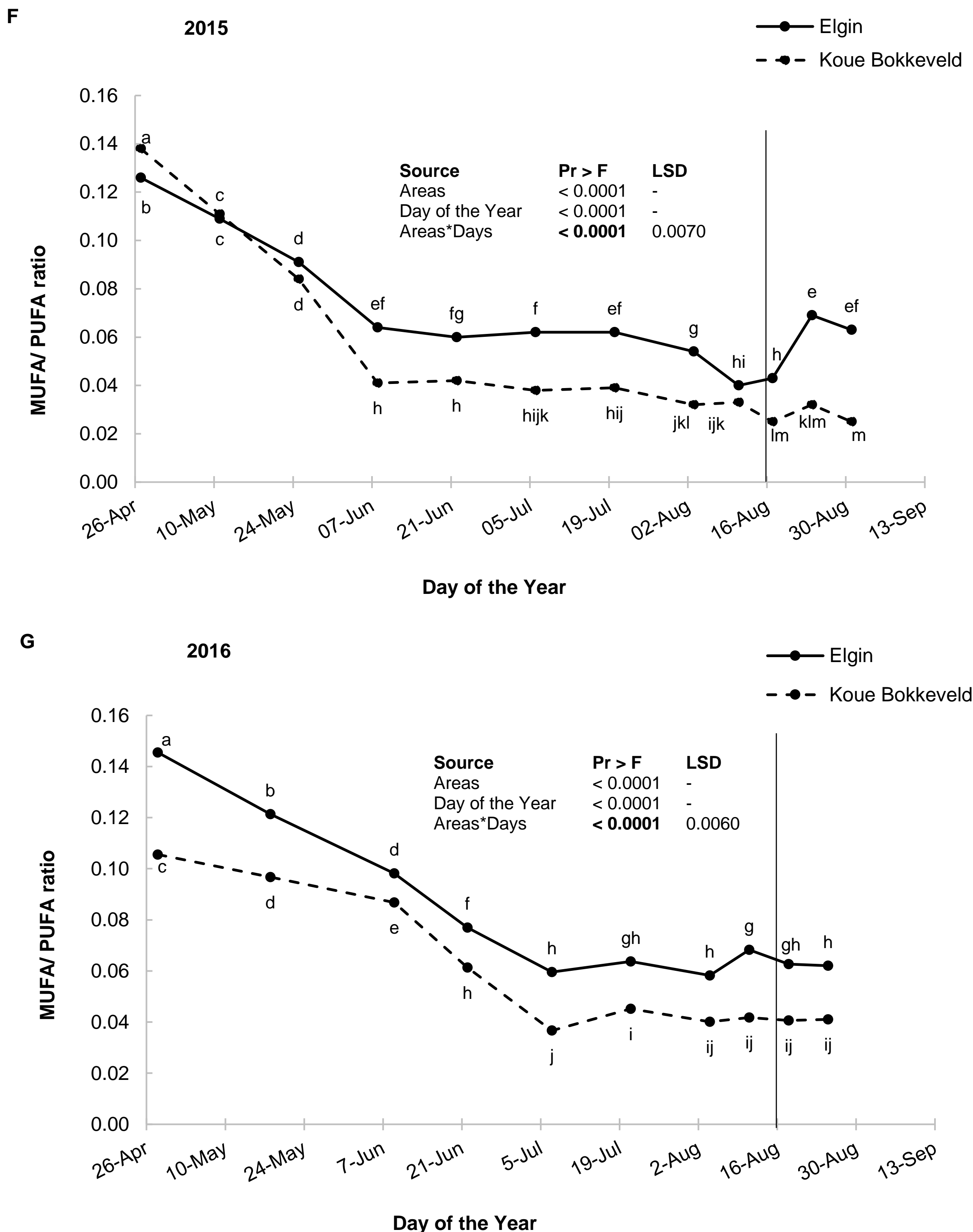


Fig. 2. Percentage monounsaturated fatty acids (MUFA) (A, B), polyunsaturated fatty acids (PUFA) (C, D, E) and monounsaturated (MUFA) / polyunsaturated (PUFA) FA ratio (F, G) present in the total polar lipids (PL) of 'Cripps Pink' buds sampled in the Koue Bokkeveld (_ _ _) and Elgin (____) in 2015 and 2016. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.

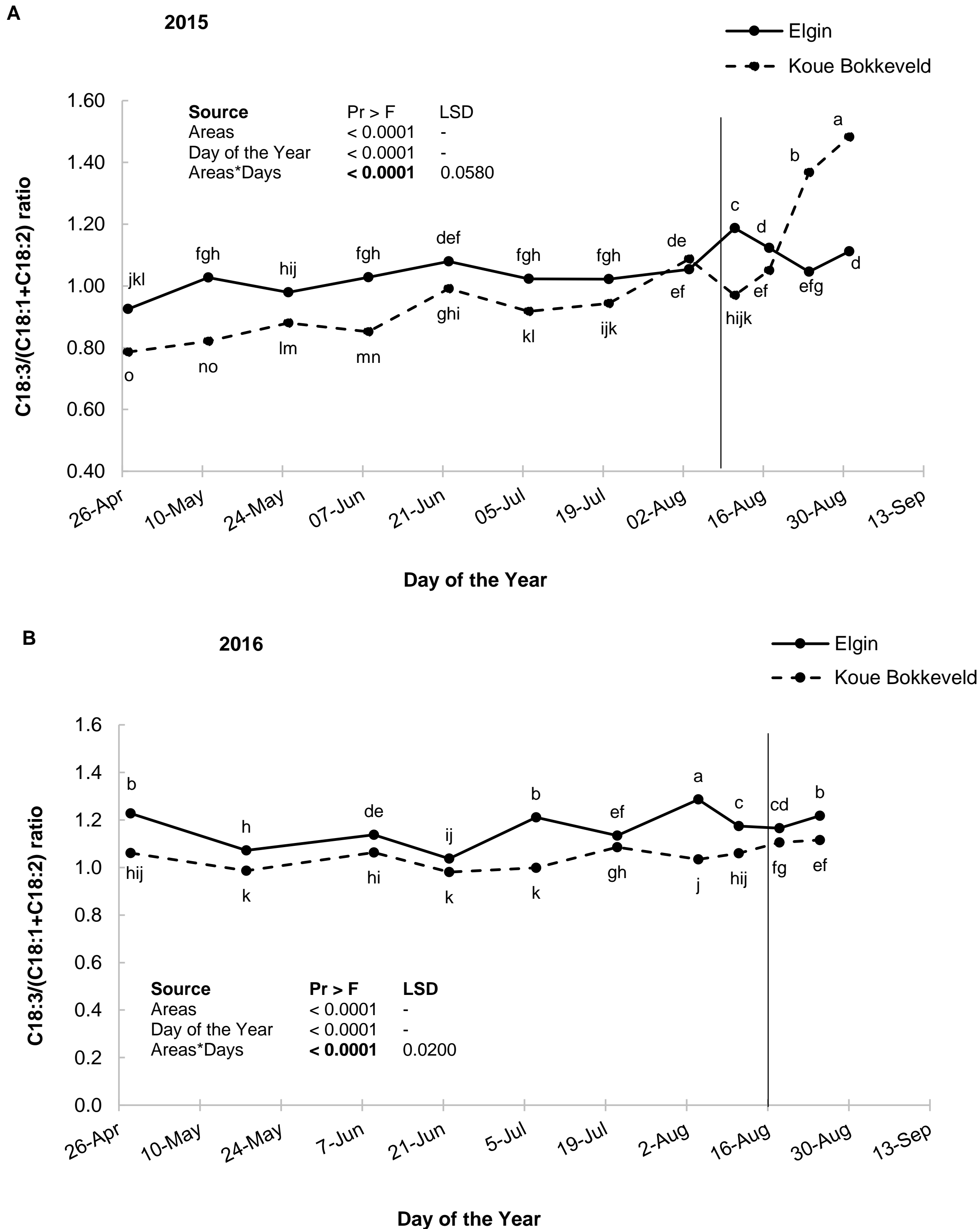
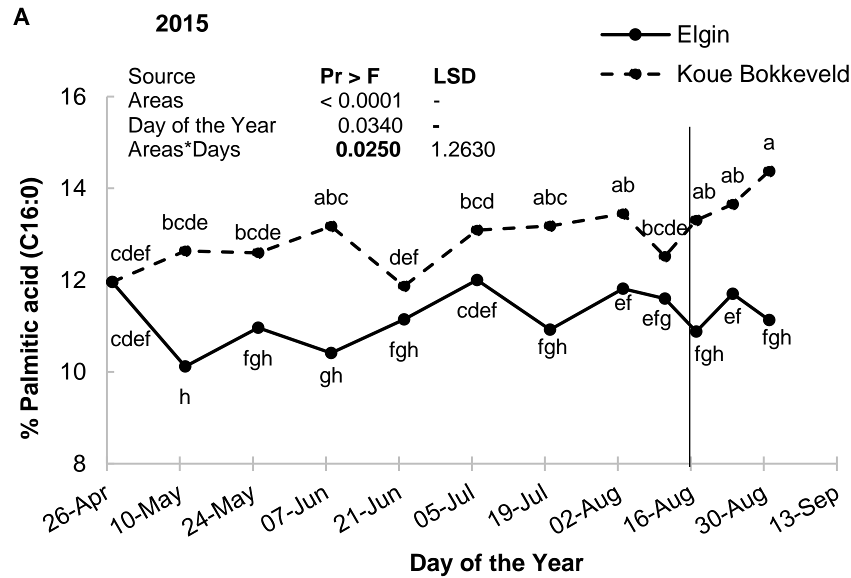
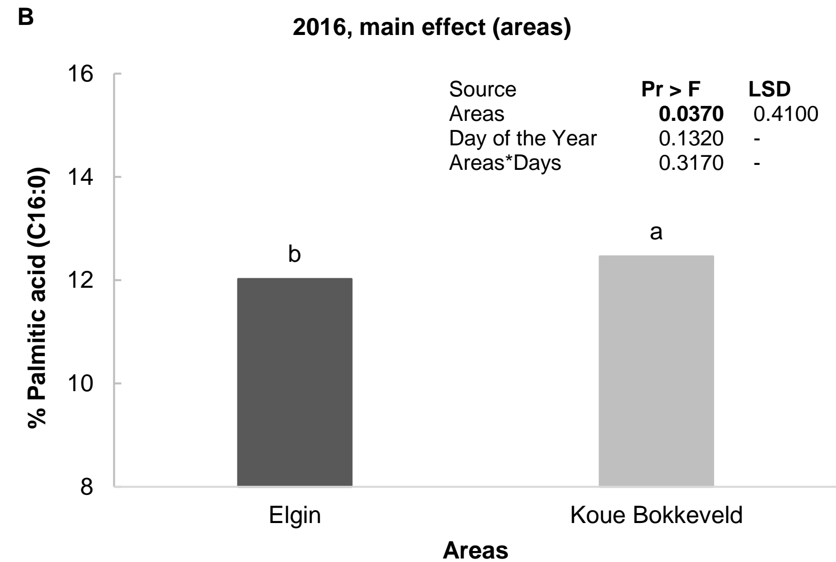
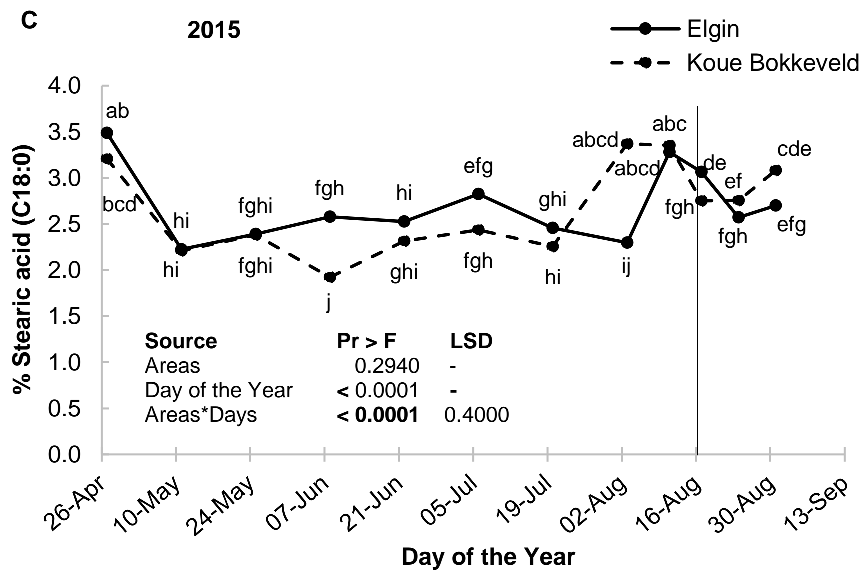


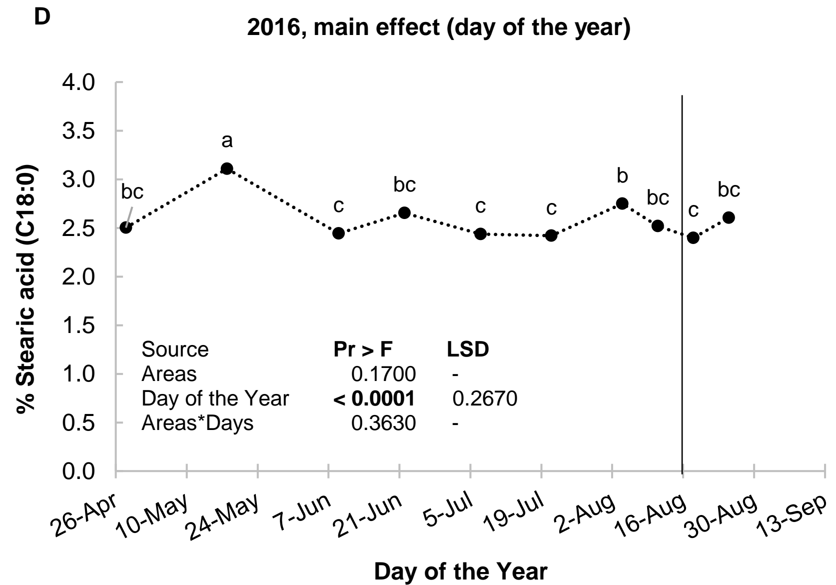
Fig. 3. The unsaturation polar lipids (PL) in 'Crips Pink' buds collected in 2015 and 2016 from the Koue Bokkeveld (_ _ _) and Elgin (_____) as given by the C18:3/ (C18:1+C18:2) ratio (A, B). The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$).

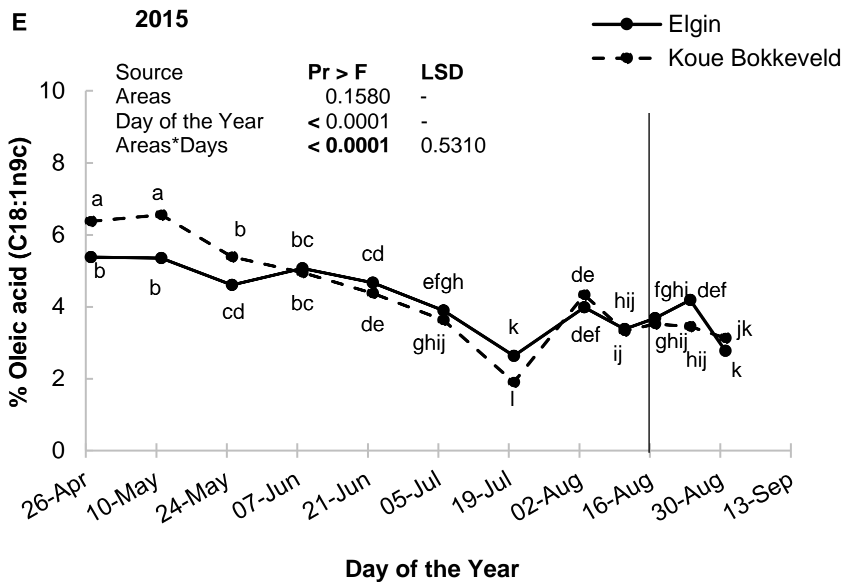
2. Fatty acids composition of the neutral lipid fraction

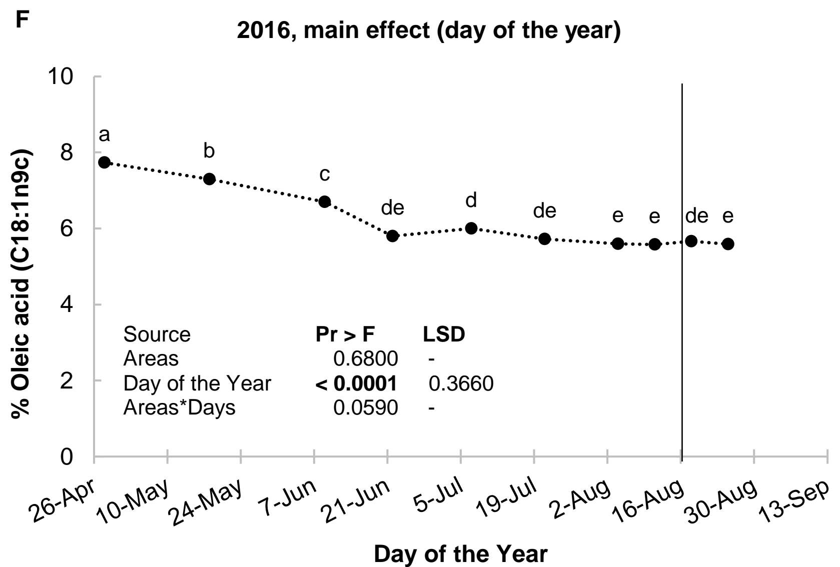


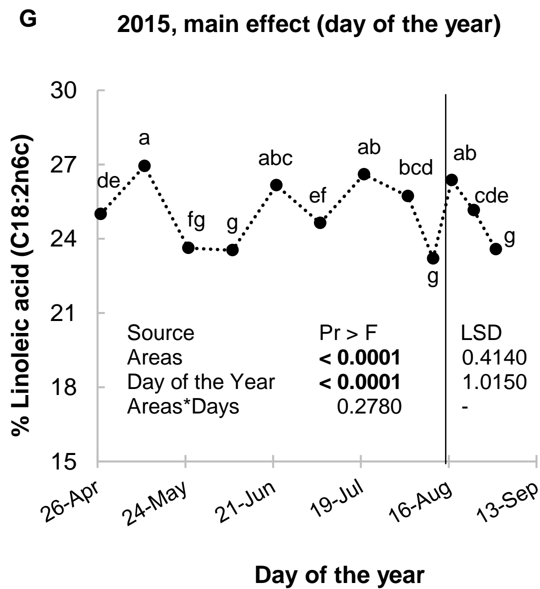


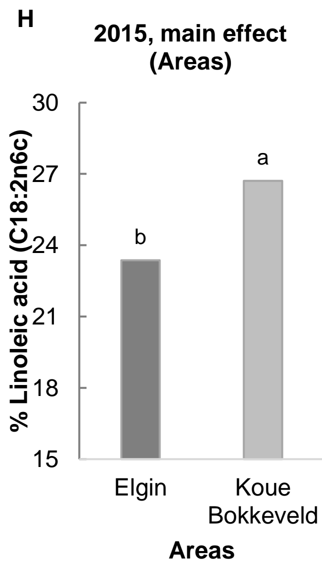


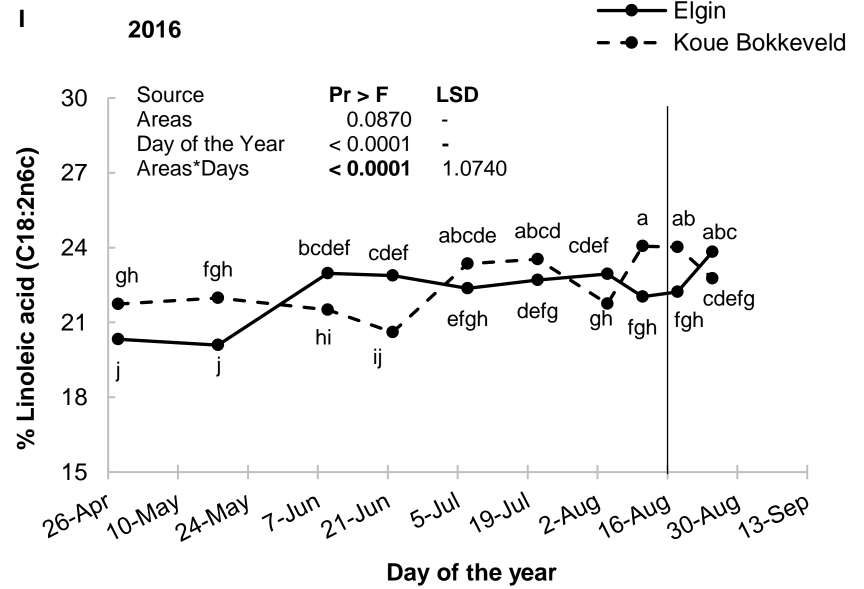


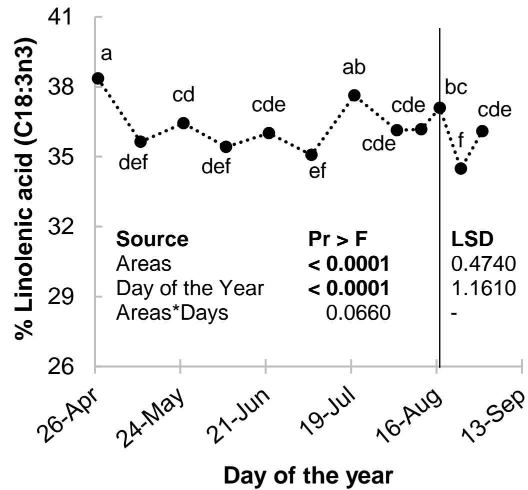

E
2015
Elgin
Koue Bokkeveld
Source Pr > F LSD
Areas 0.1580 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 0.5310
% Oleic acid (C18:1n9c)
Day of the Year

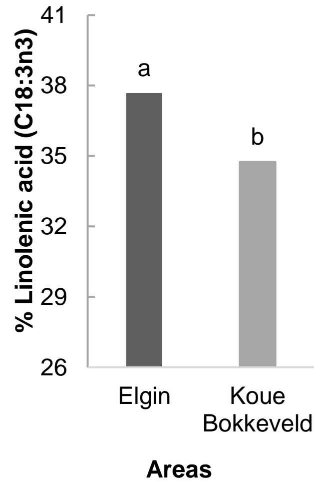

F
2016, main effect (day of the year)
Source Pr > F LSD
Areas 0.6800 -
Day of the Year < 0.0001 0.3660
Areas*Days 0.0590 -
% Oleic acid (C18:1n9c)
Day of the Year

