Critical factors concomitant to the physiological development of alternate bearing in citrus (*Citrus* spp.)

By

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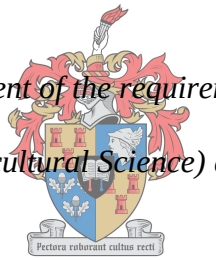

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Declaration

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Dedication

To my wife and best friend, Merise

And my boy, PJ

Summary

The significance of carbohydrates, mineral nutrients and phyto-hormones was investigated in relation to their possible roles in selected phenological events in alternate bearing 'Nadorcott' mandarin (*C. reticultata* Blanco) trees. Crop load in 'Nadorcott' mandarin trees was influenced by flowering intensity. The most important determinants of flowering intensity were the amount of new vegetative shoot growth and resulting number of new potential floral buds that developed during summer, and the influence of fruit on floral bud development during winter. The lack of development of summer vegetative shoots in "on" trees was not related to leaf carbohydrate concentration. In "off" trees, root sugar concentration peaked during full bloom and high root growth activity was observed prior to the vegetative shoot flush in summer. In "on" trees, fruit were the major carbohydrate sinks and probably disturbed the balance between vegetative shoot development and root growth. Sugar concentration in roots in "on" trees was ≈3-fold lower, root growth was absent, and shoot growth was halved. The concentration of mineral nutrients in leaves was a response to fruit load and not related to parameters of flowering or vegetative shoot growth. Measurements of phyto-hormones in leaves and roots confirmed that the inhibition of summer vegetative shoots was related to a high concentration of 1 *H*-indole-3-acetic acid (IAA) in leaves. High concentrations of dihydrophaseic acid and the abscisic acid (ABA) glucose ester suggested that IAA might have acted synergistically with ABA to create a growth inhibition in fruiting shoots. As a result, cytokinins did not contribute to the development of new summer vegetative shoots. High gibberellin concentration in leaves in May and June contributed to limited flowering in "on" trees. Consistent with this interpretation, treatment of "off" trees with 40 $mg \cdot L^{-1}$ gibberellic acid inhibited flowering, whereas soil and foliar treatments of "on" trees with 1000 $mg \cdot L^{-1}$ paclobutrazol or

uniconazole, gibberellin biosynthesis inhibitors, increased flowering and resulted in fruit development from buds of “on” shoots.

Kritieke faktore gepaardgaande met die fisiologiese ontwikkeling van alternerende drag in sitrus (*Citrus* spp.)

Opsomming

Die verband tussen die konsentrasies van koolhidrate, minerale nutriente en fito-hormone, en belangrike fenologiese gebeure is ondersoek in 'Nadorcott' mandaryn (*C. reticulata* Blanco) bome met 'n alternerende drag patroon. Vruglading was beinvloed deur blomintensiteit. Intensiteit van opvolgblom is bepaal deur die aantal beskikbare blomposisies wat gedurende die voorafgaande seisoen se somer ontwikkel het, asook deur die invloed van vrugte op blomontwikkeling gedurende winter. Die gebrek aan somer vegetatiewe lootgroei in "aan"-bome was nie verwant aan die konsentrasie van blaarkoolhidrate nie. Die suikerkonsentrasie in wortels was die hoogste in "af"-bome en tydens volblom, en wortelgroei is waargeneem voor die vegetatiewe lootgroei-stuwing in die somer. Vrugte was die sterkste koolhidraat sink in "aan"-bome en het waarskynlik die balans tussen loot- en wortelgroei versteur. Die suikerkonsentrasie in wortels van "aan"-bome was laer, wortelgroei was afwesig en lootgroei gehalveer. Die inhoud van makro-elemente in blare was'n reaksie op vruglading en nie verwant aan vegetatiewe lootgroei of blom nie. Bepaling van fito-hormoon vlakke in blare en wortels het bevestig dat indool-3-asynsuur (IAA) primêr verantwoordelik was vir die inhibisie van somer vegetatiewe lootgroei. Hoë konsentrasies van dihidrofaasuur en die absisiensuur (ABA) glukose-ester in blare kon moontlik sinergisties met IAA opgetree het om te lei tot die lootgroei-inhibisie in "aan"-bome. Gevolglik het sitokinien toedienings nie somer vegetatiewe lootgroei gestimuleer nie. Hoë gibberellien inhoud in blare gedurende die vroeë winter het bygedra tot die ontwikkeling van min of geen blomme in "aan"-bome. Behandeling van "af"-bome en lote met 40 $mg \cdot L^{-1}$ gibberelliensuur gedurende winter het opvolgblom inhibeer, terwyl behandelings met 1000

$mg \cdot L^{-1}$ paclobutrazol of unikonasool op dieselfde tyd gelei het tot blomvorming en vrugontwikkeling vanaf knoppe op “aan” lote.

This thesis is a compilation of chapters, starting with a literature review, followed by four research papers. Each paper was prepared as a scientific paper for submission to *Scientia Horticulturae*. Repetition or duplication between papers might, therefore, be necessary.

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General introduction and overall research objectives

Alternate or biennial bearing is the synchronised tendency of a fruit tree to flower profusely and produce an excess amount of fruit in one season, followed by a sparse number of flowers and fruit in the following season (Monselise and Goldschmidt, 1982). In alternate bearing fruit trees the alternate fruiting cycle repeats itself in subsequent seasons. A season of heavy fruiting is referred to as an "on" year, whereas a season of low fruit numbers is called an "off" year. In contrast, irregular bearing occurs when a tree produces flowers and fruit in an irregular pattern, with one or more seasons of low fruit yields following an "on" year, or vice versa (Monselise and Goldschmidt, 1982). In citrus (*Citrus* spp.), alternate bearing is more common than irregular bearing and can occur on an individual shoot-level, on a branch or tree, or across entire production regions (Monselise and Goldschmidt, 1982). Alternate bearing also occurs in deciduous fruit and nut trees, such as apple [*Malus* × *sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] (Guitton et al., 2012), pear (*Pyris communis* L.) (Jonkers, 1979), pecan [*Carya illinoinensis* (Wangenh.) C. Koch] (Wood et al., 2004), pistachio (*Pistachia vera* L.) (Rosecrance et al., 1998) and prune (*Prunus domestica* L.) (Davis, 1931), but is more common in evergreen fruit trees, e.g. avocado (*Persea americana* Mill.) (Garner and Lovatt, 2008), citrus (Monselise and Goldschmidt, 1982), coffee (*Caffea arabica* L.) (Vaast et al., 2005), litchi (*Litchi chinensis* Sonn.) (Menzel, 1983), mango (*Mangifera indica* L.) (Souza et al., 2004) and olive (*Olea europaea* L.) (Bustan et al., 2011).

Alternate bearing compromises the consistency of orchard management practices and leads to costly challenges in the production, harvesting, transport, packing and marketing of fruit. In citrus, alternate bearing trees generally produce fruit of low value, with the majority of fruit from "on" trees being small and high in acidity (Galliani et al., 1975; Hield and Hilgeman, 1969), or large and unattractive in "off" trees (Moss et al., 1974). In a recent poll

(CRI, 2016), South African citrus producers reported alternate bearing as a problem in grapefruit [*C. paradisi* Macf. (cv. Star Ruby)], lemon [*C. limon* L. (cv. Eureka)], mandarins [*C. reticulata* Blanco (cvs. Nules Clementine, Nova, Orri, Nadorcott and Mor)] and in 'Valencia' sweet oranges [*C. sinensis* Osbeck (cvs. Midknight and Delta)].

Factors responsible for the initiation and maintenance of alternate bearing are complex and of a combinative nature, and the fundamental cause(s) is an enigma (Bangerth, 2009). In certain citrus cultivars with a high tendency for alternate bearing, the phenomenon has conspicuous causal factors, e.g. a high seed count and a late time of harvest (Monselise and Goldschmidt, 1982). Alternate bearing has, however, been reported in some seedless, e.g. 'Shamouti' sweet orange (Schaffer et al., 1985) and early-maturing, e.g. 'Satsuma' mandarin (*C. unshiu* Marc.) (Iwasaki and Owada, 1960; Okuda, 2000) citrus cultivars. Therefore, the supposed causal factors, i.e. a high seed count and late time of harvest cannot be accepted as the rule, since in other cultivars with the same attributes, alternate bearing can be non-prolific or absent (Sanderson and Treeby, 2014).

The mechanism perpetuating alternate bearing, however, appears to be similar in different fruit crops, as well as in different citrus cultivars. The mechanism relies on the subsequent flowering response determined by the intensity of fruiting, which coincides with specific phenological events, particularly in the "on" year (Monselise and Goldschmidt, 1982). The alternate bearing habit in citrus is sustained by a lack of flowering following an "on" year (Davenport, 1990; Goldschmidt and Golomb, 1982; Hield and Hilgeman, 1969) and not due to low or poor fruit set, despite adequate flowering (Goldschmidt and Golomb, 1982). Thereafter, fruit impose a flowering inhibition on vegetative buds, either on the sprouting of new and potential flowering sites (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009), or during the period of flower induction (Krajewski and Rabe, 1995a;

Koshita et al., 1999; Muñoz-Fambuena et al., 2011). Fruit are therefore limiting the number of new vegetative shoots and their potential to undergo flower induction.

Previous studies on how fruit regulates an inhibition on flowering have produced two generalised theories of alternate bearing – the nutritional theory and the hormonal theory (Bangerth, 2009; Barnett and Mielke, 1981; Davenport, 1990; Goldschmidt, 1999). The nutritional theory of alternate bearing proposes that flowering response is dependent on mineral nutrient availability and plant metabolic energy as determined by fruit load, viz. carbohydrates. In the absence of fruit, mineral nutrients and carbohydrates accumulate in the leaves, bark and roots, and are available for bud sprouting and flower development in the subsequent spring (Dovis et al., 2014, Goldschmidt and Golomb, 1982; Monerri et al., 2011). During situations of heavy flowering and fruiting, fruit limit the carbohydrate and mineral nutrient allocation to developing and competing sinks, e.g. vegetative shoots (Martínez-Alcántara et al., 2015) and roots (Smith, 1976), which can negatively impact on tree condition (Smith, 1976), subsequent reproductive development (Dovis et al., 2014) and consistent production of fruit in the long-term.

Effects observed after girdling and fruit removal treatments corroborate the significance of the nutritional theory, since an increase in flower number usually correlates with high carbohydrate concentration (Cohen, 1981; García-Luís et al., 1995b; Goldschmidt et al., 1985; Schaffer et al., 1985). The correlative evidence resulting from studies on this theory, however, is not convincing, since the use of treatments such as girdling or de-fruiting, could also have hormonal effects on flowering or vegetative responses, or effects that are coincidental and unrelated to changes in levels of carbohydrates or mineral nutrients (Erner, 1988; García-Luís et al. 1995b; Goldschmidt et al., 1985; Koshita et al., 1999). The direct control of flowering and other roles for carbohydrates in the nutritional theory of alternate bearing have therefore not been unequivocally established.