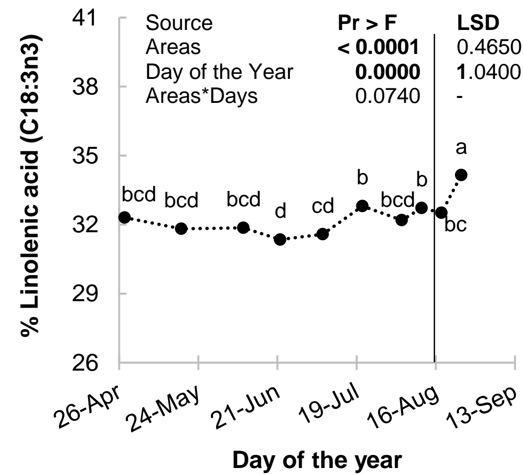

G
2015, main effect (day of the year)
Source Pr > F LSD
Areas < 0.0001 0.4140
Day of the Year < 0.0001 1.0150
Areas*Days 0.2780 -
% Linoleic acid (C18:2n6c)
Day of the year

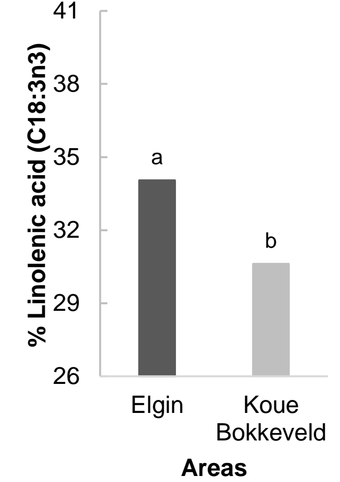

H
2015, main effect (Areas)
% Linoleic acid (C18:2n6c)
Elgin
Koue Bokkeveld
Areas

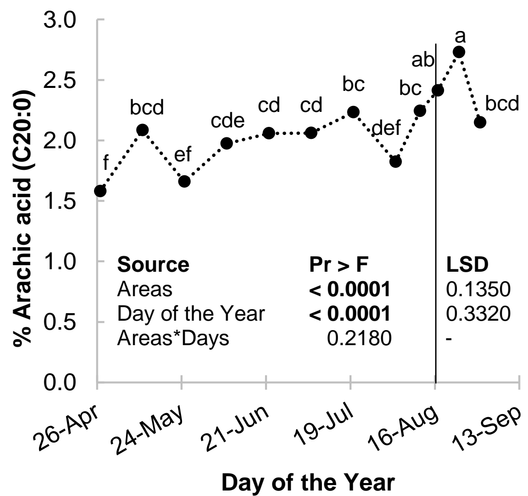

I
2016
Elgin
Koue Bokkeveld
Source Pr > F LSD
Areas 0.0870 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 1.0740
% Linoleic acid (C18:2n6c)
Day of the year

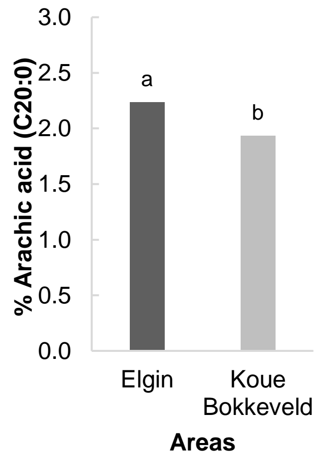

J
2015, main effect (day of the year)
% Linolenic acid (C18:3n3)
Day of the year
Source Pr > F LSD
Areas < 0.0001 0.4740
Day of the Year < 0.0001 1.1610
Areas*Days 0.0660 -
26-Apr
24-May
21-Jun
19-Jul
16-Aug
13-Sep

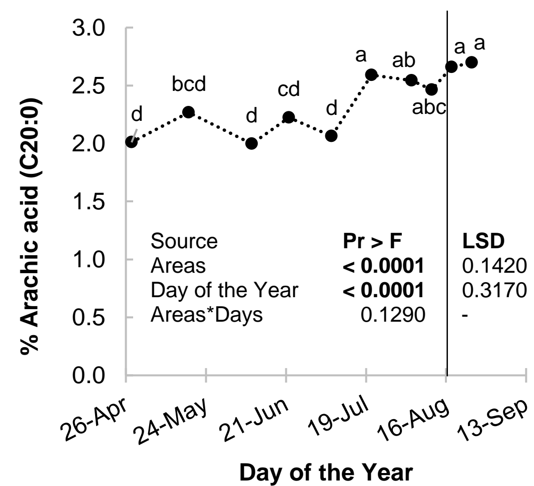

K
2015, main effect (Areas)
% Linolenic acid (C18:3n3)
Elgin
Koue Bokkeveld
Areas

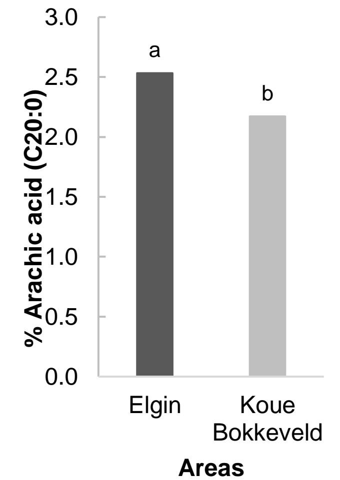

L
2016, main effect (day of the year)
% Linolenic acid (C18:3n3)
Source Pr > F LSD
Areas < 0.0001 0.4650
Day of the Year 0.0000 1.0400
Areas*Days 0.0740 -
Day of the year

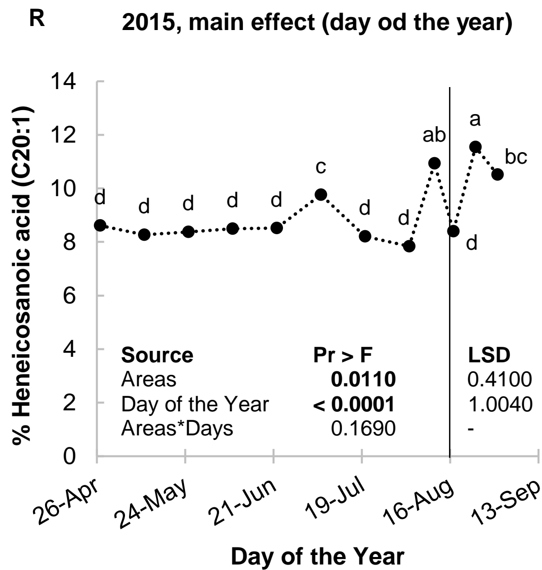

M
2016, main effect (Areas)
% Linolenic acid (C18:3n3)
Elgin
Koue Bokkeveld
Areas

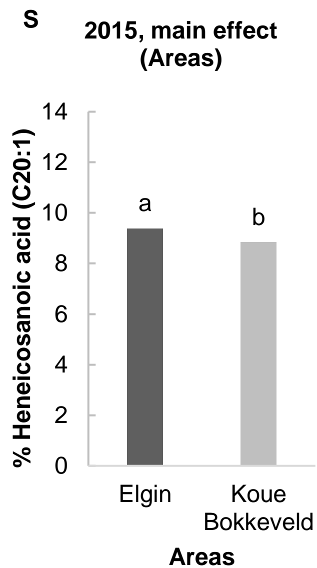

N
2015, main effect (day of the year)
% Arachic acid (C20:0)
Source Pr > F LSD
Areas < 0.0001 0.1350
Day of the Year < 0.0001 0.3320
Areas*Days 0.2180 -
Day of the Year

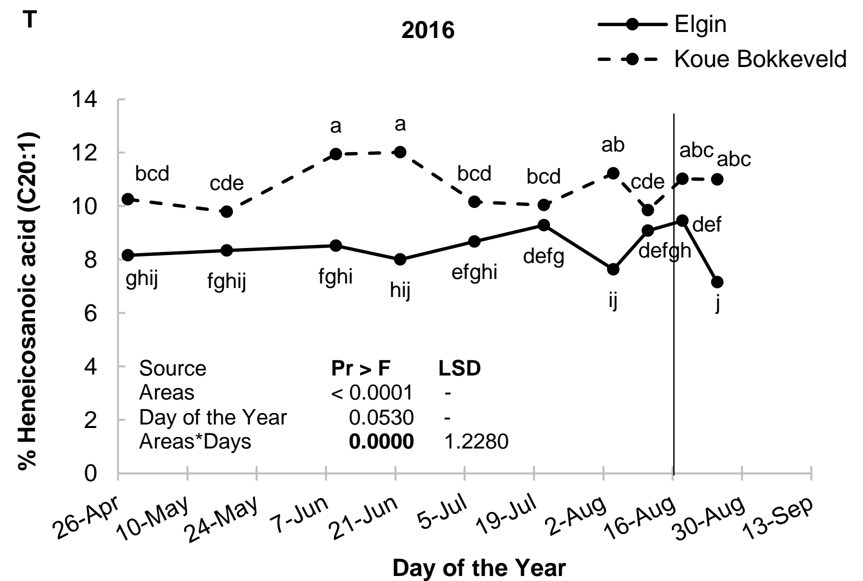

O
2015, main effect (Areas)
% Arachic acid (C20:0)
Elgin
Koue Bokkeveld
Areas

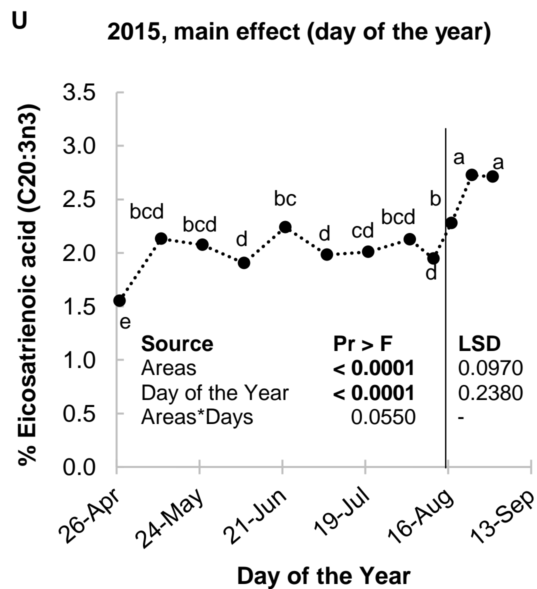

P
2016, main effect (day of the year)
% Arachic acid (C20:0)
Source Pr > F LSD
Areas < 0.0001 0.1420
Day of the Year < 0.0001 0.3170
Areas*Days 0.1290 -
Day of the Year

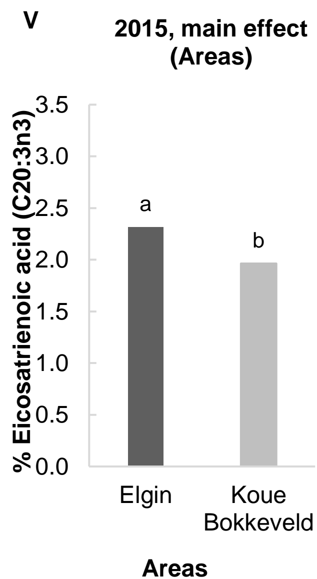

Q
2016, main effect (Areas)
% Arachic acid (C20:0)
Elgin
Koue Bokkeveld
Areas

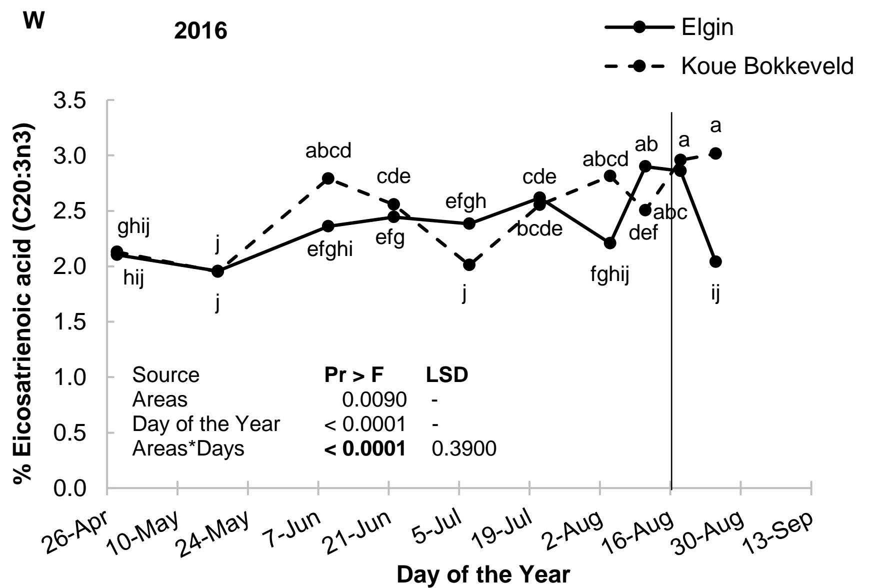

R
2015, main effect (day od the year)
% Heneicosanoic acid (C20:1)
Day of the Year
Source Pr > F LSD
Areas 0.0110 0.4100
Day of the Year < 0.0001 1.0040
Areas*Days 0.1690 -


S
2015, main effect (Areas)
% Heneicosanoic acid (C20:1)
Elgin
Koue Bokkeveld
Areas


T
2016
Elgin
Koue Bokkeveld
% Heneicosanoic acid (C20:1)
Day of the Year
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year 0.0530 -
Areas*Days 0.0000 1.2280


U
2015, main effect (day of the year)
% Eicosatrienoic acid (C20:3n3)
Day of the Year
Source Pr > F LSD
Areas < 0.0001 0.0970
Day of the Year < 0.0001 0.2380
Areas*Days 0.0550 -


V
2015, main effect (Areas)
% Eicosatrienoic acid (C20:3n3)
Elgin
Koue Bokkeveld
Areas


W
2016
Elgin
Koue Bokkeveld
% Eicosatrienoic acid (C20:3n3)
Day of the Year
Source Pr > F LSD
Areas 0.0090 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 0.3900

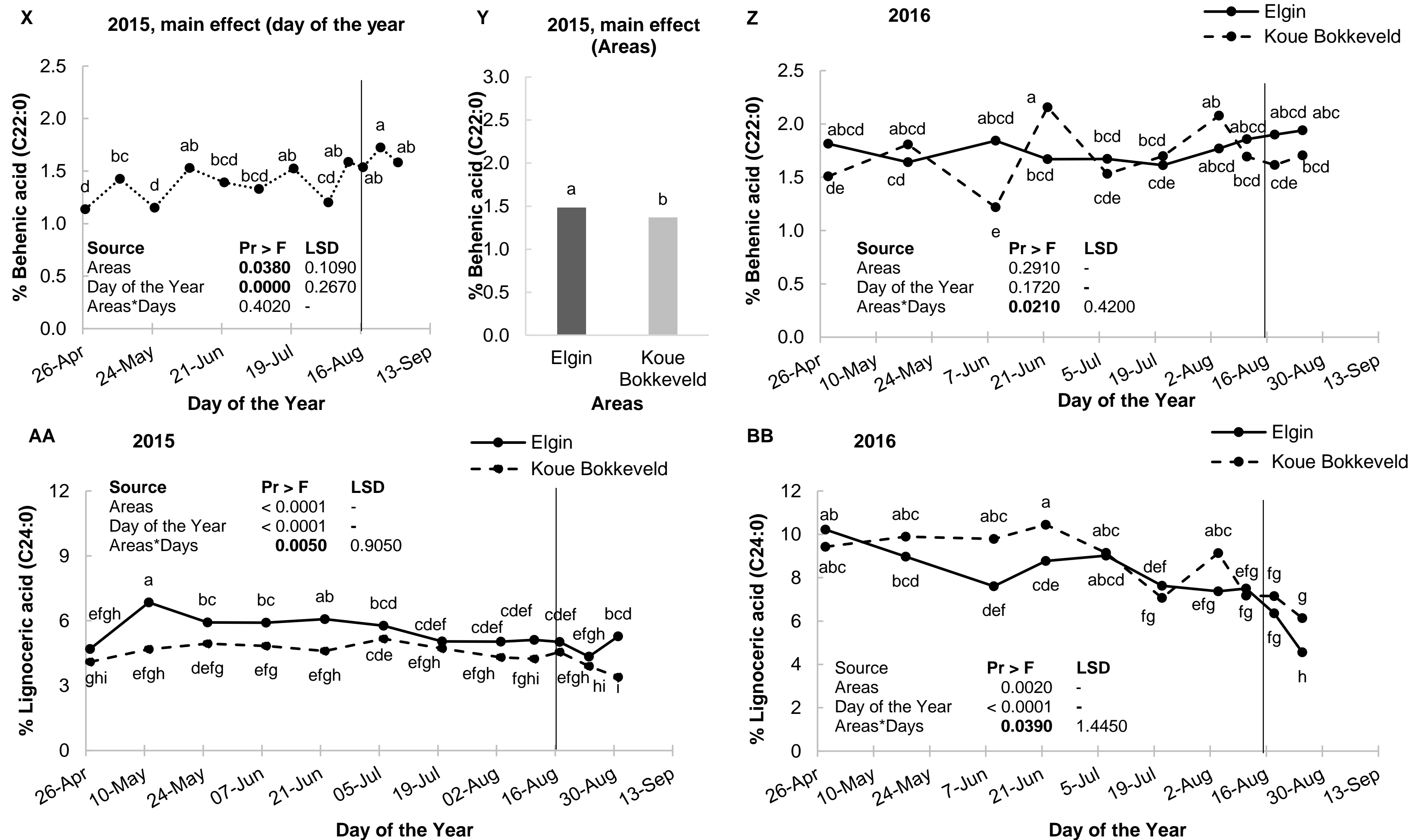
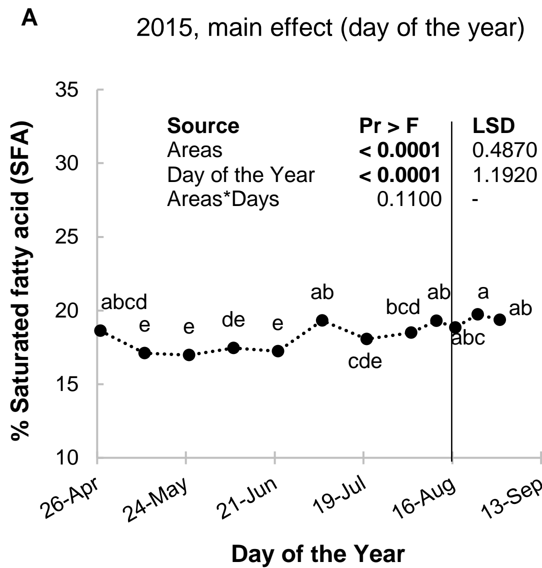
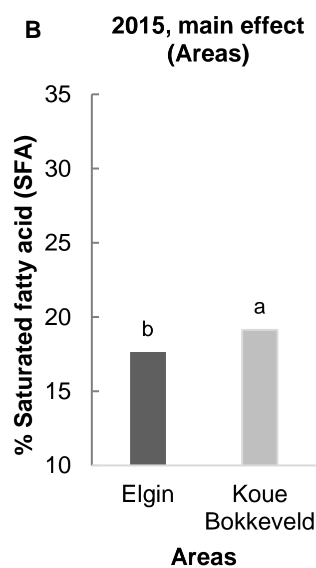
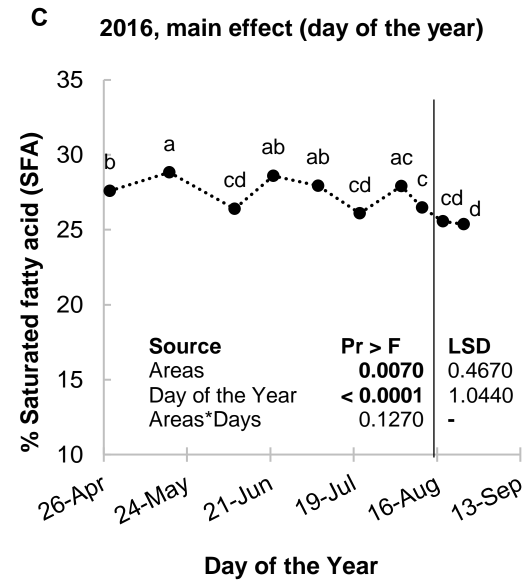
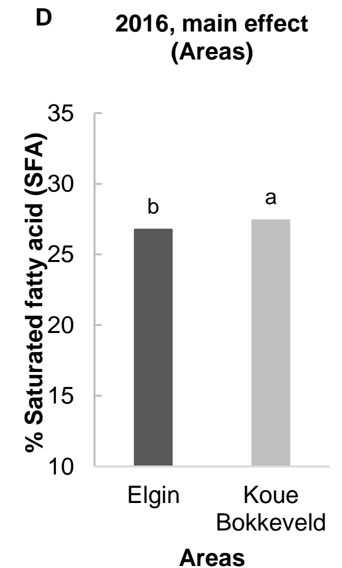


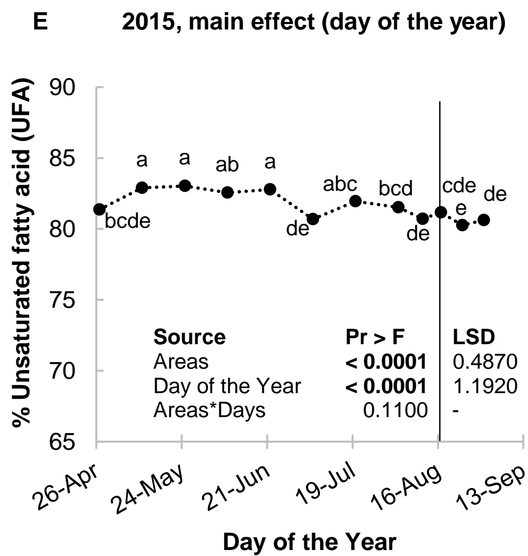
Fig. 4. Percentage Palmitic acid (C16:0) (A, B), Stearic acid (C18:0) (C, D), Oleic acid (C18:1n9c) (E, F), Linoleic acid (C18:2n6c) (G, H, I), Linolenic acid (C18:3n3) (J, K, L, M), Arachic acid (C20:0) (N, O P, Q), Heneicosanoic acid (C20:1) (R, S, T), Eicosatrienoic acid (C20:3n3) (U, V, W), Behenic acid (C22:0) (X, Y, Z) and Lignoceric acid (C24:0) (AA, BB) present in the total in the total neutral lipid fraction of 'Cripps Pink' buds sampled in Koue Bokkeveld (_ _ _) and Elgin (_____) in 2015 and 2016. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.