The hormonal theory proposes that phyto-hormones such as abscisic acid (ABA), 1 *H*-indole-3-acetic acid (IAA) and gibberellins (GAs) inhibit either the formation of new vegetative shoots and newly available flowering positions during summer (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009), and/or the expression of citrus flowering genes during flower induction (Muñoz-Fambuena et al., 2011). Shedding light on the role of phyto-hormones in alternate bearing is challenging since the physiological processes related to the alternate bearing phenomenon are closely intertwined. In the hormonal theory of alternate bearing, inhibition of vegetative shoot growth by IAA (Verreynne, 2005; Verreynne and Lovatt, 2009), and flowering and fruit development by GAs (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012) have been established and accredited to one specific plant hormone, but few studies have investigated a 'hormonal balance' concept (Goldschmidt, 1999, 2015). Studies with cytokinins have mostly been conducted in tissue-culture or in potted and non-fruiting citrus trees (Hendry et al., 1982a, 1982b; Van Staden and Davey, 1979), and the role of ABA is yet to be demonstrated in alternate bearing (Goldschmidt; 1984; Jones et al., 1976; Shalom et al., 2014).

By using a model alternate bearing citrus cultivar, 'Nadorcott' mandarin, the aim of the study was to gain more insight into the mechanism perpetuating alternate bearing in citrus by investigating two main objectives:

1) The roles of carbohydrates and mineral nutrients in the nutritional theory of alternate bearing in citrus;

2) The significance of the phyto-hormones ABA, cytokinins, GAs and IAA in the hormonal theory of alternate bearing in citrus.

'Nadorcott', also known as 'W. Murcott', is a late-maturing, sexually self-incompatible and highly parthenocarpic mandarin cultivar which developed from a seed of the highly-seeded 'Murcott' mandarin (Nadori, 2006). 'Murcott' is of unknown parentage, but is

believed to be a tangor; a mandarin hybrid between a mandarin and a sweet orange (*C. reticulata* × *C. sinensis*). Under certain commercial production conditions 'Nadorcott' mandarin is prone to alternate bearing (Stander and Cronjé, 2016; Van der Merwe, 2012) and was therefore selected as a model cultivar for the study.

The seasonal concentrations of carbohydrates, mineral nutrients and phyto-hormones in leaves and roots were measured and investigated in relation to their roles in specific phenological events in the presence or absence of fruit, at the shoot-, branch- and tree-level. To test these findings, leaf mineral nutrient and carbohydrate concentrations, and phenological events were evaluated in response to source/sink manipulations in a time-course study. Results from exogenous phyto-hormone treatments and/or fruit removal during summer and winter were compared to any significant results that were obtained from endogenous phyto-hormone measurements. An overall model is presented that integrates the nutritional and hormonal theories in alternate bearing in 'Nadorcott' mandarin.

Chapter 1: Literature review

Citrus flowering as related to alternate bearing cycles

1. Introduction

Alternate or biennial bearing is the synchronised tendency of a fruit tree to flower profusely and produce an excessive amount of fruit in one season, followed by few flowers and fruit in the following season (Monselise and Goldschmidt, 1982). In alternate bearing fruit trees, the alternate fruiting cycle repeats itself in subsequent seasons. A season of heavy fruiting is referred to as an "on" year, whereas a season of low fruit numbers is called an "off" year. Irregular bearing is when a tree produces flowers and fruit in an irregular pattern of seasonal intensity, with one or more seasons of low fruit yields following an "on" year, or vice versa (Monselise and Goldschmidt, 1982). In citrus (*Citrus* spp.), alternate bearing is more common than irregular bearing and can occur on an individual shoot-level, on a branch or tree, or across entire production regions (Monselise and Goldschmidt, 1982). Alternate bearing also occurs in deciduous fruit and nut trees such as apple [*Malus* × *sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] (Guitton et al., 2012), pear (*Pyris communis* L.) (Jonkers, 1979), pecan [*Carya illinoinensis* (Wangenh.) C. Koch] (Wood et al., 2004), pistachio (*Pistachia vera* L.) (Rosecrance et al., 1998) and prune (*Prunus domestica* L.) (Davis, 1931), but is more common in evergreen fruit trees, e.g. avocado (*Persea americana* Mill.) (Garner and Lovatt, 2008), citrus (Monselise and Goldschmidt, 1982), coffee (*Caffea arabica* L.) (Vaast et al., 2005), litchi (*Litchi chinensis* Sonn.) (Menzel, 1983), mango (*Mangifera indica* L.) (Souza et al., 2004) and olive (*Olea europaea* L.) (Bustan et al., 2011).

Alternate bearing compromises the consistency of orchard management practices and leads to costly challenges in the production, harvesting, transport, packing and marketing of fruit. In citrus, alternate bearing trees generally produce fruit of low commercial value, with

the majority of fruit from "on" trees being small and high in acidity (Galliani et al., 1975; Hield and Hilgeman, 1969), or large and unattractive in "off" trees (Moss et al., 1974).

Factors responsible for the initiation and maintenance of alternate bearing appear to be complex and of a combinative nature, and the fundamental cause(s) is an enigma (Bangerth, 2009). In certain citrus cultivars with a high tendency for alternate bearing the phenomenon first seemed to have conspicuous causal factors, e.g. a high seed count and a late time of harvest (Monselise and Goldschmidt, 1982). However, discrepancies have since been reported for these factors to be accepted as a rule, since in other cultivars with the same attributes alternate bearing can be non-prolific or totally non-prevalent (Sanderson and Treeby, 2014). In a recent poll (CRI, 2016) South African citrus producers reported alternate bearing as a problem in grapefruit [*C. paradisi* Macf. (cv. Star Ruby)], lemon [*C. limon* L. (cv. Eureka)], mandarins [*C. reticulata* Blanco (cvs. Nules Clementine, Nova, Orri, Nadorcott and Mor)] and in 'Valencia' sweet oranges [*C. sinensis* Osbeck (cvs. Midknight and Delta)]. However, on a whole-tree level, alternate bearing is most notably prevalent in easy-peeling mandarin cultivars (Monselise and Goldschmidt, 1982; Wheaton, 1992). In mandarins and their hybrids, as well as mandarin hybrids with grapefruit (*C. reticulata* × *C. paradisi*, i.e. tangelos) and sweet oranges (*C. reticulata* × *C. sinensis*, i.e. tangors) alternate bearing is typically a rule, irrespective of their level of seediness (Monselise and Goldschmidt, 1982).

Alternate bearing has been reported in cultivars with many seeds, viz. 'Murcott' (unknown parentage, but believed to be a tangor) (Smith, 1976), 'Moncada' [*C. reticulata* hybrid (*C. clementina* Hort. × (*C. unshiu* × *C. nobilis* Lour.)] (Muñoz-Fambuena et al., 2011), 'Wilking' (*C. reticulata* hybrid) (Goldschmidt and Golomb, 1982) and 'Kinnow' [(*C. reticulata* hybrid) (*C. nobilis* × *C. deliciosa* Ten.)] (Mirsoleimani et al., 2014); whereas in low- to medium-seeded mandarins, alternate bearing has been reported in the cultivars 'Michal' (*C. reticulata* hybrid) (Monselise et al., 1983), 'Nadorcott' [a chance 'Murcott'

seedling (*C. reticulata*)] (Stander et al., 2017; Van der Merwe, 2012), 'Orri' [an induced mutation of 'Orah' mandarin (*C. reticulata* hybrid) and progeny of 'Kinnow' (Barry et al., 2015)] (Goldberg-Moeller et al., 2013), 'Pixie' [second generation seedling of 'Kincy' mandarin (*C. nobilis* × *C. reticulata*)] (Tang, 2017; Verreynne and Lovatt, 2009) and 'Ponkan' (unknown parentage) (Mataa et al., 1996). In sweet oranges, alternate bearing has been reported in low-seeded 'Salustiana' (Monerri et al., 2011) and 'Shamouti' sweet orange (Schaffer et al., 1985), as well as in various seeded 'Valencia' cultivars (Dovis et al., 2014; Jones et al., 1974; Martínez-Fuentes et al., 2010; Plummer et al., 1989). Alternate bearing occurs in some of the earliest maturing citrus cultivars, i.e. 'Satsuma' (*C. unshiu* Marc.) and 'Pixie', as well as in some of the latest maturing cultivars, i.e. 'Murcott' and 'Valencia', and therefore, as a whole, appears to manifest irrespective of the timing of a cultivar's period of fruit growth and maturity (Table 1).

The mechanism perpetuating alternate bearing, however, appears to be similar for different fruit crops and citrus cultivars, with the subsequent flowering response determined by the intensity of fruiting. In the majority of alternate bearing trees, fruiting coincides with specific phenological events (Monselise and Goldschmidt, 1982) and alternate bearing in citrus perpetuates due to a lack of flowering following an "on" year (Davenport, 2000; Goldschmidt and Golomb, 1982; Hield and Hilgeman, 1969), and not due to low or poor fruit set, despite adequate flowering (Goldschmidt and Golomb, 1982). In citrus, fruit impose a flowering inhibition on vegetative buds, either on the sprouting of new and potential flowering sites (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009), or during the period of flower induction (Krajewski and Rabe, 1995a; Koshita et al., 1999; Muñoz-Fambuena et al., 2011) (Fig. 1). Fruit therefore limit the number of new vegetative shoots with the potential to undergo flower induction.

Studies on how fruit regulates the inhibition on flowering have produced two generalized theories of alternate bearing – the hormonal theory and the nutritional theory (Bangerth, 2009; Barnett and Mielke, 1981; Davenport, 2000; Goldschmidt, 1999). The hormonal theory of alternate bearing proposes that phyto-hormones such as abscisic acid (ABA), 1 *H*-indole-3-acetic acid (IAA) and gibberellins (GAs) inhibit either the formation of new vegetative shoots and newly available flowering positions during summer (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009), and/or the expression of citrus flowering genes during flower induction (Muñoz-Fambuena et al., 2011; Tang, 2017).

The nutritional theory of alternate bearing, on the other hand, proposes that flowering is dependent on mineral nutrient availability and plant metabolic energy as determined by fruit load, viz. carbohydrates. In the absence of fruit, mineral nutrients and carbohydrates accumulate in the leaves, bark and roots, and are available for bud sprouting and flower development in the subsequent spring (Dovis et al., 2014, Goldschmidt and Golomb, 1982; Monerri et al., 2011). In heavy flowering and fruiting situations, fruit limit carbohydrate and mineral nutrient allocation to developing and competing sinks, e.g. vegetative shoots (Martínez-Alcántara et al., 2015) and roots (Smith, 1976), which can negatively impact on tree condition (Smith, 1976), subsequent reproductive development (Dovis et al., 2014), and consistent production of fruit in the long-term.

In the following review the general phenology of vegetative shoot flushing and flowering of a citrus tree will be discussed, as well as the roles of important factors considered within the two different models of alternate bearing – carbohydrates and mineral nutrients in the nutritional theory of alternate bearing, and the endogenous hormones, ABA, cytokinin, GAs and IAA in the hormonal theory of alternate bearing.

2. Development of flower bearing shoots

Citrus trees grown under subtropical climates sustain a complex evergreen structure by sprouting new vegetative shoots during one to three distinctive vegetative shoot flushes per season (Abbott, 1935; Monselise, 1985; Sauer, 1951). The first shoot flush occurs in spring, when buds normally produce flowers, new and purely leafy vegetative shoots, or a combination of flowers and leaves (Abbott, 1935; Mullins et al., 1989; Sauer, 1951). New vegetative shoots originate by pushing through the terminal or lateral buds on one-year-old parent shoots, and elongate in a strongly apical dominant manner (Schneider, 1968; Spiegel-Roy and Goldschmidt, 1996). Growth of vegetative shoots in citrus follows a sympodial growth habit, meaning that the apical meristem of the shoot terminates upon cessation of the current period of vegetative shoot flush (Schneider, 1968). Subsequent vegetative shoot flushes arise by bud sprouting of lateral meristems on already-developed parent shoots from the previous season, or from previous vegetative shoot flushes (Monselise, 1985; Mullins et al., 1989; Schneider, 1968).