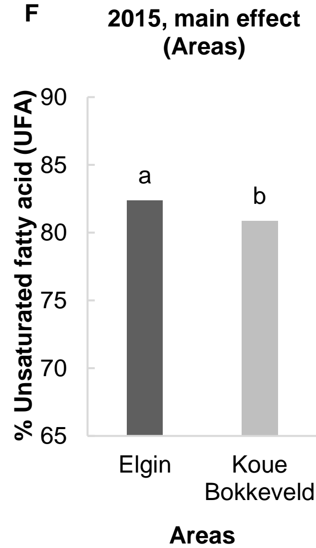

A
2015, main effect (day of the year)
% Saturated fatty acid (SFA)
Source Pr > F LSD
Areas < 0.0001 0.4870
Day of the Year < 0.0001 1.1920
Areas*Days 0.1100 -
Day of the Year

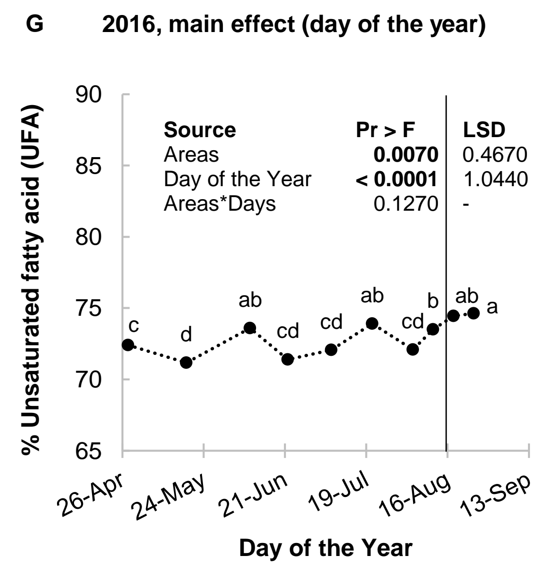

B
2015, main effect (Areas)
% Saturated fatty acid (SFA)
Elgin
Koue Bokkeveld
Areas

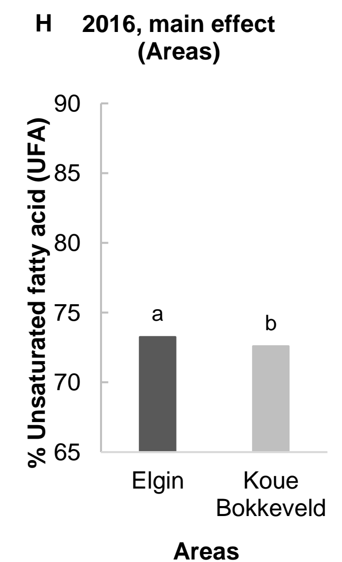

C
2016, main effect (day of the year)
% Saturated fatty acid (SFA)
Source Pr > F LSD
Areas 0.0070 0.4670
Day of the Year < 0.0001 1.0440
Areas*Days 0.1270 -
Day of the Year


D
2016, main effect (Areas)
% Saturated fatty acid (SFA)
Elgin
Koue Bokkeveld
Areas


E
2015, main effect (day of the year)
% Unsaturated fatty acid (UFA)
Source Pr > F LSD
Areas < 0.0001 0.4870
Day of the Year < 0.0001 1.1920
Areas*Days 0.1100 -
Day of the Year


F
2015, main effect (Areas)
% Unsaturated fatty acid (UFA)
Elgin
Koue Bokkeveld
Areas


G
2016, main effect (day of the year)
% Unsaturated fatty acid (UFA)
Source Pr > F LSD
Areas 0.0070 0.4670
Day of the Year < 0.0001 1.0440
Areas*Days 0.1270 -
Day of the Year


H
2016, main effect (Areas)
% Unsaturated fatty acid (UFA)
Elgin
Koue Bokkeveld
Areas

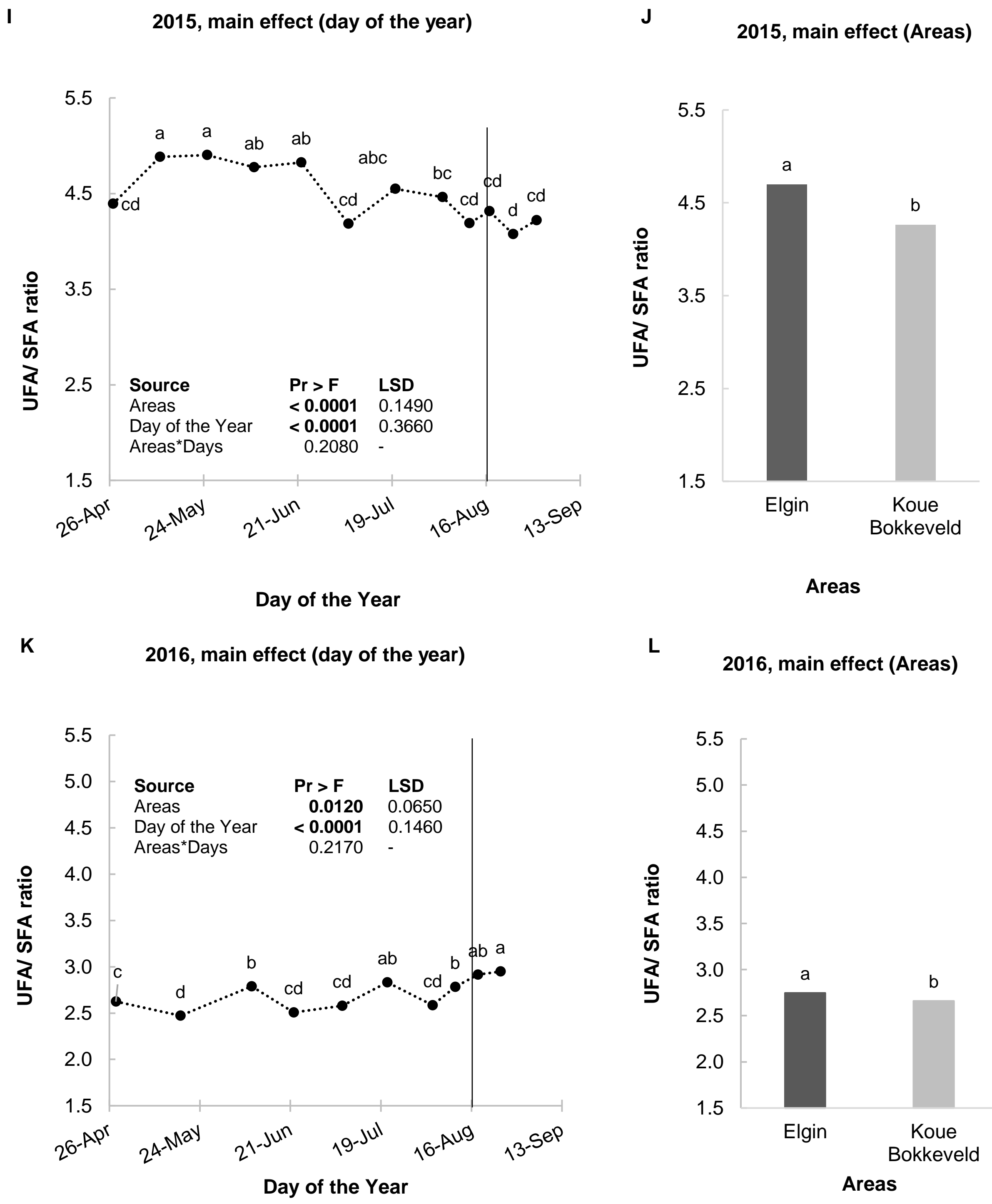
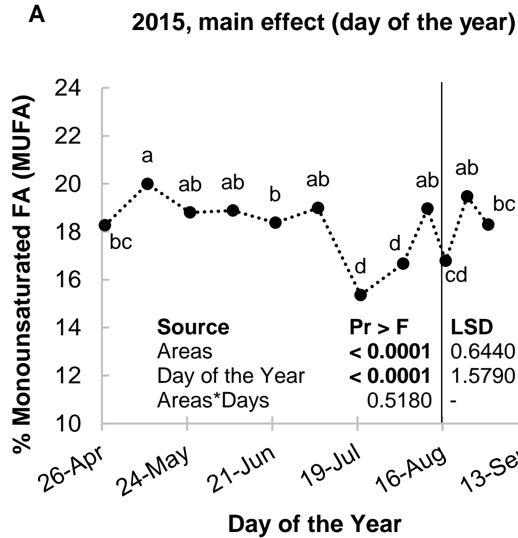
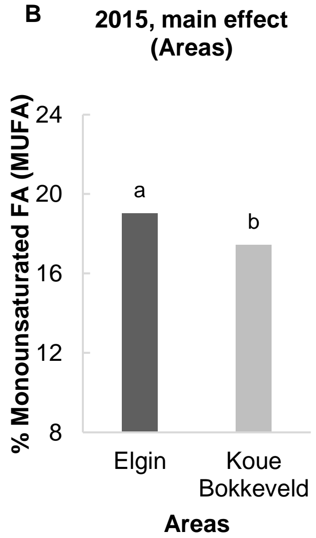
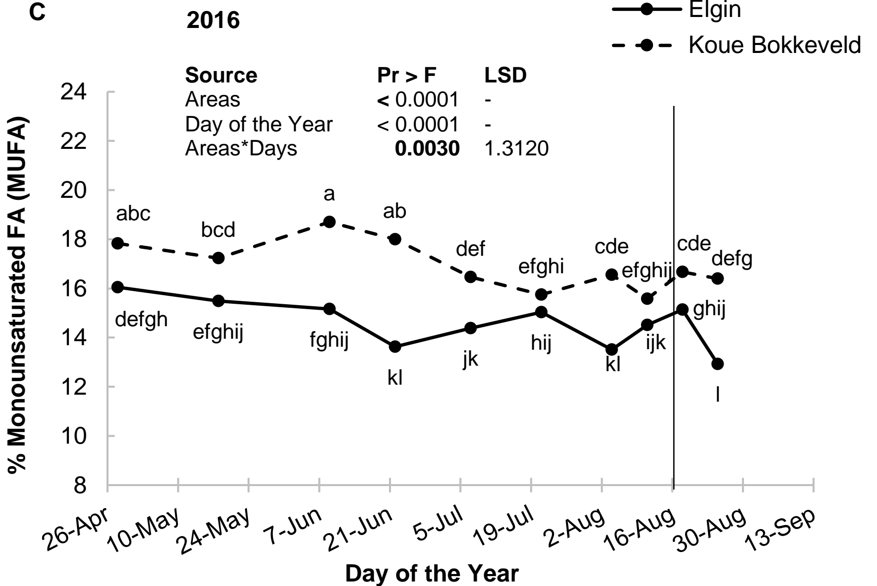
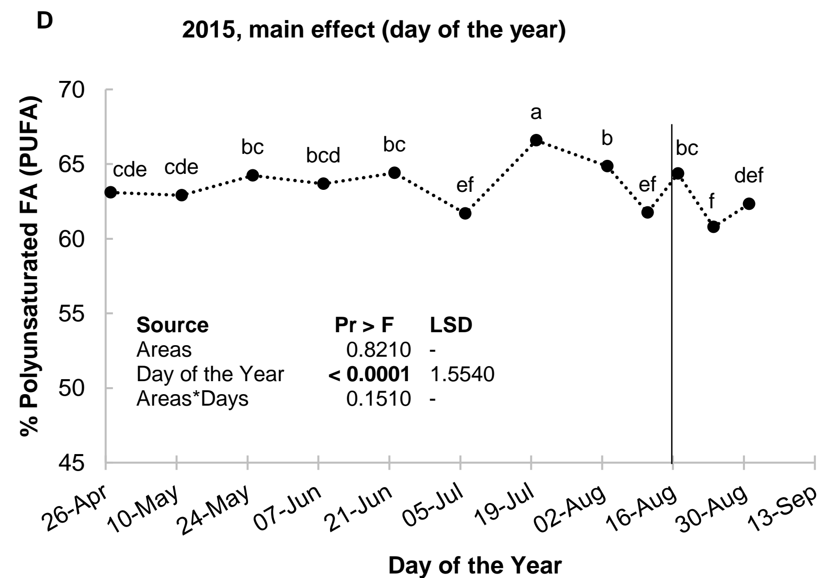


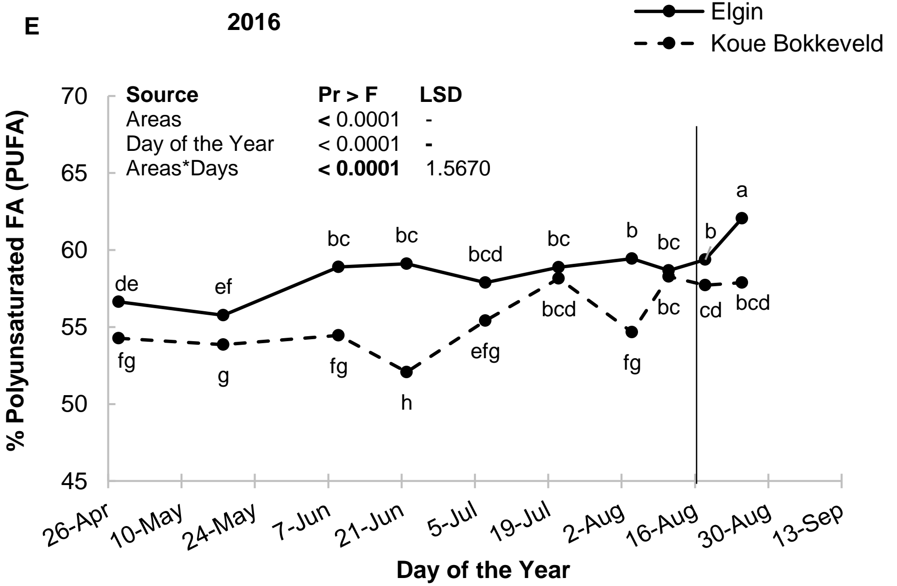
Fig. 5. Percentage Saturated fatty acids (SFAs) (A, B, C, D), unsaturated fatty acids (UFAs) (E, F, J, H), and UFAs/ SFAs ratio (I, J, K, L) present in the total neutral lipid of dormant buds sampled at Koue Bokkeveld (_ _ _) and Elgin (____) in 2015 and 2016. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.


A
2015, main effect (day of the year)
% Monounsaturated FA (MUFA)
Source Pr > F LSD
Areas < 0.0001 0.6440
Day of the Year < 0.0001 1.5790
Areas*Days 0.5180 -
Day of the Year


B
2015, main effect (Areas)
% Monounsaturated FA (MUFA)
Elgin
Koue Bokkeveld
Areas


C
2016
Elgin
Koue Bokkeveld
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year < 0.0001 -
Areas*Days 0.0030 1.3120
% Monounsaturated FA (MUFA)
Day of the Year


D
2015, main effect (day of the year)
% Polyunsaturated FA (PUFA)
Source Pr > F LSD
Areas 0.8210 -
Day of the Year < 0.0001 1.5540
Areas*Days 0.1510 -
Day of the Year


E
2016
Elgin
Koue Bokkeveld
Source Pr > F LSD
Areas < 0.0001 -
Day of the Year < 0.0001 -
Areas*Days < 0.0001 1.5670
% Polyunsaturated FA (PUFA)
Day of the Year

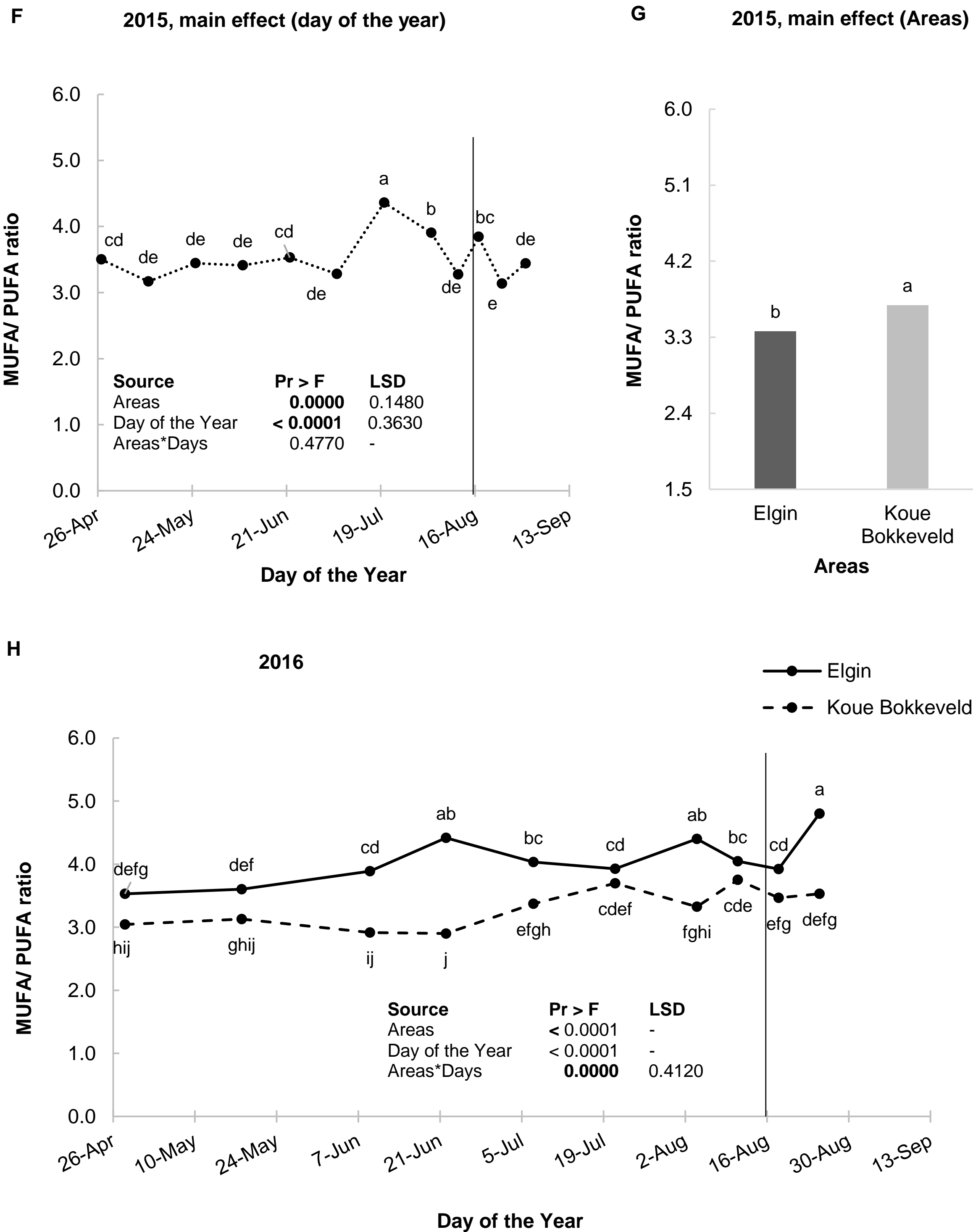
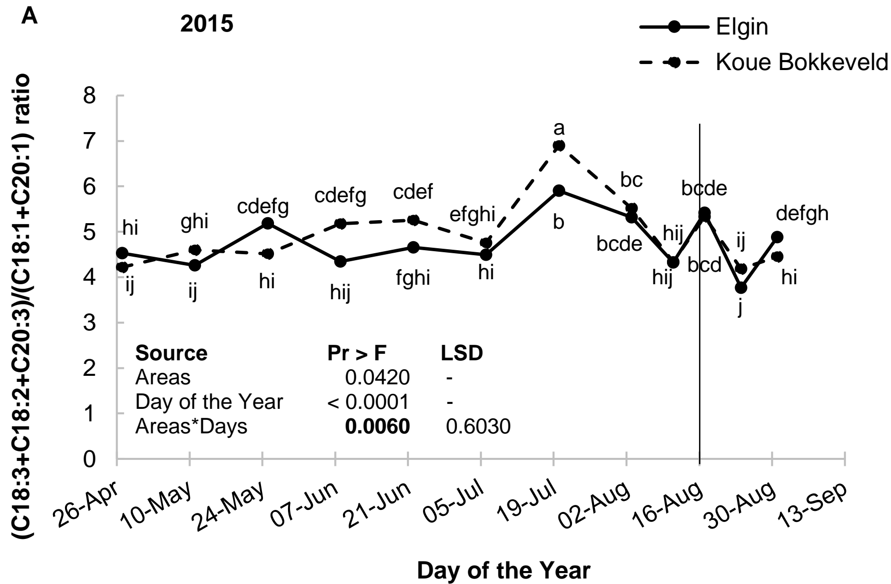
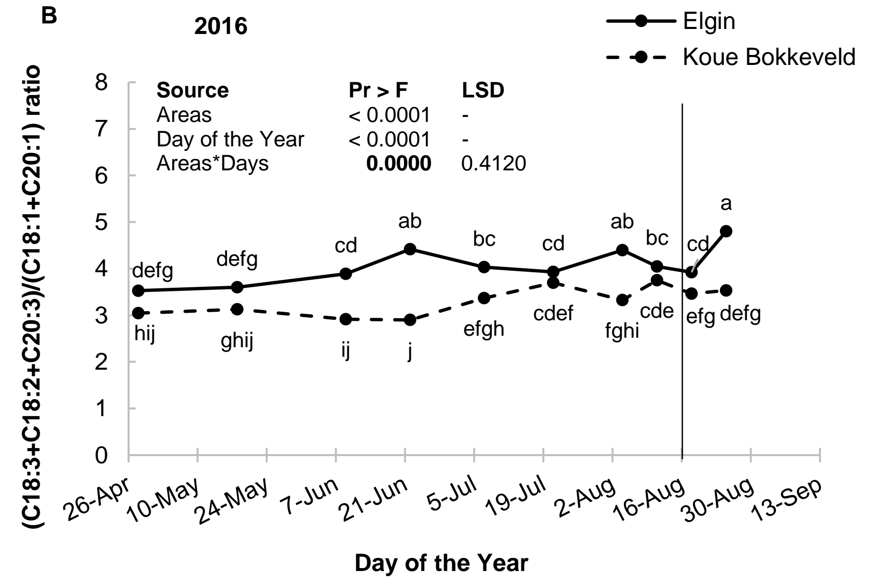
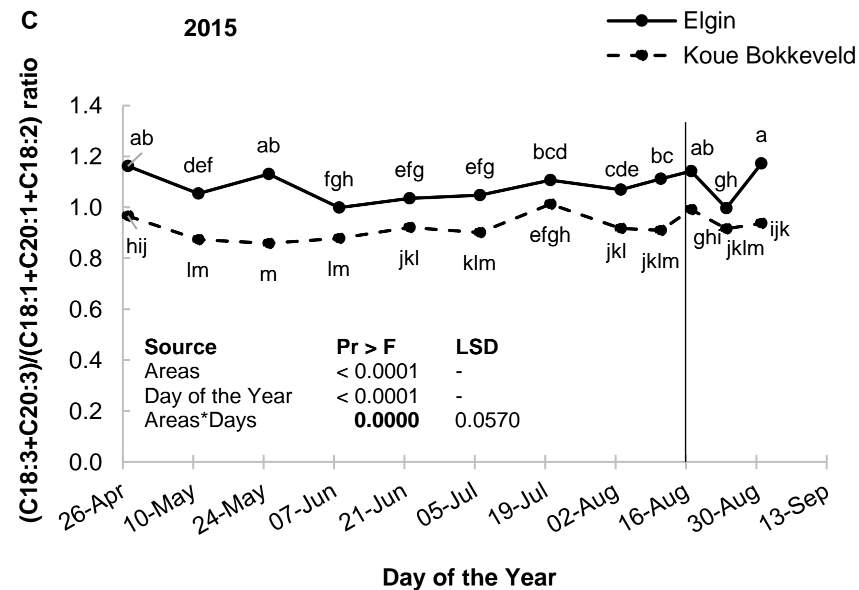
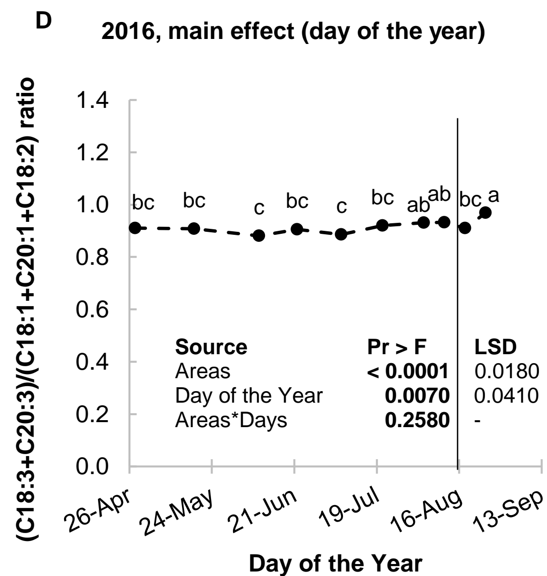


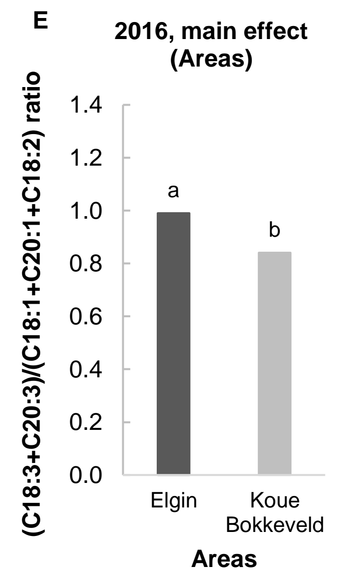
Fig. 6. Percentage monounsaturated fatty acids (MUFAs) (A, B, C), polyunsaturated fatty acids (PUFAs) (D, E) and ratio of MUFA/PUFA (F, G, H) present in the total neutral lipid of dormant buds sampled at Koue Bokkeveld (_ _ _) and Elgin (____) during 2015 and 2016 seasons. The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.


A
2015
Elgin
Koue Bokkeveld
(C18:3+C18:2+C20:3)/(C18:1+C20:1) ratio
Source
Areas
Day of the Year
Areas*Days
Pr > F
0.0420
< 0.0001
0.0060
LSD
-
-
0.6030
Day of the Year


B
2016
Elgin
Koue Bokkeveld
(C18:3+C18:2+C20:3)/(C18:1+C20:1) ratio
Source
Areas
Day of the Year
Areas*Days
Pr > F
< 0.0001
< 0.0001
0.0000
LSD
-
-
0.4120
Day of the Year


C
2015
Elgin
Koue Bokkeveld
(C18:3+C20:3)/(C18:1+C20:1+C18:2) ratio
Source
Areas
Day of the Year
Areas*Days
Pr > F
< 0.0001
< 0.0001
0.0000
LSD
-
-
0.0570
Day of the Year


D
2016, main effect (day of the year)
(C18:3+C20:3)/(C18:1+C20:1+C18:2) ratio
Source
Areas
Day of the Year
Areas*Days
Pr > F
< 0.0001
0.0070
0.2580
LSD
0.0180
0.0410
-
Day of the Year


E
2016, main effect (Areas)
(C18:3+C20:3)/(C18:1+C20:1+C18:2) ratio
Elgin
Koue Bokkeveld
Areas

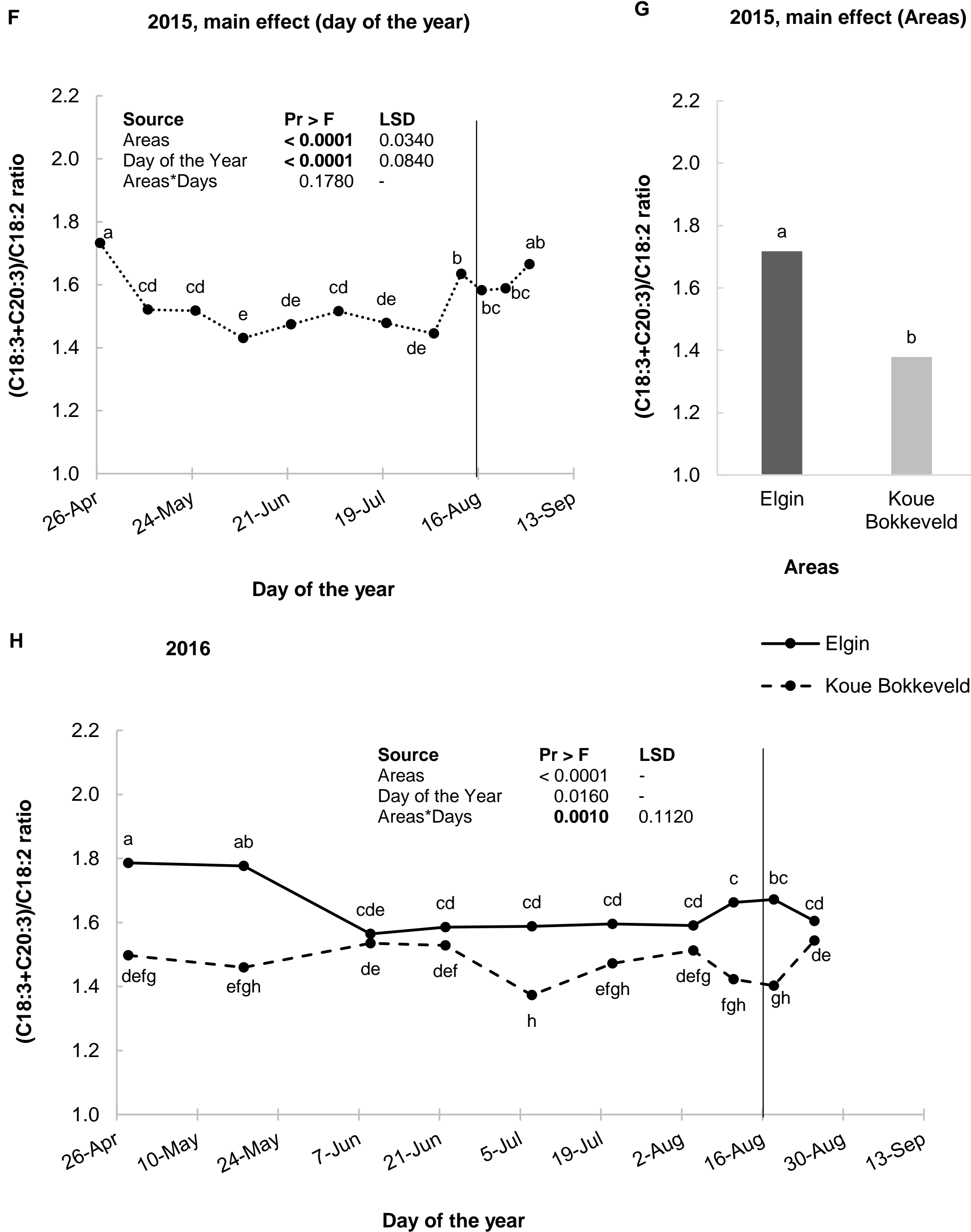


Fig. 7. The unsaturation of neutral lipids (NL) in 'Crips Pink' buds collected in 2015 and 2016 from the Koue Bokkeveld (_ _ _) and Elgin (_____) as given by the (C18:3+C18:2)/ C18:1 ratio (A, B), the C18:3/ (C18:1+C18:2) ratio (C, D, E), and the C18:3/ C18:2 ratio (F, G, H). The vertical line indicates the end of the dormant phase and the start of the growth resumption phase. Letters show significant differences between the means ($p < 0.05$) and if the interaction was not significant, the significant main effects are presented.

3. Fatty acids composition of the neutral lipid fraction in lab-chilled buds

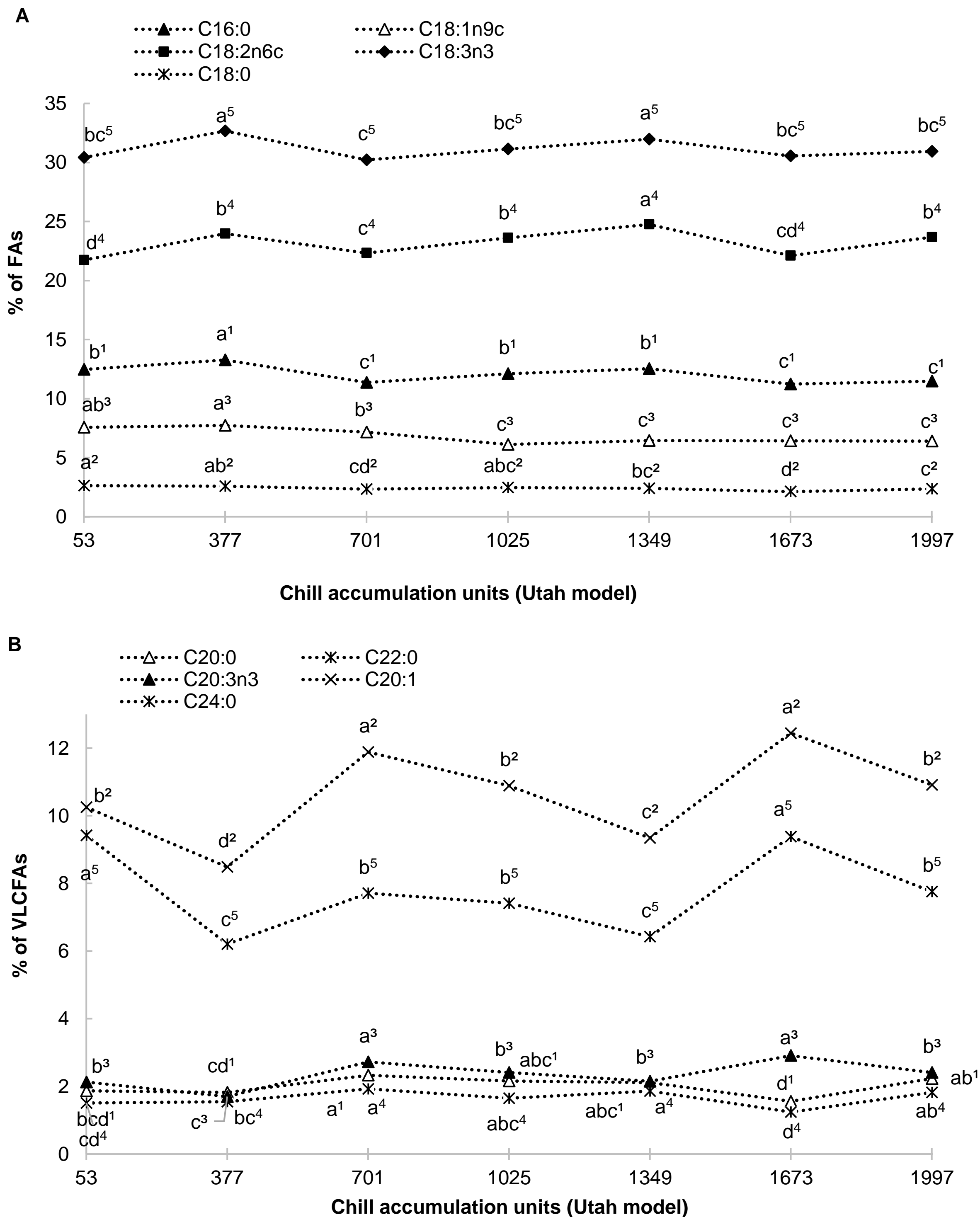
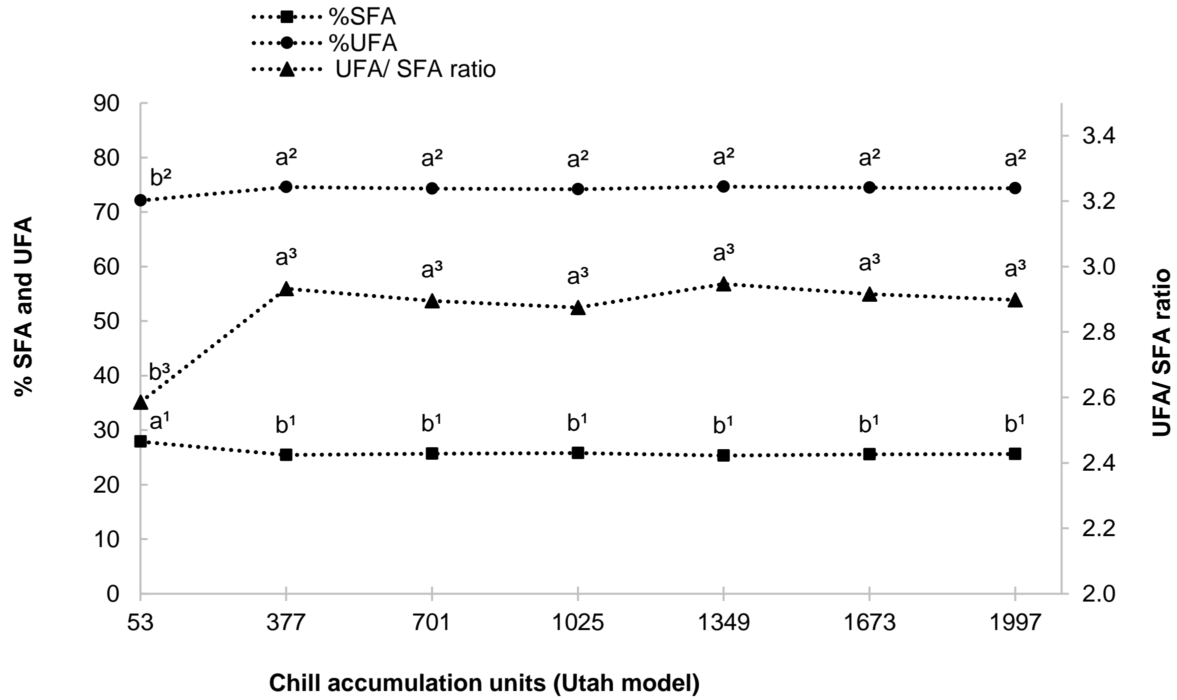
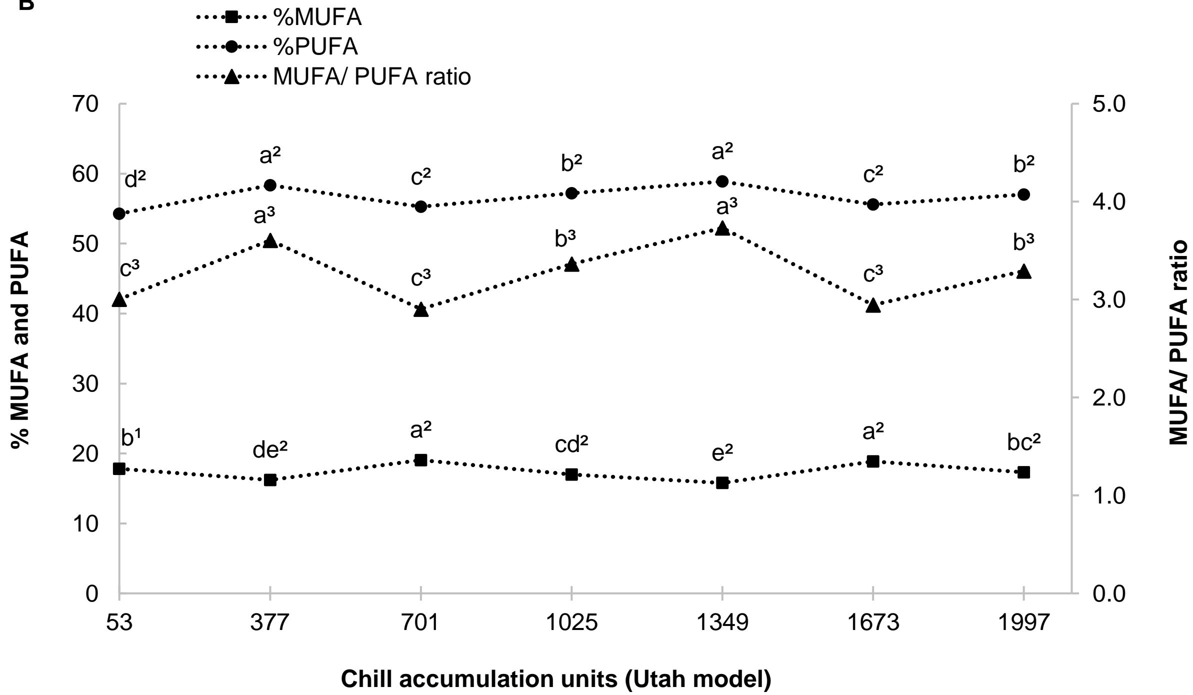


Fig. 8: Percentage of palmitic acid (C16:0) ([1]), stearic acid (C18:0) ([2]), oleic acid (C18:1n9c) ([3]), linoleic acid (C18:2n6c) ([4]) and linolenic acid (C18:3n3) ([5]) (A) and percentage of VLCFAs arachic acid (C20:0) ([1]), heneicosanoic acid (C20:1) ([2]), eicosatrienoic acid (C20:3n3) ([3]), behenic acid (C22:0) ([4]), lignoceric acid (C24:0) ([5]) (B) of 'Cripps Pink' dormant buds that received progressively more chill units (Utah model). Letters indicate statistical differences between the means ($p = 0.05$). Captions ([1, 2, 3, 4, 5]) indicate individual parameter evaluated alongside chilling accumulation.

A



B



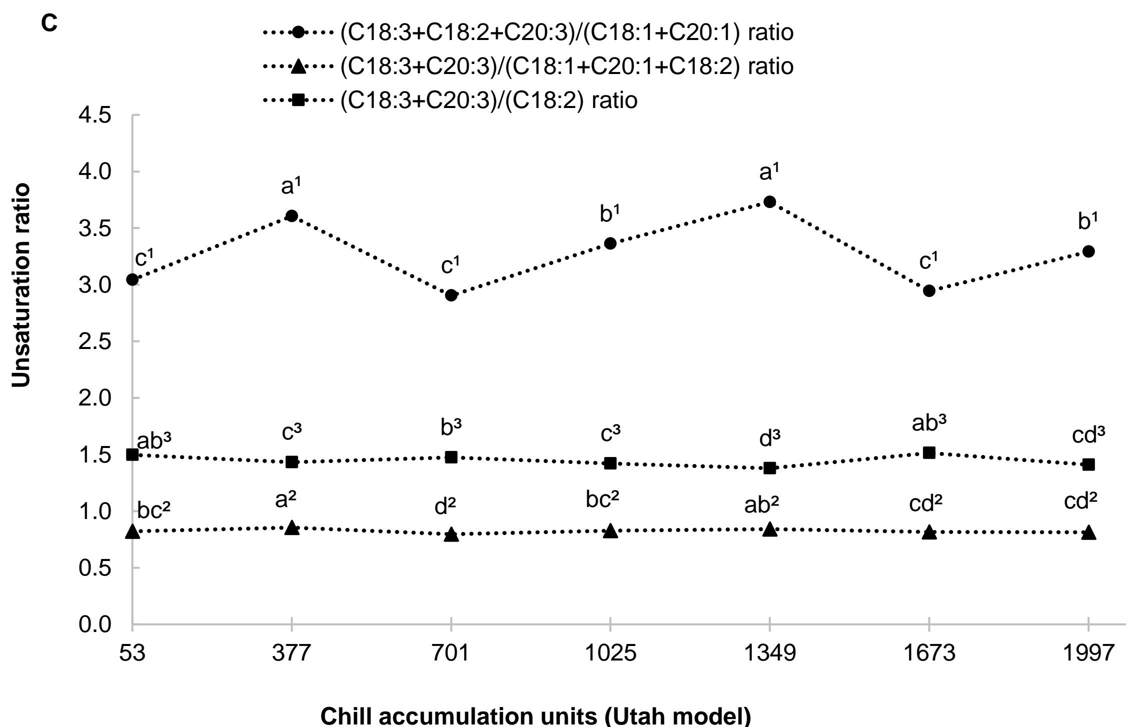


Fig. 9: Percentage of saturated fatty acids (SFAs), unsaturated fatty acids (UFAs) and ratio UFAs/ SFAs (A), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) and ratio of MUFAs/PUFAs (B) ratios ((C18:3+C18:2+C20:3)/ (C18:1+C20:1)), ((C18:3+C20:3)/ (C18:2)) and ((C18:3+C20:3)/ (C18:1+C20:1+C18:2)) (C) in total neutral lipids of 'Cripps Pink' dormant buds that received progressively more chill units. Letters indicate statistical differences between the means (p = 0.05). Captions ([1, 2, 3]) indicate individual parameter evaluated alongside chilling accumulation.