In citrus (Monselise and Goldschmidt, 1982; Verreynne and Lovatt, 2009) and other evergreen fruit trees such as olive (Dag et al., 2010) and avocado (Ziv et al., 2014), new vegetative shoots provide the sites from which flowers develop in the subsequent spring, i.e. new flower bearing units (Table 2). Flower bearing units tend to have a length of approximately six to eight nodes (Ehara et al., 1981), triangular internodes in cross-section compared to older non-flowering shoots that are typically round, thicker and shorter (Schneider, 1968), and have an age of 5 to 12 months, i.e. vegetative shoots from the previous season (Albrigo and Chica, 2011; Krajewski and Rabe, 1995b; Verreynne and Lovatt, 2009). The inhibition of the development of new vegetative shoots in citrus is an impediment to return bloom flowering and can increase the potential for the manifestation of

alternate bearing (Lenz, 1967; Monselise and Goldschmidt, 1981, 1982; Verreynne and Lovatt, 2009).

The growth of vegetative shoots and roots in citrus follow a strong cyclical nature (Bevington and Castle, 1985; Eissenstat and Duncan, 1992) as a result of strong correlative responses of shoots and roots to low and high soil temperature, or to water deficit stress (Bueno et al., 2011; Cossmann, 1939; Marloth, 1949; Reed and MacDougal, 1938; Ribeiro et al., 2012). Furthermore, the number and length of vegetative shoot growth has a strong inverse relationship with intensity in fruiting (Ehara et al., 1981; García-Luís et al., 1995b; Lenz, 1967; Plummer et al., 1989; Verreynne and Lovatt, 2009). Most of the research on relationships between the growth of vegetative shoots and other tree organs provides evidence for the involvement of carboydrates in the inhibition or upregulation of vegetative shoot development (Goldschmidt and Golomb, 1982; Monerri et al., 2011; Martínez-Alcántara et al., 2015; Smith, 1976). New vegetative shoots are strong sinks for carbohydrate supply from mature leaves (Ruan, 1993), and only act as a carbohydrate source three to four months after bud sprouting (Ruan, 1993; Spiegel-Roy and Goldschmidt, 1996) (Fig. 2). For this reason, the first vegetative shoot flush that develops during spring and in the absence of fruit, mainly uses reserve carbohydrates from the previous season (Monerri et al., 2011; Reed and MacDougal, 1938). On the other hand, the second vegetative shoot flush occurs after flowering and subsequent to physiological fruit drop in early summer, when fruit is the major carbohydrate sink and compete with new vegetative shoot growth (García-Luís et al., 1988; Guardiola, 1988; Van Rensburg et al., 1996) (Fig. 2).

Martínez-Alcántara et al. (2015) recently reported that in heavy-flowering and -fruiting ‘Moncada’ mandarin trees, fruit presence and fruit growth during summer limited the carbohydrate and mineral nutrient allocation to buds and developing vegetative shoots, which was the main cause of a lack of return bloom flowering following an “on” year. Furthermore,

in heavy-fruiting trees, strong inter-sink competition for carbohydrates between roots and fruit inhibits root growth (Goldschmidt and Golomb, 1982; Smith, 1976), and in a severe case this competition resulted in death of feeder roots and tree collapse of heavy-fruiting 'Murcott' mandarin trees (Smith, 1976) – apparently due to the strong dependency of vegetative shoot growth on roots (Bevington and Castle, 1985) (Fig. 2).

In addition to fruit being dominant carbohydrate sinks, fruit, on the other hand, are also major sources of phloem-transported hormones, which influences the development of vegetative shoots and new and potential flower bearing units (Erner et al., 1976; Talon et al., 1990b; Verreynne, 2005) (Figs. 2 and 3). Verreynne (2005) showed that the lack of summer vegetative shoot development and flowering in "on" 'Pixie' mandarin trees was attributed to a high IAA concentration combined with low cytokinin concentration in buds caused by the presence of fruit at shoot tip – a mechanism of inhibition of vegetative shoot growth similar to the correlative inhibition of a terminal shoot tip on lateral or axillary bud sprouting, called apical dominance (Bangerth, 1989; Cline, 1991; Dun et al., 2006). However, in other studies (Bower et al., 1990; Goldschmidt, 1984; Jones et al., 1976; Shalom et al., 2014), an inhibition of new vegetative shoots was related to high concentrations of ABA in leaves and buds (Figs. 2 and 3).