APPENDIX B

This appendix contains the results from the analysis (tables) of polar and neutral lipids seasons (2015 and 2016) refered to in Paper 4.

1. Fatty acids composition of the polar lipid fraction

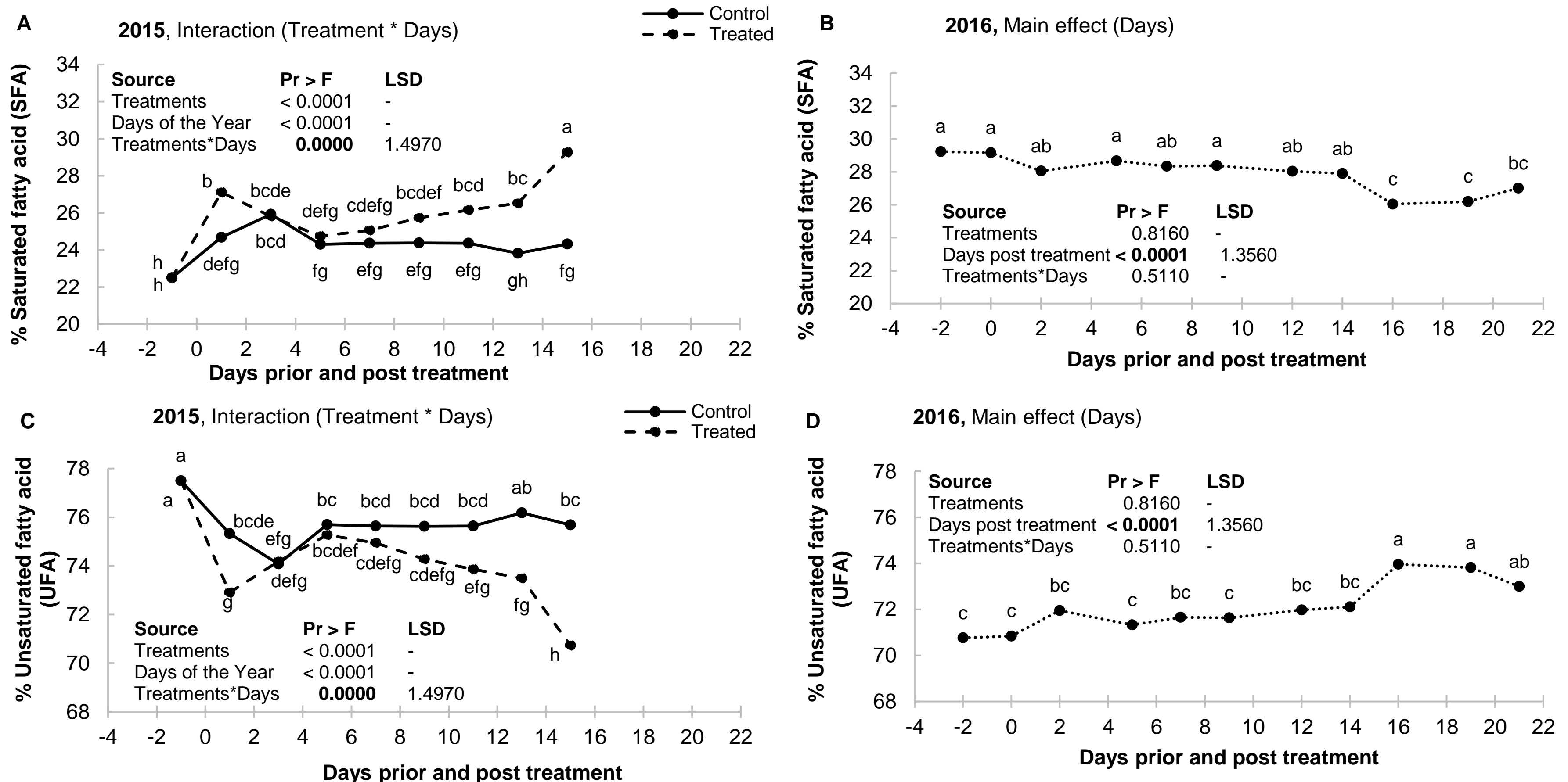


Figure 1: Percentage of saturated fatty acid (SFA) (A, B) and unsaturated fatty acid (UFA) (C, D) extracted from treated and control buds during 2015 and 2016 season. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$.

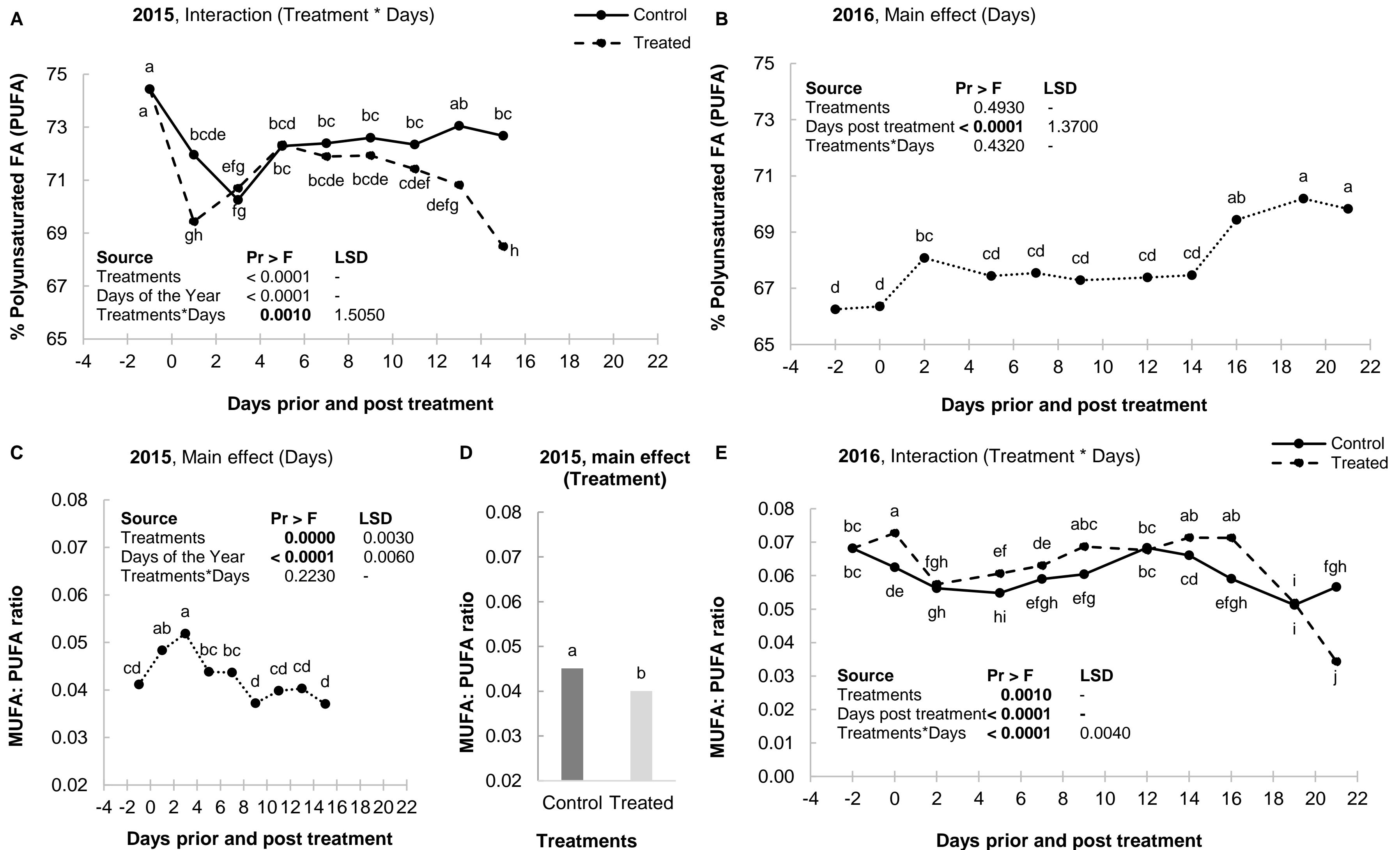


Figure 2: Percentage of polyunsaturated FA (PUFA) (A, B), as well as the MUFA: PUFA ratio (C, D, E) present in polar lipids (PLs) fractions extracted from treated and control buds during 2015 and 2016 season. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$ and in cases where the interaction between the main effects (Treatments*Days) was not significant, the significant main effects are presented.

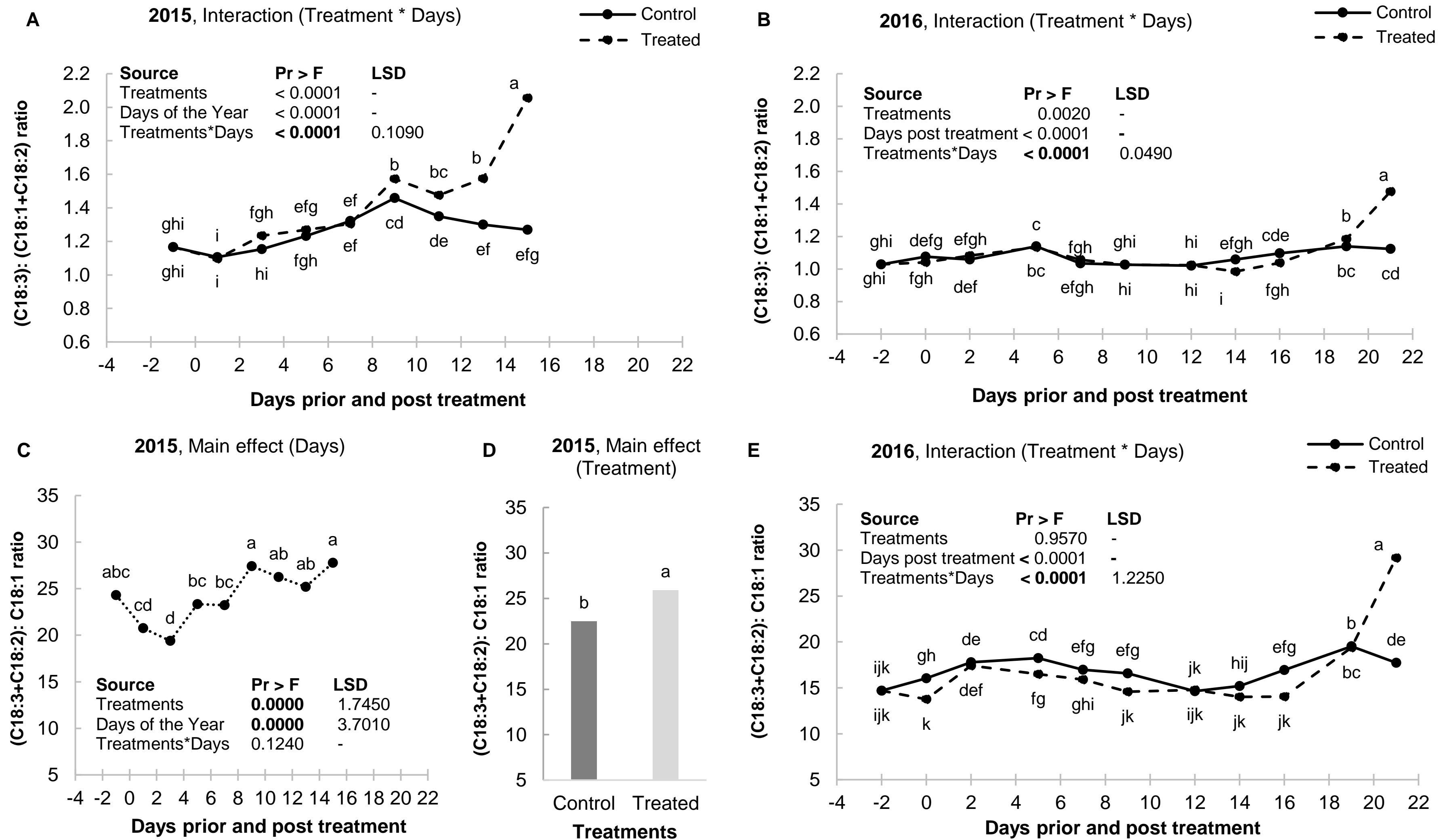


Figure 3: The unsaturation of polar lipids (PL) in treated and control buds for 2015 and 2016 as given by the (C18:3+C18:2): C18:1 ratio (A, B) and the C18:3: (C18:1+C18:2) ratio (C, D, E). Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$, in cases where the interaction between the main effects (Treatments*Days) was non-significant ($p > 0.05$), the significant main effects are presented.

2. Total free sterols and phospholipids in HCo treated and control buds

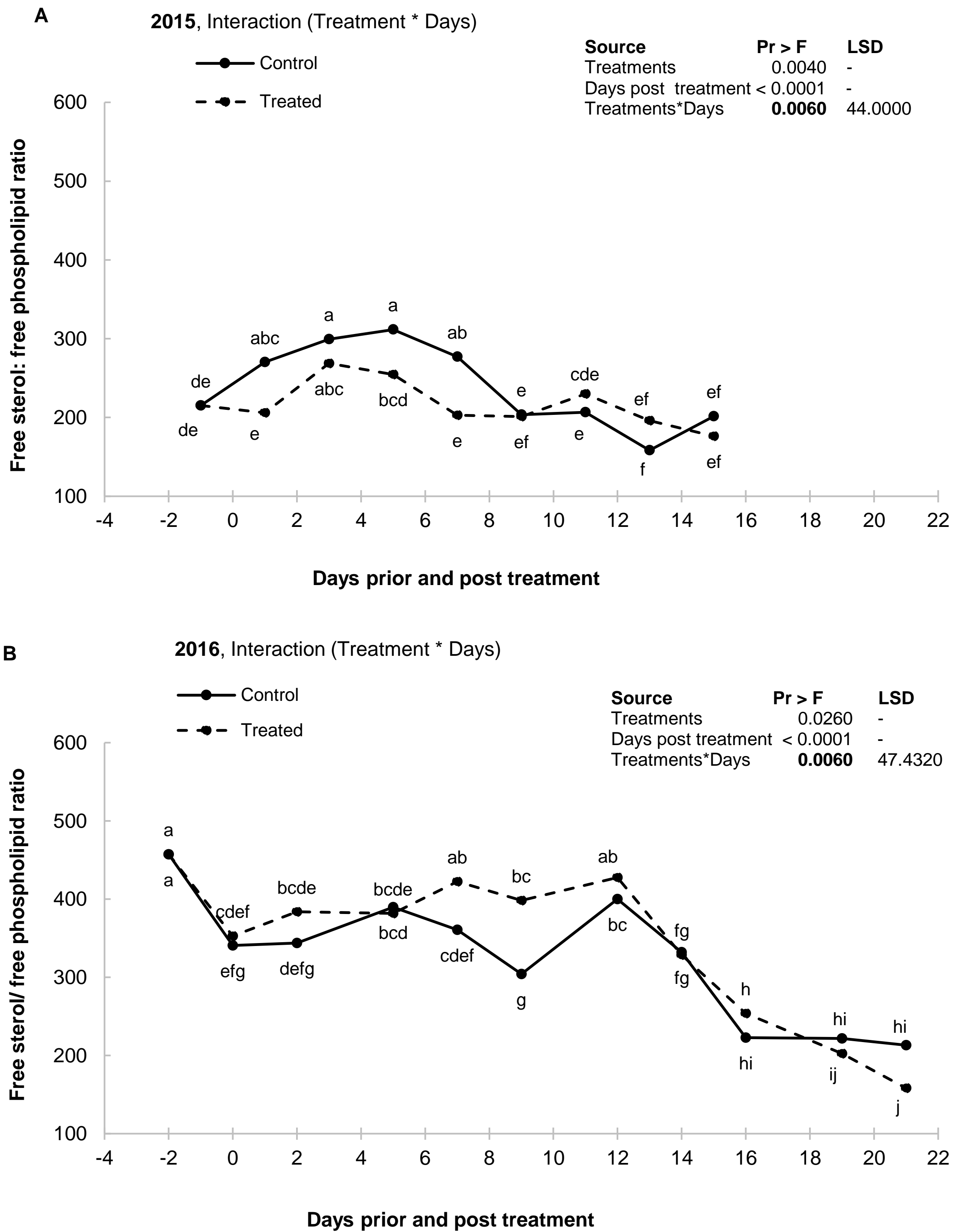
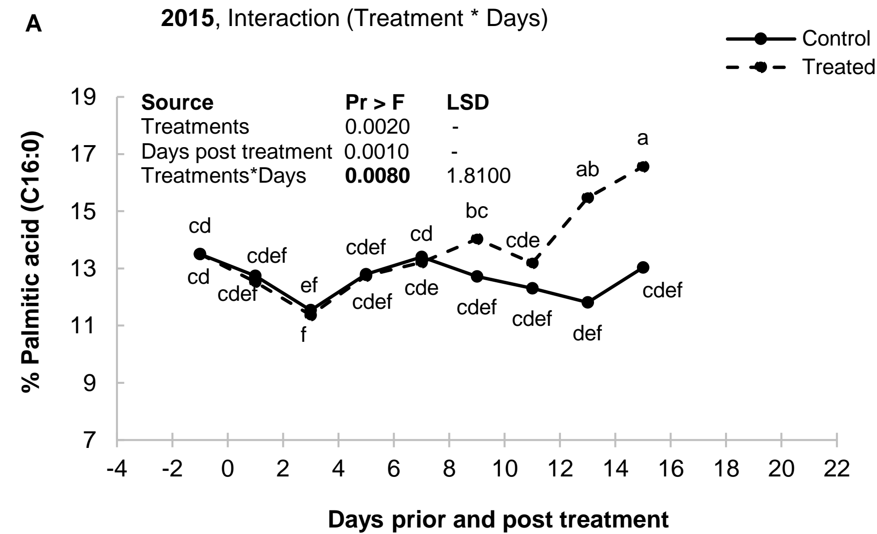
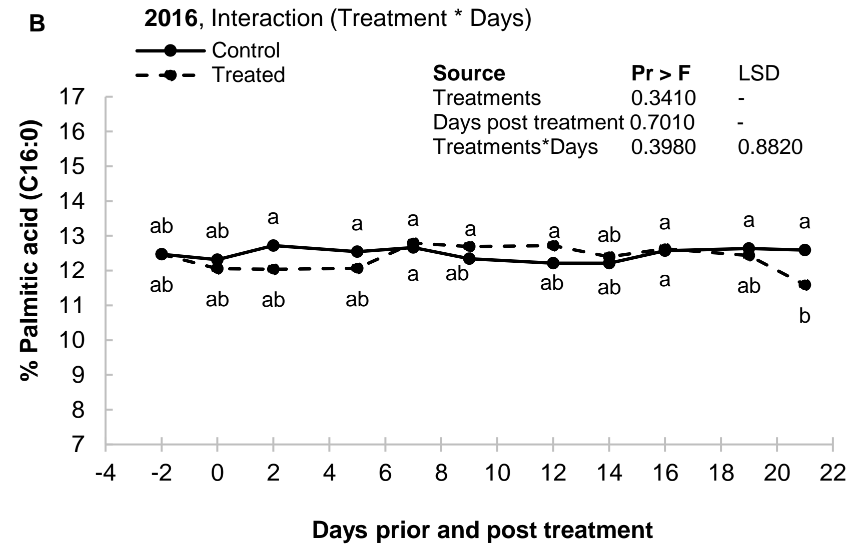
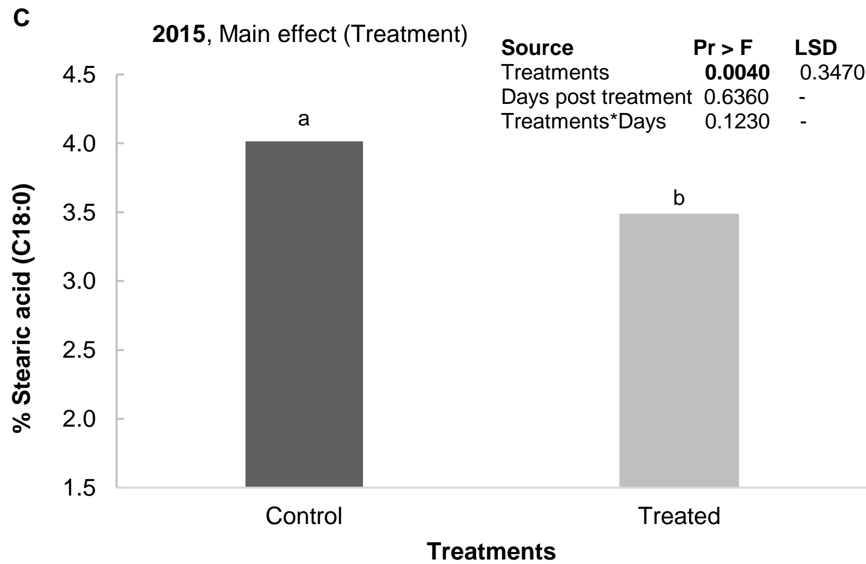


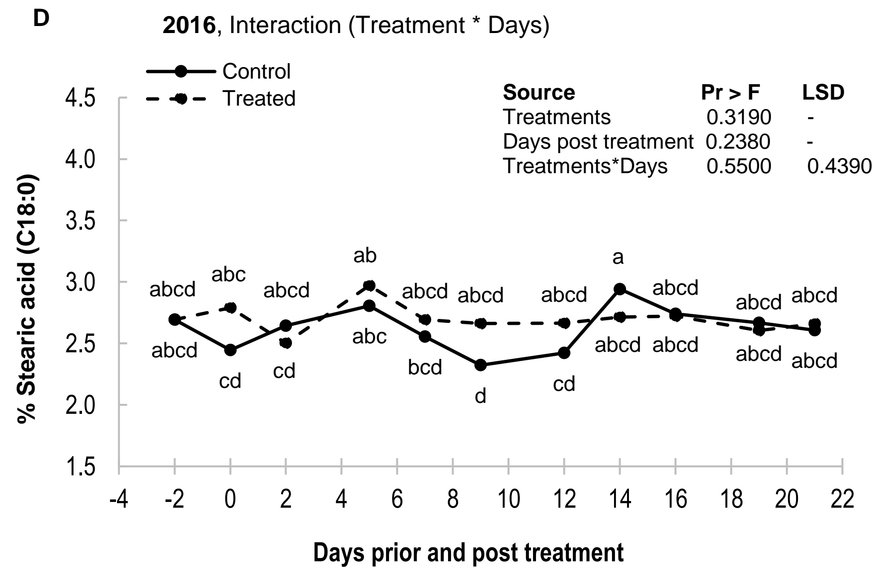
Figure 4: Free sterol: phospholipid ratio (A, B) in treated and control buds during 2015 and 2016. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$.