3. Flowering in *Citrus* spp.

In subtropical and Mediteranean-type climates, citrus flowering occurs during spring, but is preceded by an intricate and synchronised flower development process during the previous autumn and winter (Davenport, 1990; Krajewski and Rabe, 1995a) (Fig. 1). Flower induction is the first and essential step in flower development. Citrus flowering is daylength neutral (Davenport, 1990; Moss, 1969) and the main stimuli promoting flower induction in citrus trees are a continuous period of water deficit stress (Chica and Albrigo, 2013; Moss,

1969; Reuther et al., 1973; Southwick and Davenport, 1986), and low ambient temperatures (Lenz, 1969; Moss, 1976; Nishikawa et al., 2007; Valiente and Albrigo, 2004) (Fig. 1). In most citrus species grown commercially, flower induction starts at the onset of autumn and terminates towards the end of winter (Lenz, 1969; Moss, 1969; Nishikawa, 2013; Reuther et al., 1973; Valiente and Albrigo, 2004). Furr and Armstrong (1956) determined time of flower induction for 'Marsh' grapefruit grown in California as the period extending from September to December, by measuring flowering response to leaf removal and girdling treatments. From these findings, Monselise and Halevy (1964) determined with foliar gibberellic acid (GA_3) treatments that time of flower induction in 'Shamouti' sweet orange extends from November to January in Israel. More recently, time of flower induction has been established in a similar period for 'Moncada' mandarin (Muñoz-Fambuena et al., 2011) and 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012) grown in Spain, and 'Orri' mandarin grown in Israel (Goldberg-Moeller et al., 2013). Shalom et al. (2012) in Israel, and Tang (2017) in California, however, recently determined that flower induction occurs much earlier in 'Murcott' (Shalom et al., 2012), and in 'Nules clementine' and 'Pixie' mandarins (Tang, 2017).

In the classic model for flower induction (Bangerth, 2009), a signal is detected by leaves, which then express the flowering gene, the *FLOWERING LOCUS T* (*FT*) (Komeda, 2004). The *FT* protein and so-called "florigen", is subsequently transported from leaves to buds where it regulates flower developmental events (Corbesier et al., 2007). The contribution of leaves to flowering has been illustrated using horticultural manipulations (Furr and Armstrong, 1956; Monselise and Halevy, 1964; Krajewski and Rabe, 1995b), but more recently in studies using more advanced and mostly molecular approaches (Chica and Albrigo, 2013; Muñoz-Fambuena et al., 2012; Nishikwa et al., 2007, 2013). Defoliation experiments in 'Moncada' mandarin, for example, revealed that the absence of leaves during

flower induction reduced expression of most of the flowering-related genes, and completely prevented blossoming (Muñoz-Fambuena et al., 2012).

In contrast, a different study in 'Moncada' mandarin showed that presence of fruit affected flowering by altering gene expression within the bud (Muñoz-Fambuena et al., 2012). Nishikawa et al. (2013) showed that defoliation of 'Satsuma' mandarin trees did not completely suppress flower induction, which suggested that events in the bud also importantly contributed to flowering, and not only events within the leaf. In fact, in a study in 'Orri' mandarin, mRNA levels of the *CiFT* gene were considerably higher in buds than in leaves (Goldberg-Moeller et al., 2013), and Nishikawa et al. (2007) showed that mRNA levels of the *CiFT* gene in stems correlated stronger with flowering than in leaves, and therefore played a more important role. Malik et al. (2015) reported that cold treatment of defoliated 'Satsuma' mandarin, grapefruit and sweet orange trees resulted in the majority of buds sprouting flowers. Since flower development from buds occurred in the absence of leaves, it indicated that metabolic processes that led to flowering occurred within the resting bud itself, and independent of the presence of leaves (Malik et al., 2015). Citrus trees can therefore have a hysteranthous flowering response, i.e. they can sprout flowers in the absence of leaves (Fig. 3). Although it appears that both leaves and buds can generate a flowering signal and not necessarily only either of the two, the discrepancies in this area of research on alternate bearing in citrus require more attention.

Flower initiation in citrus follows flower induction, and involves the transition of bud meristematic tissue from a vegetative to reproductive state (Davenport, 1990) in response to levels of sufficiently accumulated *CiFT* proteins in the bud (Nishikawa, 2013; Tang, 2017). Flower initiation is the process during which the organs of a citrus flower start to develop at a molecular level into a state significantly distinguishable from vegetative or non-induced buds (Lord and Eckard, 1985). Finally, flower differentiation occurs at the onset of growth-

promoting conditions (Randhawa and Dinsa, 1947) and manifests in bud transformation into either a reproductive state, or remains vegetative (Davenport, 1990; Lord and Eckard, 1985; Randhawa and Dinsa, 1947). With the onset of flower differentiation, buds enter a state of irreversible commitment to either flower or remain vegetative, and are unable to undergo a change in their morphological fate (Guardiola et al., 1982). Non-differentiated buds, however, can remain dormant as a result of sprouting inhibition induced by the presence of fruit (Martínez-Fuentes et al., 2010), insufficient growth-promoting conditions (García-Luís et al., 1995a; Moss, 1969; Randhawa and Dinsa, 1947), or age of the bud or shoot (García-Luís et al., 1995a; Schneider, 1968). In isolation of normal flowering conditions, if a bud has been sufficiently induced, inflorescences could sprout out-of-season, when inhibition of bud differentiation is removed (Furr and Armstrong, 1956).

4. Regulators of citrus flowering

The majority of research on citrus flowering and the subsequent development of alternate bearing acknowledge a self-regulatory model that involves one or more endogenous signal(s) transmitted within citrus trees, i.e. hormones, which control flower developmental events. In this model, the intensity of transmission of these signals may result in the complete inhibition of flowering and lead to an "off" year, or excessive flowering and an "on" year (Davenport, 2000; Koshita et al., 1999; Muñoz-Fambuena et al., 2011; Shalom et al., 2012; 2014) (Fig. 1). Flowering response to these signals furthermore appears to be dependent on the availability of plant metabolic energy, viz. carbohydrates, and the prevalence and intensity of factors influencing the availability thereof (Davenport, 2000; García-Luís et al, 1995b; Goldschmidt et al., 1985; Goldschmidt and Koch, 1996) (Figs. 2 and 3). In the following sections, the proposed roles of carbohydrates and mineral nutrients

in flowering and the nutritional theory of alternate bearing will be discussed, as well as that of endogenous hormones in the hormonal theory of alternate bearing.

3.1. The nutritional theory of alternate bearing

3.1.1. Carbohydrates

The principal carbohydrate components in citrus leaves are the non-reducing disaccharide sugar, sucrose, followed by the more complex starch molecule (Goldschmidt, 1997; Jones and Steinacker, 1951; Koch, 1984). Small amounts of glucose, fructose, malic acid and myo-inositol are also present in citrus leaves (Jones et al., 1974), in addition to complex chains of polysaccharides (Lenz and Küntzel, 1974; Stander and Cronjé, 2016). Allocation of carbohydrates from photosynthesising leaves as sources, to heterotrophic non-photosynthetic organs as sinks, relies on an efficient and highly controlled phloem transport system (Koh et al., 2012; Wang and Ruan, 2015) (Fig. 4). Among all the photosynthetically-fixed carbohydrates in the leaf, only few are able to be transported over a long distance (Lemoine et al., 2013). In this context, sucrose is the primary translocated form due to its non-reducing nature (Iglesias et al., 2003; Koch, 1984; Ruiz et al., 2001; Yildiz et al., 2013). Accumulation of sucrose in the phloem of the source leaves attracts water osmotically, which creates high turgor pressure in the phloem. This drives mass flow of sucrose towards lower turgor pressure at the sinks (Ruan et al.,1996), at rates much higher than that of active transport of hormones, for example (150 $cm \cdot h^{-1}$, compared to 16 $cm \cdot h^{-1}$ for IAA) (Wang and Ruan, 2015).

In citrus, flowers (Dovis et al., 2014) and fruit (Koch, 1984; Martínez-Alcántara et al., 2015) are the major carbohydrate sinks apart from developing vegetative shoots (Ruan, 1993) and non-photosynthesising tree organs such as roots (Bueno et al., 2011; Monerri et al., 2011; Ribeiro et al., 2012) and the bark and wood (Bester and Rabe, 1996; Monerri et al., 2011).

Consequently, sugars are detected in most organs of a citrus tree, and their concentrations undergo particular patterns of change as determined mostly by seasonal variation in temperature (Bueno et al., 2011; Yelonosky and Guy, 1977) and variation in intensity of fruiting, e.g. an "on" or "off" year (Dovis et al., 2014; Goldschmidt, 1997; Monerri et al., 2011; Yildiz et al., 2013).

In addition to the use of sugars from current photosynthesis supply, sugars are also available from stored carbohydrate reserves (Dovis et al., 2014; Monerri et al., 2011; Ruiz et al., 2001). A certain measure of carbohydrate reserve accumulation occurs naturally in citrus organs (Fishler et al., 1983), but carbohydrates generally accumulate in specific tree organs when photo-assimilate supply exceeds the current demand, i.e. during sink-limitation (Dovis et al., 2014; Goldschmidt and Golomb, 1982; Loescher et al., 1990; Monerri et al., 2011; Nebauer et al., 2014; Schaffer et al., 1986). Starch is the main storage carbohydrate in citrus, as well as the major carbohydrate component in roots (Eissenstat and Duncan, 1992; Loescher et al., 1990; Monerri et al., 2011; Nebauer et al., 2014) and trunk wood (Bester and Rabe, 1996), as opposed to sucrose in citrus leaves. Climatic factors such as low temperature (Mataa et al., 1996; Yelenosky and Guy, 1977), structural interference of the transport pathway (Cohen, 1981; Koh et al., 2012; Schaffer et al., 1986) and over-production of photosynthates (Goldschmidt and Golomb, 1982; Monerri et al., 2011; Nebauer et al., 2014) lead to starch build-up. This form of carbohydrate can be used during periods when carbohydrate supply by current photosynthesis cannot meet current active sink demand, i.e. during source-limited periods (Bustan and Goldschmidt, 1998; Dovis et al., 2014; Goldschmidt, 1997; Monerri et al., 2011; Ruan, 1993).

In citrus, a fruit over-load seems to restrict a tree's capacity to build up starch to use for flowering, and root and vegetative shoot growth in the subsequent season (Goldschmidt and Golomb, 1982; Smith, 1976). "On" trees accumulate most of their carbohydrates in the fruit,

while no accumulation occurs in roots (Koch, 1984; Monerri et al., 2011). In 'Washington Navel' sweet orange, for example, allocation of dry matter and root growth were reduced by fruit (Cary, 1970), whereas in 'Valencia' sweet orange fruit removal in spring increased carbohydrate concentration in roots, but also resulted in a root mass density increase of 51% during summer (Duncan and Eissenstadt, 1993). In "off" trees, on the other hand, starch accumulates in leaves (Van der Merwe, 2012) and roots (Monerri et al., 2011), and this is apparently important for the initiation and maintenance of new growth in the subsequent spring (Dovis et al., 2014; Monerri et al., 2011; Nebauer et al., 2014).

Reed and MacDougal (1938) reported that for sweet orange, the first vegetative shoot flush in spring is maintained by carbohydrate reserves that accumulated in permanent structural tree organs during the previous season. In 'Valencia' sweet orange trees, approximately two thirds of the total carbohydrate pool used by flowering and maintenance of spring vegetative shoot growth were contributed by carbohydrate reserves in roots (Dovis et al., 2014). Various sugars are therefore also often detected in the trunk wood (Bester and Rabe, 1996; Mataa et al., 1996) and xylem sap (Schill et al., 1996; Secchi and Zwieniecki, 2012) as a consequence of an acropetal xylem transport of re-mobilised sugars from reserve carbohydrates in roots, to shoots and leaves (Dovis et al., 2014; Monerri et al., 2011; Nebauer et al., 2014).