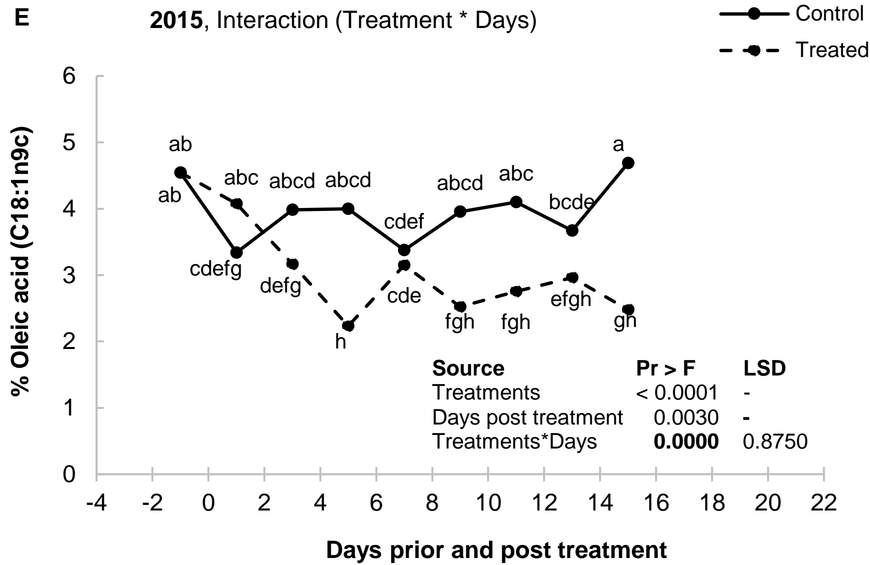
3. Fatty acids composition of the neutral lipids fraction

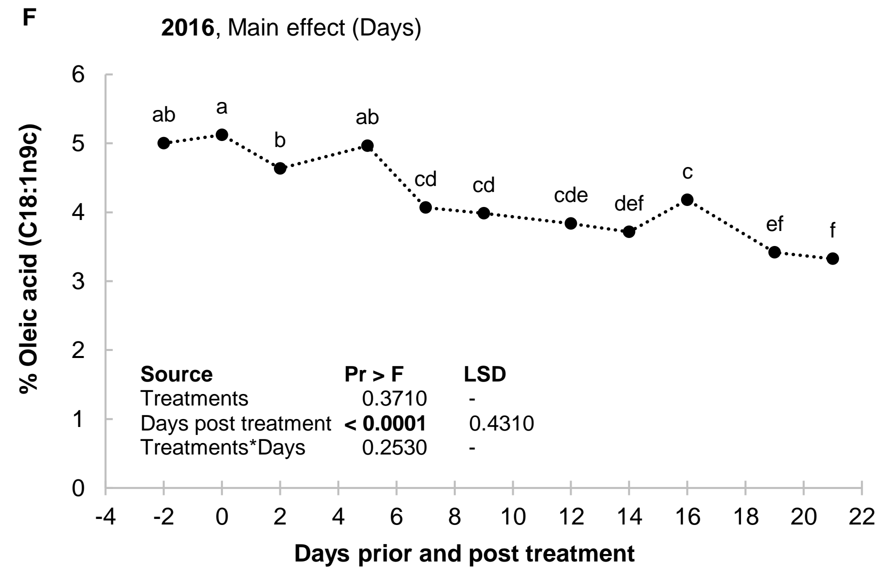


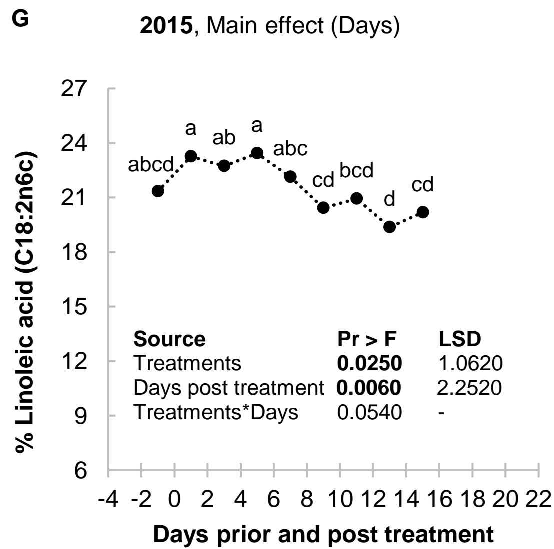


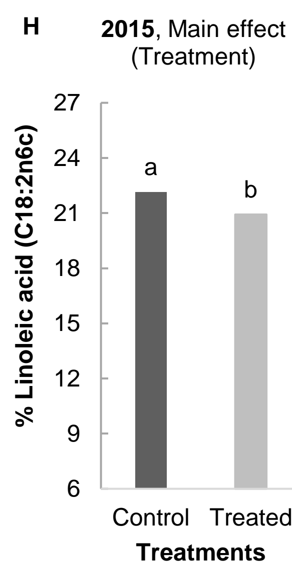


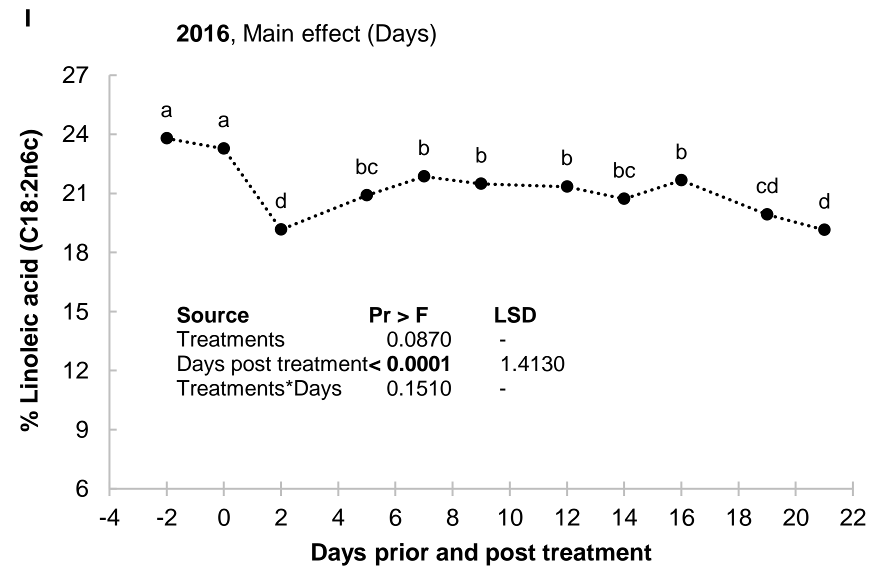


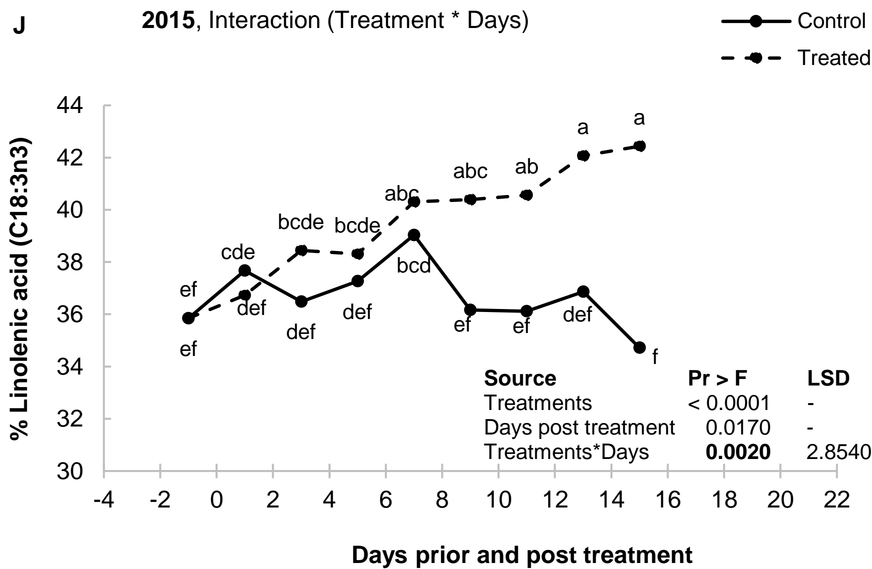

E
2015, Interaction (Treatment * Days)
Control
Treated
% Oleic acid (C18:1n9c)
Days prior and post treatment
Source Pr > F LSD
Treatments < 0.0001 -
Days post treatment 0.0030 -
Treatments*Days 0.0000 0.8750

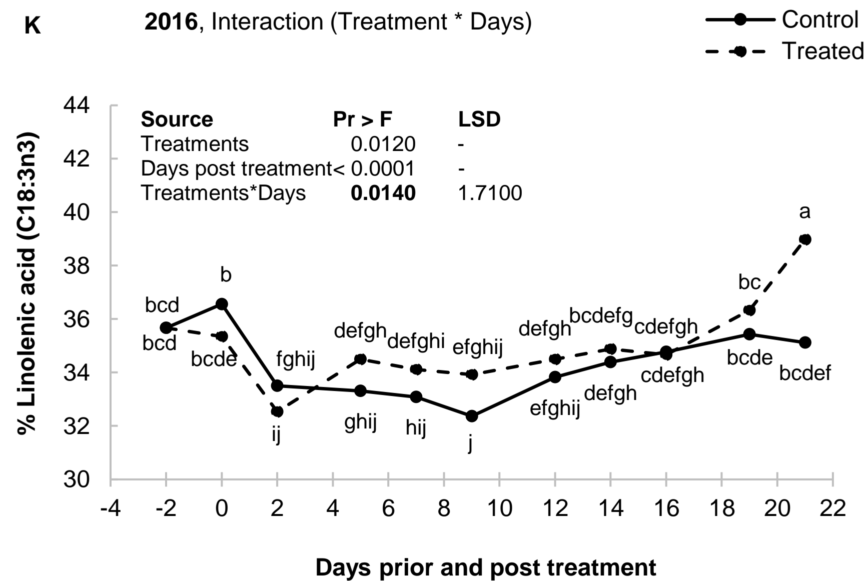

F
2016, Main effect (Days)
% Oleic acid (C18:1n9c)
Days prior and post treatment
Source Pr > F LSD
Treatments 0.3710 -
Days post treatment < 0.0001 0.4310
Treatments*Days 0.2530 -

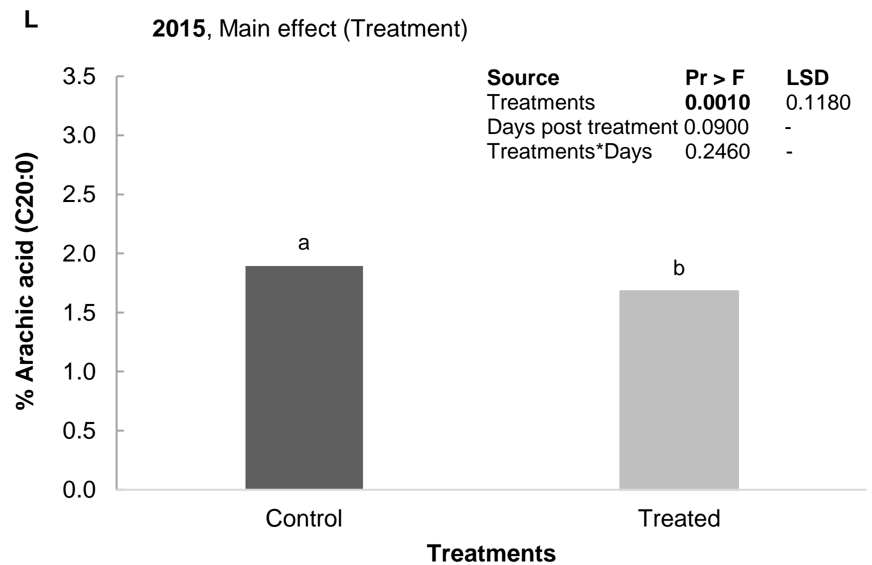

G
2015, Main effect (Days)
% Linoleic acid (C18:2n6c)
Days prior and post treatment
Source Pr > F LSD
Treatments 0.0250 1.0620
Days post treatment 0.0060 2.2520
Treatments*Days 0.0540 -

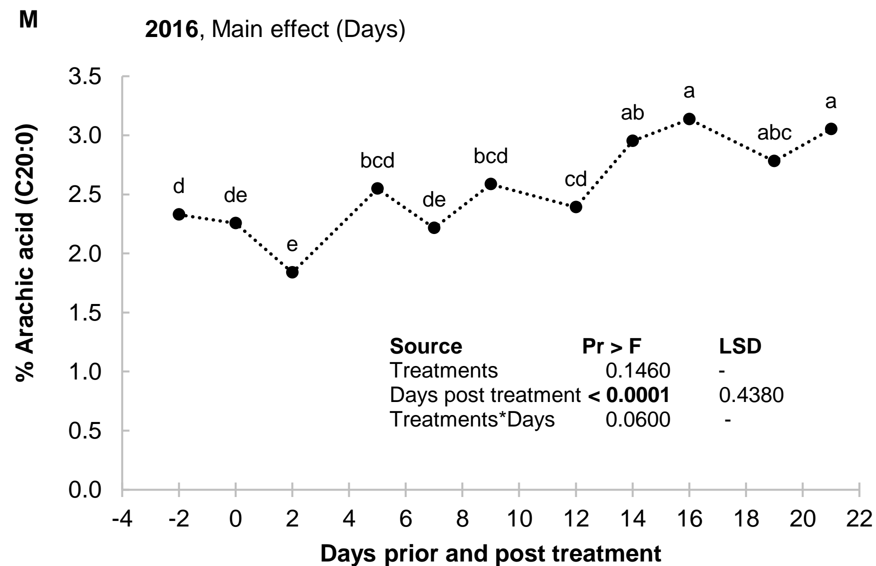

H
2015, Main effect (Treatment)
% Linoleic acid (C18:2n6c)
Control
Treated
Treatments

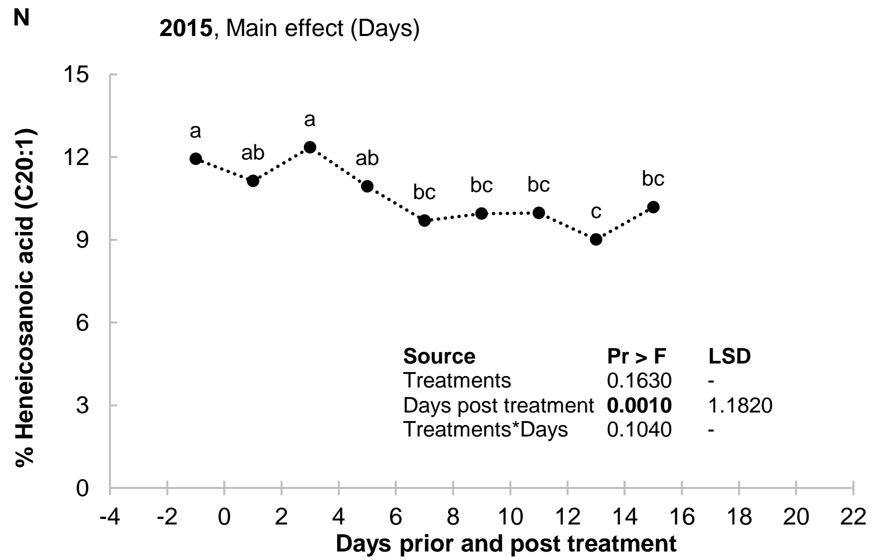

I
2016, Main effect (Days)
% Linoleic acid (C18:2n6c)
Days prior and post treatment
Source Pr > F LSD
Treatments 0.0870 -
Days post treatment < 0.0001 1.4130
Treatments*Days 0.1510 -

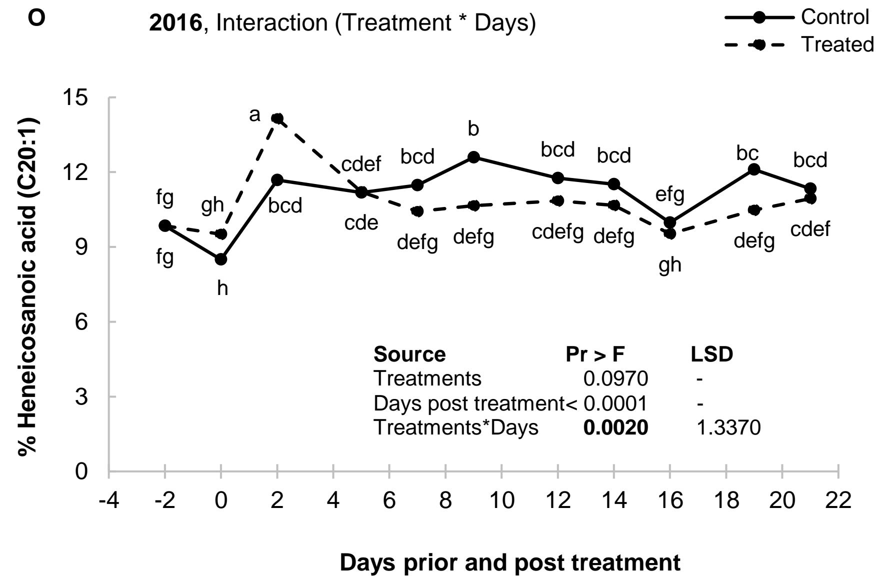

J
2015, Interaction (Treatment * Days)
Control
Treated
% Linolenic acid (C18:3n3)
Days prior and post treatment
Source Pr > F LSD
Treatments < 0.0001 -
Days post treatment 0.0170 -
Treatments*Days 0.0020 2.8540

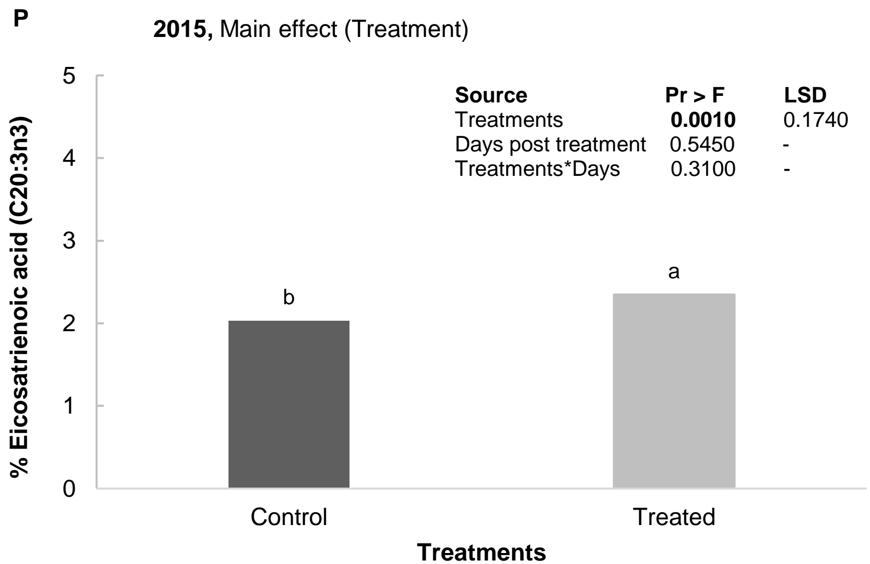

K
2016, Interaction (Treatment * Days)
Control
Treated
% Linolenic acid (C18:3n3)
Days prior and post treatment
Source Pr > F LSD
Treatments 0.0120 -
Days post treatment < 0.0001 -
Treatments*Days 0.0140 1.7100

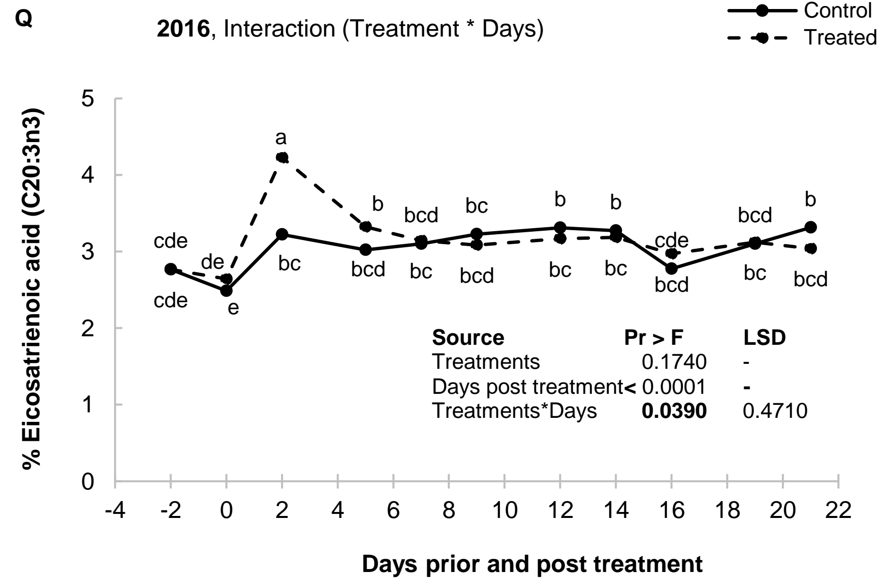

L
2015, Main effect (Treatment)
% Arachic acid (C20:0)
Control
Treated
Treatments
Source Pr > F LSD
Treatments 0.0010 0.1180
Days post treatment 0.0900 -
Treatments*Days 0.2460 -

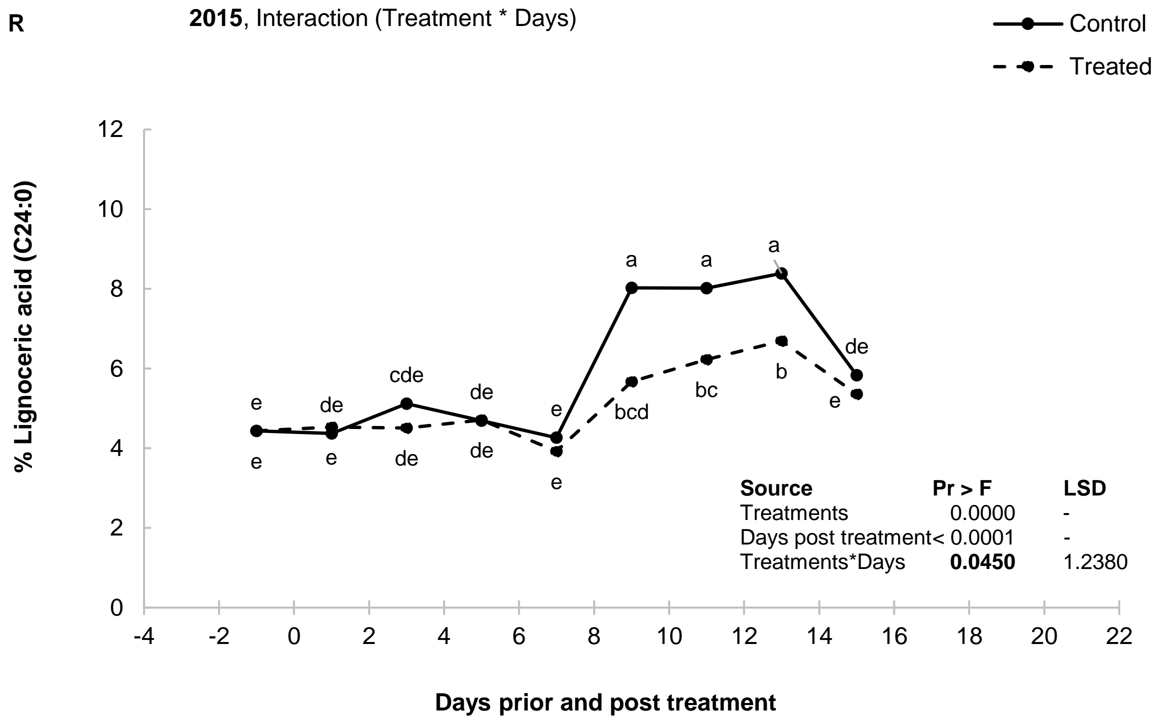

M
2016, Main effect (Days)
% Arachic acid (C20:0)
Days prior and post treatment
Source Pr > F LSD
Treatments 0.1460 -
Days post treatment < 0.0001 0.4380
Treatments*Days 0.0600 -


N
2015, Main effect (Days)
% Heneicosanoic acid (C20:1)
Days prior and post treatment
a
ab
a
ab
bc
bc
bc
c
bc
Source Pr > F LSD
Treatments 0.1630 -
Days post treatment 0.0010 1.1820
Treatments*Days 0.1040 -


O
2016, Interaction (Treatment * Days)
Control
Treated
% Heneicosanoic acid (C20:1)
Days prior and post treatment
Source Pr > F LSD
Treatments 0.0970 -
Days post treatment< 0.0001 -
Treatments*Days 0.0020 1.3370


P
2015, Main effect (Treatment)
% Eicosatrienoic acid (C20:3n3)
Source Pr > F LSD
Treatments 0.0010 0.1740
Days post treatment 0.5450 -
Treatments*Days 0.3100 -
b
a
Control
Treated
Treatments


Q
2016, Interaction (Treatment * Days)
Control
Treated
% Eicosatrienoic acid (C20:3n3)
Days prior and post treatment
Source Pr > F LSD
Treatments 0.1740 -
Days post treatment< 0.0001 -
Treatments*Days 0.0390 0.4710



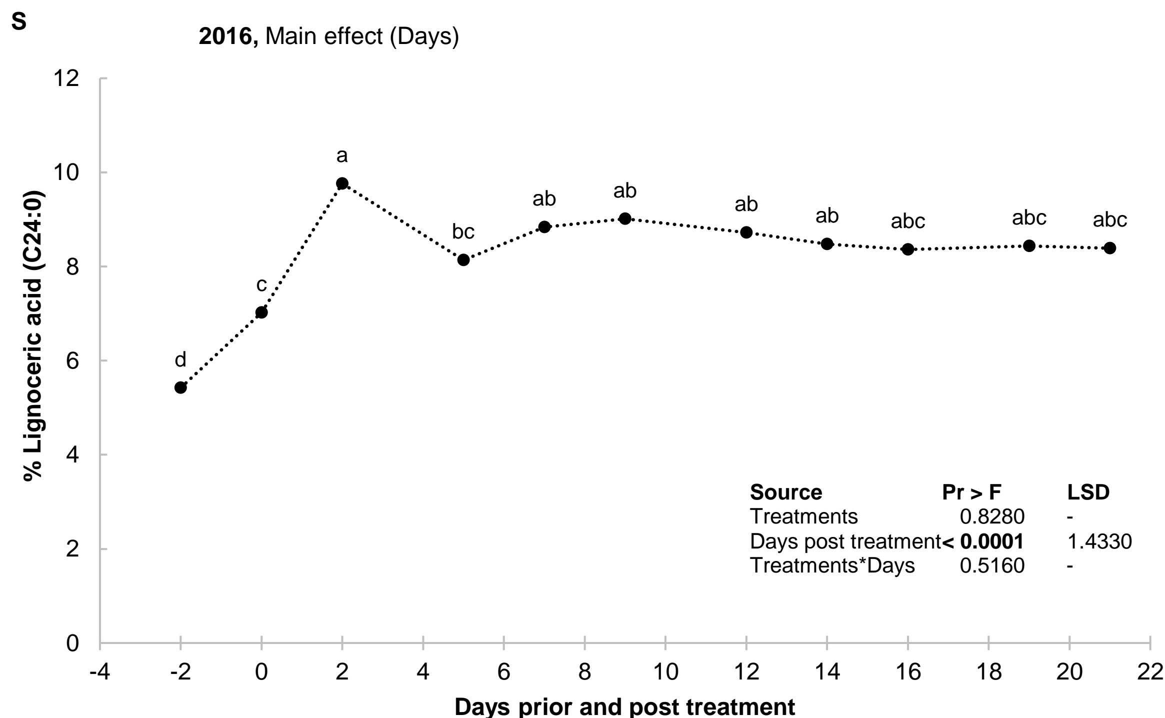
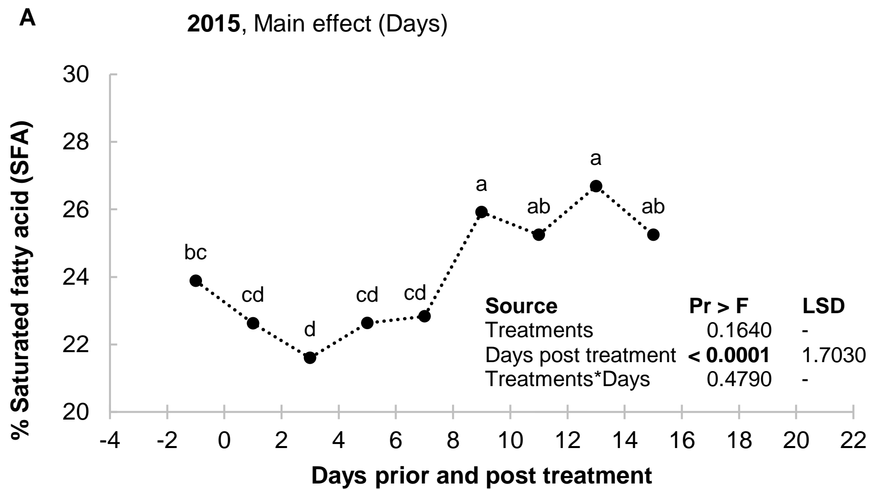
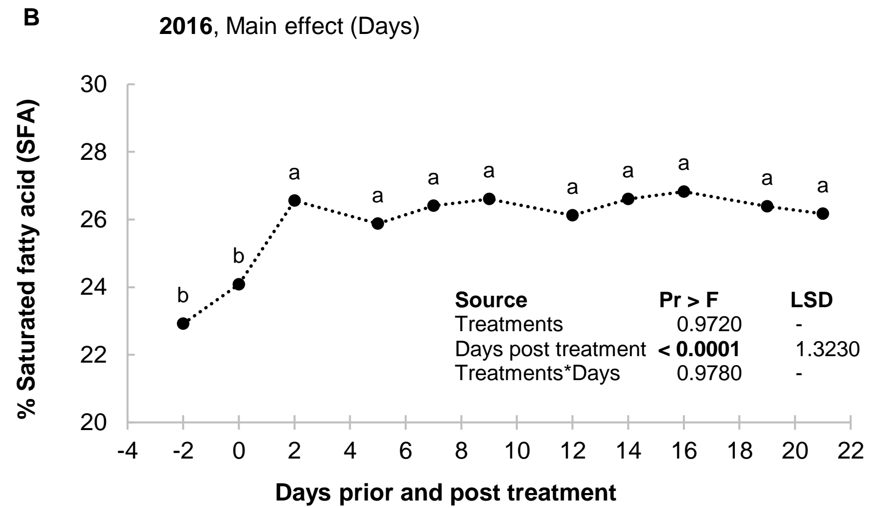
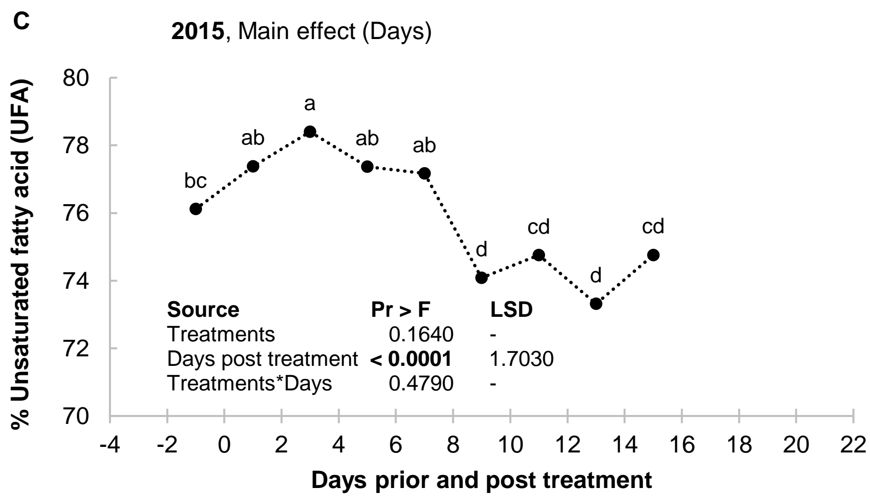
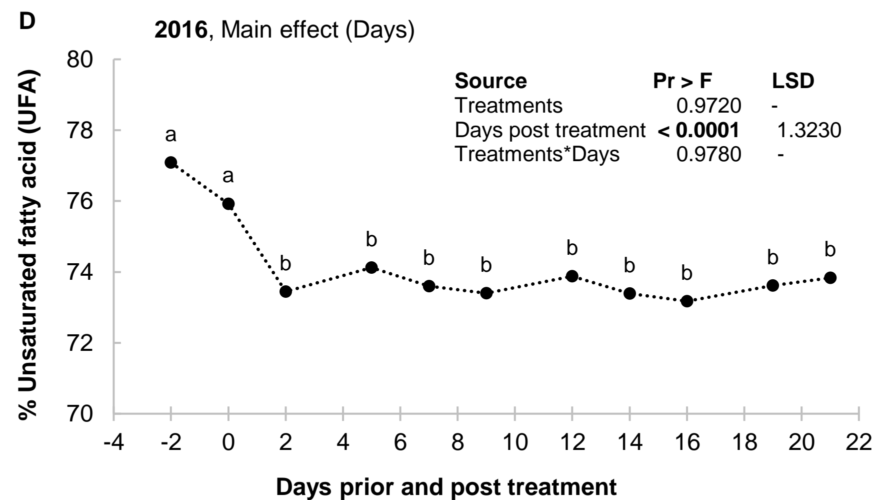


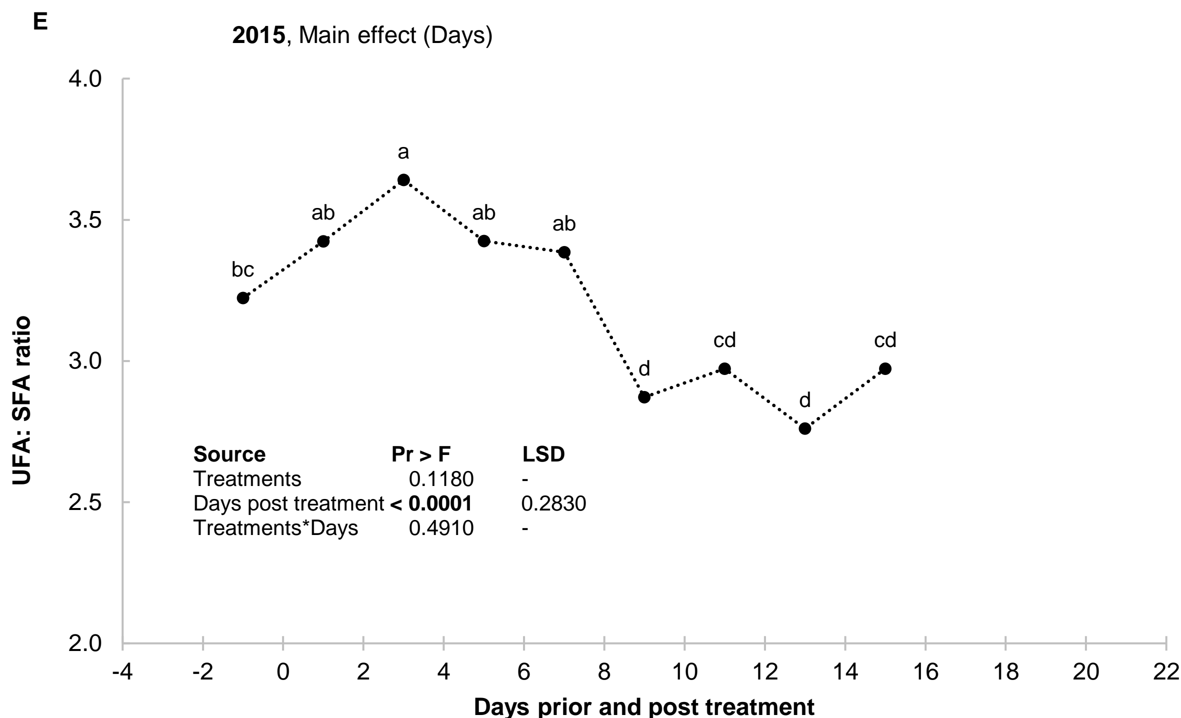
Figure 5: Percentage of palmitic acid (C16:0) (A, B), stearic acid (C18:0) (C, D), oleic acid (C18:1n9c) (E, F), linoleic acid (C18:2n6c) (G, H, I), linolenic acid (C18:3n3) (J, K), arachic acid (C20:0) (L, M), heneicosanoic acid (C20:1) (N, O), eicosatrienoic acid (C20:3n3) (P, Q), and lignoceric acid (C24:0) (R, S) present in the total neutral lipid (NL) fractions extracted from treated and control buds during 2015 and 2016 season. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$, in cases where the interaction between the main effects (Treatments*Days) was non-significant ($p > 0.05$), the significant main effects are presented.


A
2015, Main effect (Days)
% Saturated fatty acid (SFA)
Days prior and post treatment
bc
cd
d
cd
cd
a
ab
a
ab
Source Pr > F LSD
Treatments 0.1640 -
Days post treatment < 0.0001 1.7030
Treatments*Days 0.4790 -


B
2016, Main effect (Days)
% Saturated fatty acid (SFA)
Days prior and post treatment
b
b
a
a
a
a
a
a
a
a
a
Source Pr > F LSD
Treatments 0.9720 -
Days post treatment < 0.0001 1.3230
Treatments*Days 0.9780 -


C
2015, Main effect (Days)
% Unsaturated fatty acid (UFA)
Days prior and post treatment
bc
ab
a
ab
ab
d
cd
d
cd
Source Pr > F LSD
Treatments 0.1640 -
Days post treatment < 0.0001 1.7030
Treatments*Days 0.4790 -


D
2016, Main effect (Days)
% Unsaturated fatty acid (UFA)
Days prior and post treatment
a
a
b
b
b
b
b
b
b
b
b
Source Pr > F LSD
Treatments 0.9720 -
Days post treatment < 0.0001 1.3230
Treatments*Days 0.9780 -



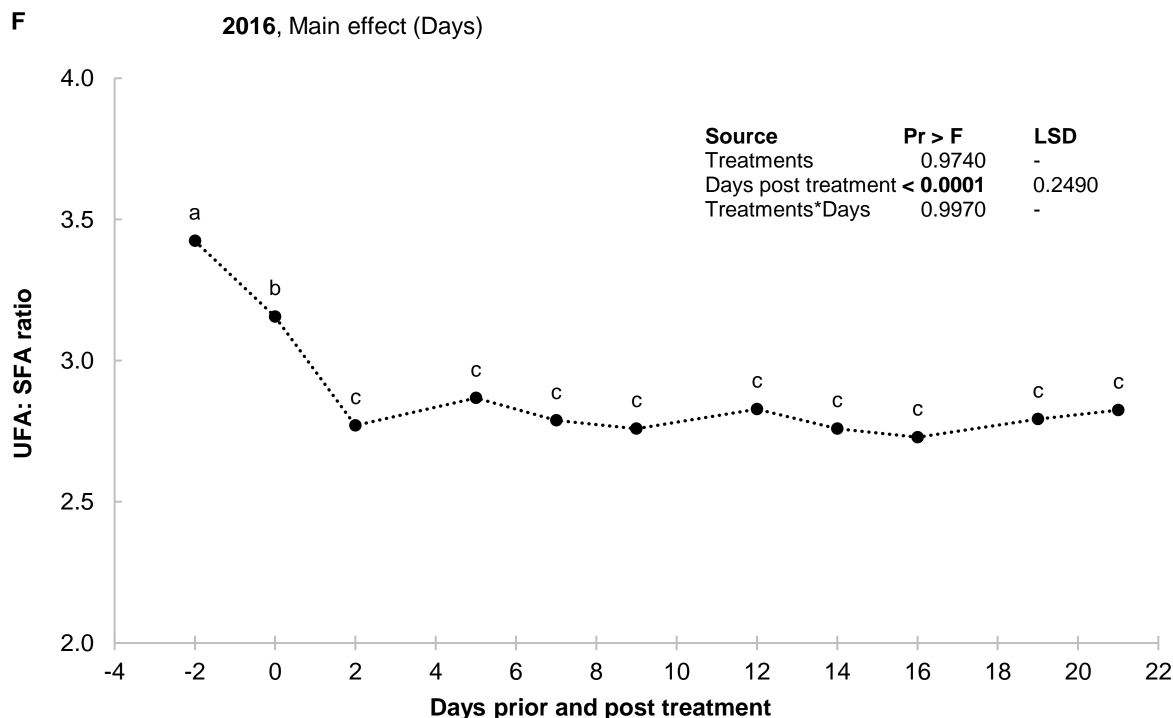
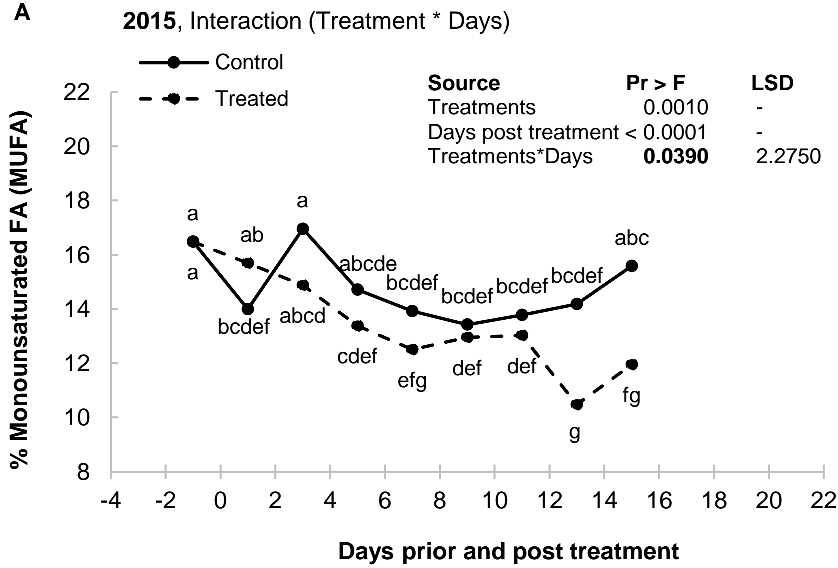
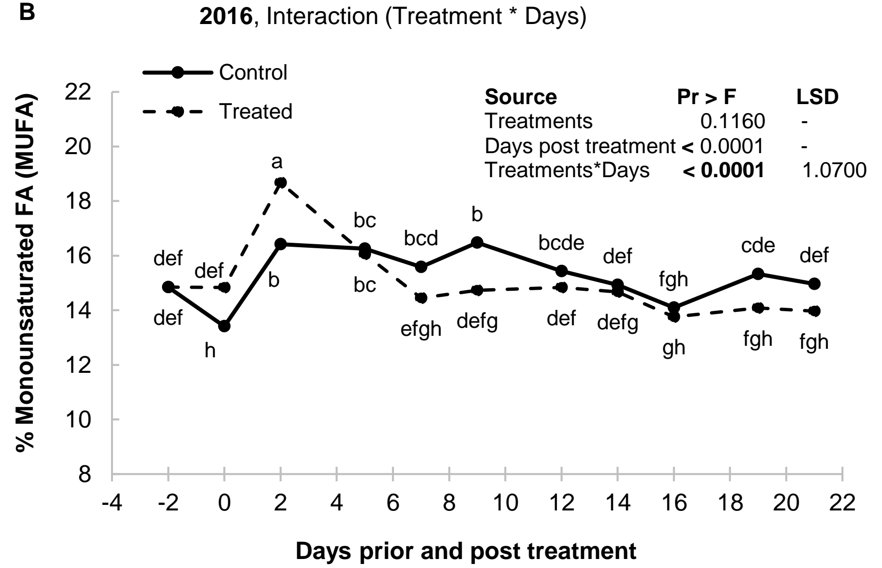
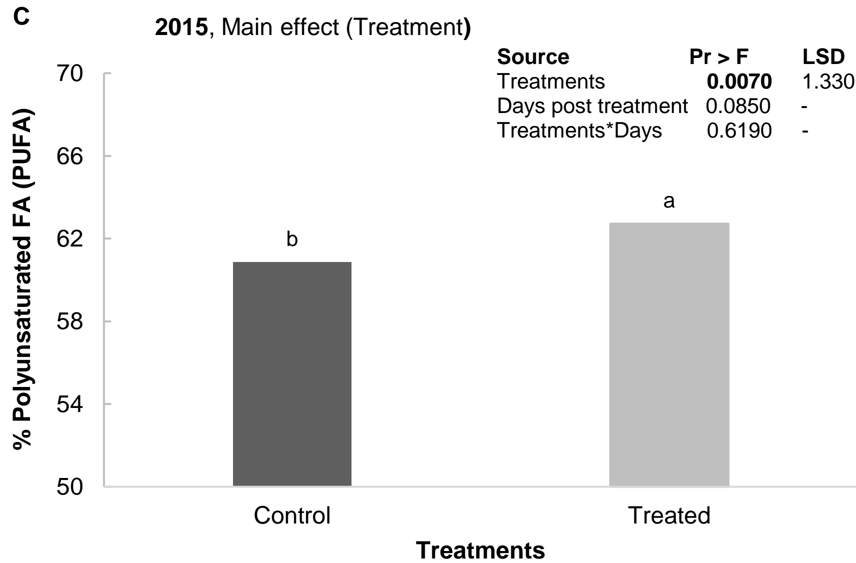
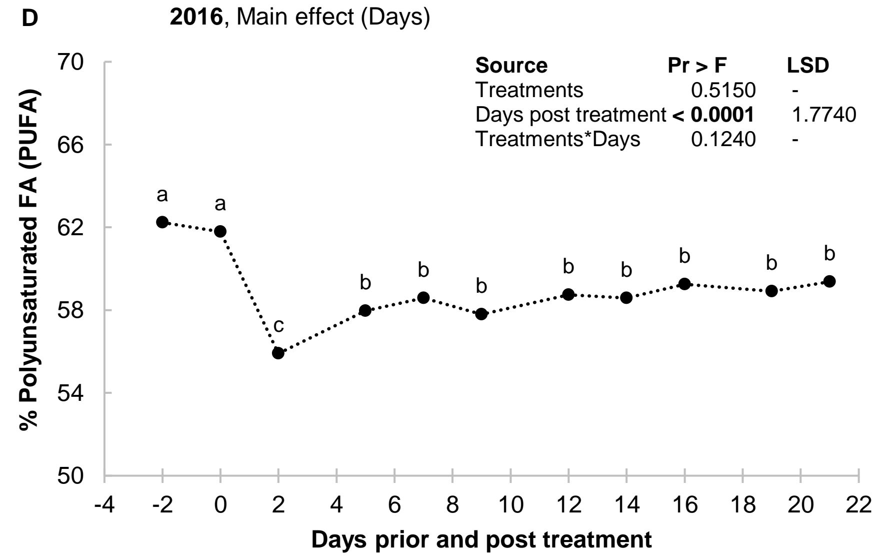


Figure 6: Percentage of saturated fatty acid (SFA) (A, B), unsaturated fatty acid (UFA) (C, D) and ratio of UFA/ SFA (E, F) in the total neutral lipid (NL) fraction of treated and control buds during 2015 and 2016 seasons. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$, in cases where the interaction between the main effects (Treatments*Days) was non-significant ($p > 0.05$), the significant main effects are presented.


A
2015, Interaction (Treatment * Days)
Control
Treated
Source Pr > F LSD
Treatments 0.0010 -
Days post treatment < 0.0001 -
Treatments*Days 0.0390 2.2750
% Monounsaturated FA (MUFA)
Days prior and post treatment


B
2016, Interaction (Treatment * Days)
Control
Treated
Source Pr > F LSD
Treatments 0.1160 -
Days post treatment < 0.0001 -
Treatments*Days < 0.0001 1.0700
% Monounsaturated FA (MUFA)
Days prior and post treatment


C
2015, Main effect (Treatment)
Source Pr > F LSD
Treatments 0.0070 1.330
Days post treatment 0.0850 -
Treatments*Days 0.6190 -
% Polyunsaturated FA (PUFA)
Control
Treated
Treatments


D
2016, Main effect (Days)
Source Pr > F LSD
Treatments 0.5150 -
Days post treatment < 0.0001 1.7740
Treatments*Days 0.1240 -
% Polyunsaturated FA (PUFA)
Days prior and post treatment

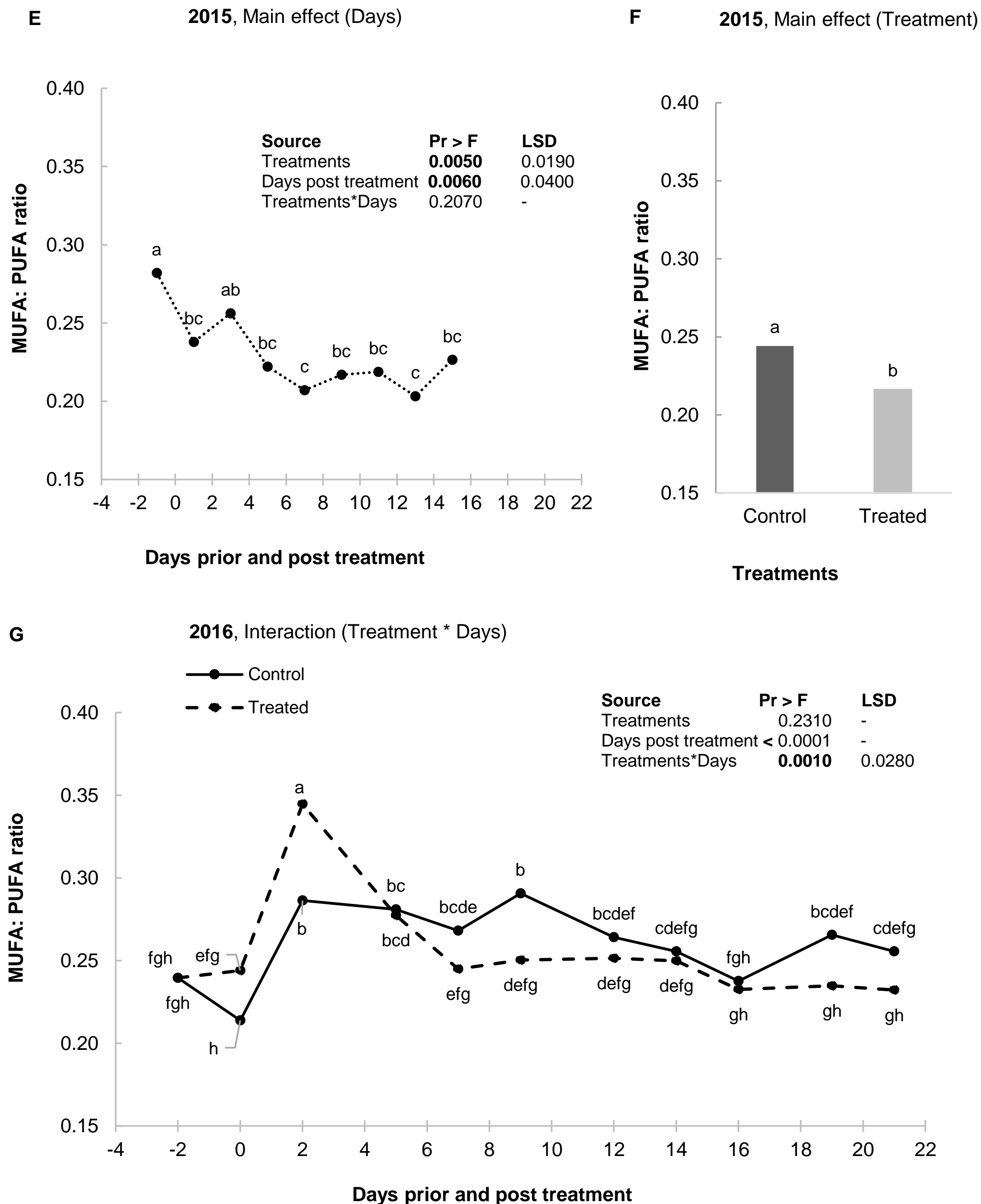
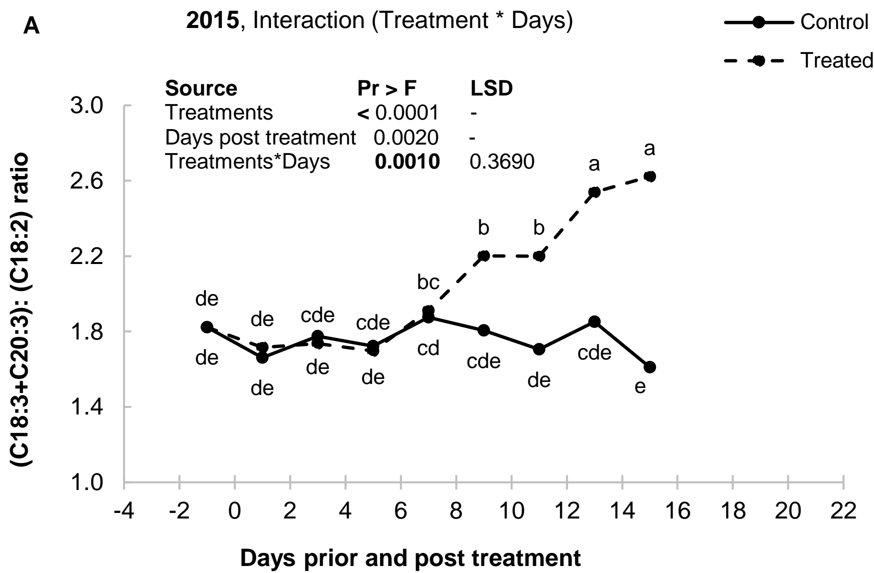
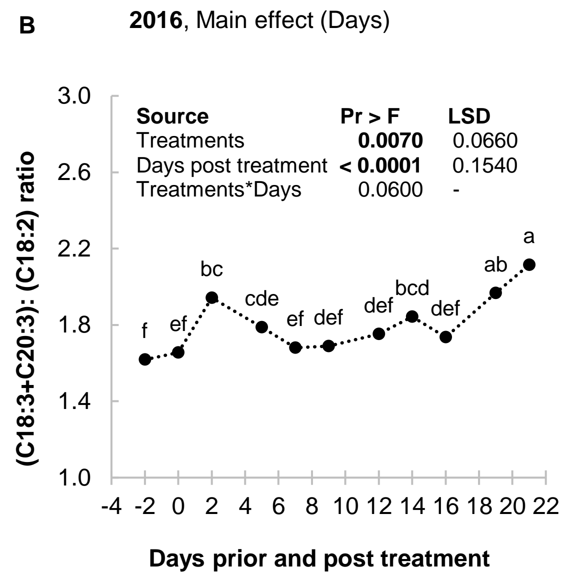


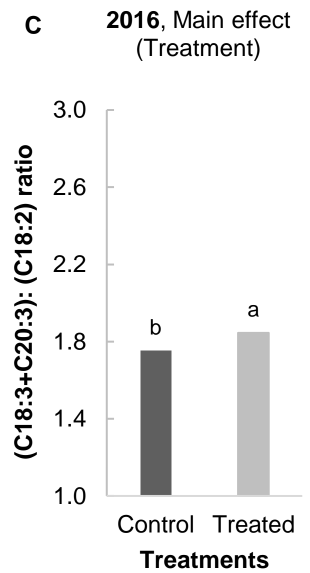
Figure 7: Percentage of monounsaturated fatty acids (MUFA) (A, B, C) and polyunsaturated fatty acids (PUFA) (D, E), as well as the MUFA: PUFA ratio (F, G, H) present in the neutral lipids (NL) of hydrogen cyanamide + oil (HCo) treated and control buds for 2015 and 2016 seasons. Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$, in cases where the interaction between the main effects (Treatments*Days) was non-significant ($p > 0.05$), the significant main effects are presented.



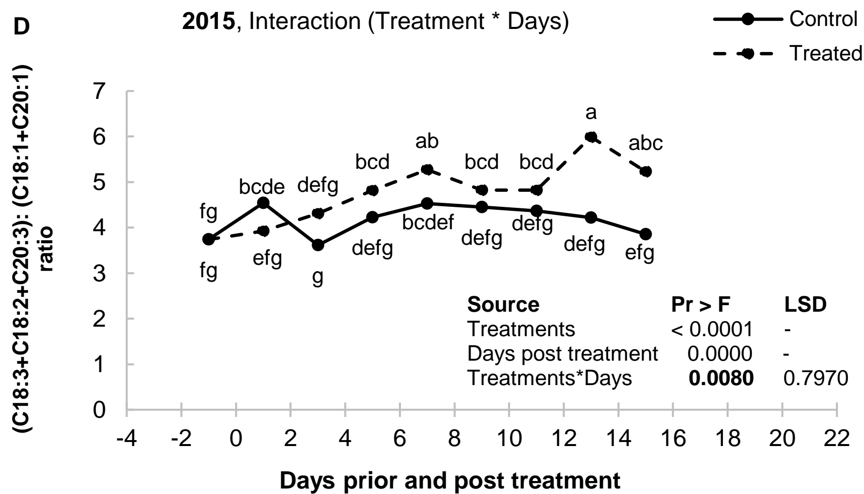
A
2015, Interaction (Treatment * Days)
Control
Treated
Source Pr > F LSD
Treatments < 0.0001 -
Days post treatment 0.0020 -
Treatments*Days 0.0010 0.3690
(C18:3+C20:3): (C18:2) ratio
3.0
2.6
2.2
1.8
1.4
1.0
-4 -2 0 2 4 6 8 10 12 14 16 18 20 22
Days prior and post treatment
de de cde cde bc b b a a
de de de de cd cde de cde e



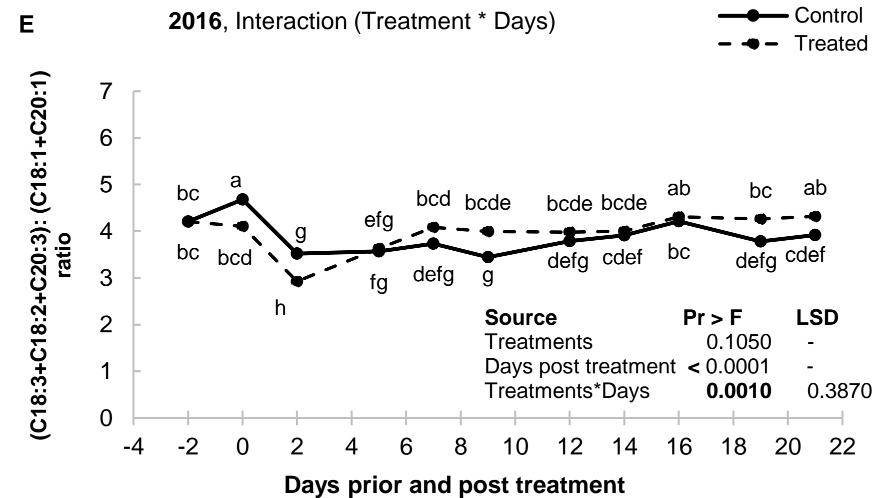
B
2016, Main effect (Days)
Source Pr > F LSD
Treatments 0.0070 0.0660
Days post treatment < 0.0001 0.1540
Treatments*Days 0.0600 -
(C18:3+C20:3): (C18:2) ratio
3.0
2.6
2.2
1.8
1.4
1.0
-4 -2 0 2 4 6 8 10 12 14 16 18 20 22
Days prior and post treatment
f ef bc cde ef def def bcd def ab a



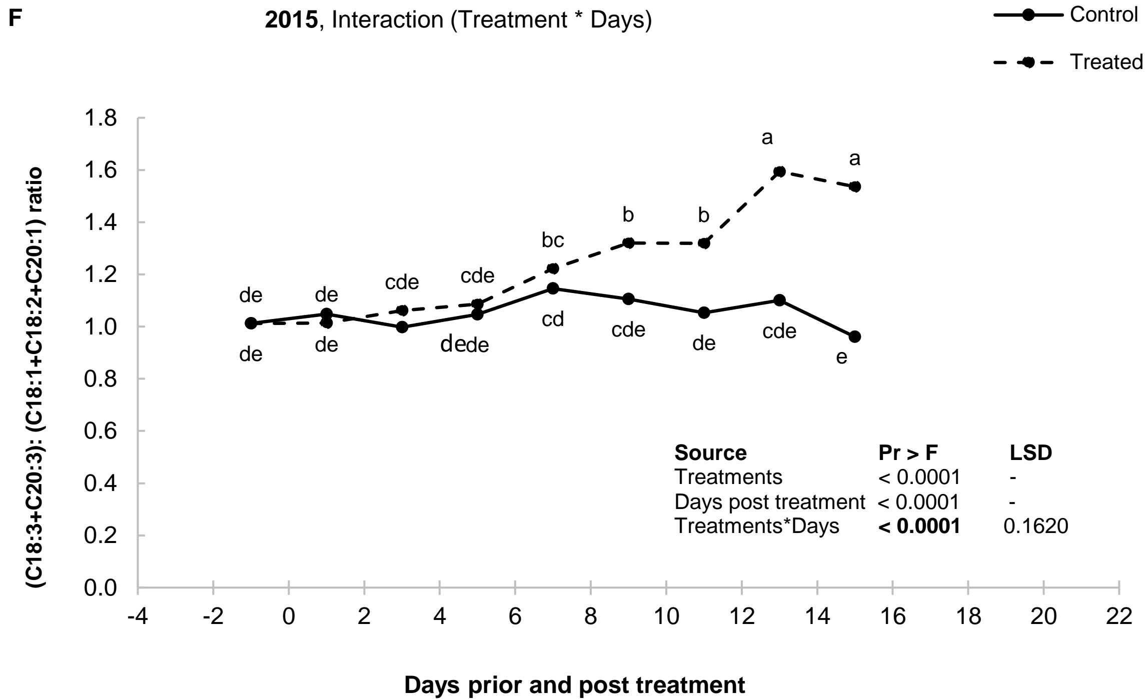
C
2016, Main effect (Treatment)
(C18:3+C20:3): (C18:2) ratio
3.0
2.6
2.2
1.8
1.4
1.0
b
a
Control Treated
Treatments



D
2015, Interaction (Treatment * Days)
Control
Treated
(C18:3+C18:2+C20:3): (C18:1+C20:1) ratio
7
6
5
4
3
2
1
0
-4 -2 0 2 4 6 8 10 12 14 16 18 20 22
Days prior and post treatment
fg bcde defg bcd ab bcd bcd a abc
fg efg g defg bcdef defg defg defg efg
Source Pr > F LSD
Treatments < 0.0001 -
Days post treatment 0.0000 -
Treatments*Days 0.0080 0.7970



E
2016, Interaction (Treatment * Days)
Control
Treated
(C18:3+C18:2+C20:3): (C18:1+C20:1) ratio
7
6
5
4
3
2
1
0
-4 -2 0 2 4 6 8 10 12 14 16 18 20 22
Days prior and post treatment
bc a g efg bcd bcde bcde bcde ab bc ab
bc bcd h fg defg g defg cdef bc defg cdef
Source Pr > F LSD
Treatments 0.1050 -
Days post treatment < 0.0001 -
Treatments*Days 0.0010 0.3870



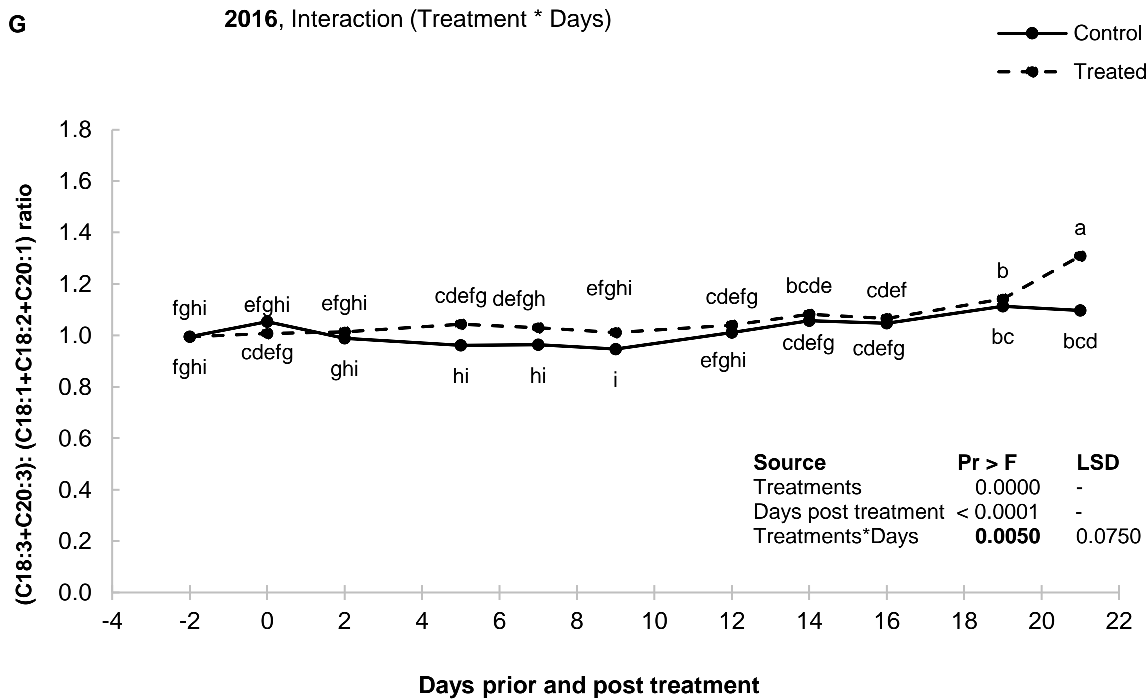


Figure 8: The unsaturation of neutral lipids (NL) in treated and control buds during 2015 and 2016 as given by the (C18:3+C20:3): (C18:2) ratio (A, B, C), (C18:3+C18:2+C20:3): (C18:1+C20:1) ratio (D, E) and the (C18:3+C20:3): (C18:1+C18:2+C20:1) ratio (F, G). Negative values represent days prior to treatment, 0 indicates the day of treatment and positive values denote days post treatment. Letters indicate significant differences at $p < 0.05$, in cases where the interaction between the main effects (Treatments*Days) was non-significant ($p > 0.05$), the significant main effects are presented.