There is uncertainty as to whether carbohydrates have a role in the perpetuatio of alternate bearing in citrus. In a classic study in severe alternate bearing 'Wilking' mandarin trees, leaf and root starch concentrations were found to be 3.6 and 17.4 times higher for "off" trees compared to "on" trees. In actual dry-matter content, "off" trees accumulated 13 kg starch and 10 kg soluble sugars compared to only 3 kg and 7 kg in "on" trees (Goldschmidt and Golomb, 1982). Removal of fruit from "on" trees by mid-summer altered this tendency in 'Wilking' and 'Murcott' mandarins (Goldschmidt and Golomb, 1982; Smith, 1976) and

permitted starch build-up in the various tree organs, which correlated with increased flowering (Goldschmidt and Golomb, 1982; Jones et al., 1974; Smith, 1976). In other studies, winter girdling of vegetative/"off" branches resulted in significant accumulation of carbohydrates in leaves above the girdle (Fig. 4), and the numbers of buds sprouting in spring, as well as the numbers of flowers and eventual fruit increased significantly (Cohen, 1981; Schaffer et al., 1986). Fruit removal and girdling resulted in the same response and increased flowering in 'Murcott' (Goldschmidt et al., 1985) and in 'Satsuma' (García-Luís et al., 1995b) mandarins, but the response was different in the presence of fruit. Elevating leaf carbohydrate concentration in the presence of fruit consistently revealed an overriding effect of fruit, which reduced flowering response compared to flowering in girdled "off", or de-fruited branches (García-Luís et al., 1995b; Goldschmidt et al., 1985; Koshita et al., 1999). In addition to fruit being dominant carbohydrate sinks, fruit are also sources of phloem-transported hormones (Erner et al., 1976; Talon et al., 1990b; Verreynne, 2005), and girdling also influences the hormonal balance in all plant tissues above the girdle as a result of accumulation of GAs and specifically IAA (Fig. 4), of which the roles will be discussed in later sections.

Apart from a possible direct involvement for carbohydrates in alternate bearing, there is also proof that carbohydrates influence fruit load in citrus by determining the extent to which certain tree organs experience growth, as well as the success of energy-requiring flowering and fruiting processes such as budbreak, flower differentiation and fruit set. Martínez-Alcántara et al. (2015) recently reported that in heavy-flowering and -fruiting 'Moncada' mandarin trees, fruit presence and growth limitted the carbohydrate and mineral nutrient allocation to developing vegetative shoots during summer, which was the main cause of a lack of return bloom flowering following an "on" year. Furthermore, in heavy-fruiting trees, a very strong inter-sink competition between roots and fruit for carbohydrates inhibitted root

growth (Goldschmidt and Golomb, 1982; Smith, 1976), and resulted in death of feeder roots and tree collapse of heavy-fruiting 'Murcott' trees (Smith, 1976) – apparently due to the strong dependency of vegetative shoot growth on roots (Bevington and Castle, 1985).

With flowers and fruit being the major carbohydrate sinks, carbohydrate availability during flowering and fruit set also seem to play a major role in determining or limiting a flower's successful morphological transition to a fruit (Iglesias et al., 2003; Monerri et al., 2011; Ruiz et al., 2001; Schaffer et al., 1985). This was illustrated by Schaffer et al. (1985) who showed that the high natural fruit set obtained in 'Murcott' mandarin, a strong alternate bearer, is a result of high level of available leaf carbohydrates during flowering. In 'Shamouti' sweet orange, a generally moderate and regular fruit bearer, on the other hand, fruit set percentage was twice as low, and similarly the leaf carbohydrate concentration during flowering. A girdling treatment of 'Shamouti' sweet orange trees during flowering increased leaf starch concrentration and also fruit set (Schaffer et al., 1985). The importance of carbohydrates in fruit set was also confirmed in 'Washington Navel' sweet orange (Ruiz et al., 2001), 'Ponkan' mandarin (Mataa et al., 1996), 'Salustiana' sweet orange (Monerri et al., 2011) and 'Satsuma' mandarin (Iglesias et al., 2003), but in citrus, alternate bearing is sustained by the lack of flowering (Davenport, 1990; Goldschmidt and Golomb, 1982; Hield and Hilgeman, 1969) and not due to low or poor fruit set despite adequate flowering (Goldschmidt and Golomb, 1982).

In summary, no evidence is currently available acknowledging a direct role of carbohydrates in the regulation of a meristem's transition from a vegetative to reproductive state in citrus. Carbohydrate content and availability as a source of energy seem to affect growth of other tree organs such as roots, as well as determine the intensity of flowering response as a factor of flower differentiation and bud sprouting in the absence of fruit-transmitted hormones. This concept was initially supported by Cohen (1981), García-Luís et

al. (1995b) and Goldschmidt et al. (1985) and recently confirmed (Dovis et al., 2014; Martínez-Alcántara et al., 2015; Monerri et al., 2011). The current role for carbohydrates in the model for alternate bearing therefore appears to be that of a secondary role, with its positive effects being experienced in the absence of fruit and flowering inhibiting plant hormones.

3.1.2. Mineral nutrients

In citrus, initiation of new vegetative growth during spring mainly uses mineral nutrient reserves that accumulated in old leaves, shoots and roots during the previous season, as opposed to mineral nutrient uptake from the soil (Dasberg et al., 1983; Legaz et al., 1995; Martínez-Alcántara et al., 2015). Upon mobilisation, large amounts of mineral nutrients are translocated to developing shoots, flowers and fruit (Dasberg, 1988; Sanz et al., 1987), with consumption by new growth peaking in mid-summer (Martínez-Alcántara et al., 2015). Fruit are strong sinks and if mineral nutrients are not supplemented under heavy-fruiting conditions, permanent structural tree organs can experience a gradual, but substantial depletion of the major mineral nutrient constituents from time of flower differentiation during spring, until fruit maturity the following winter (Golomb and Goldschmidt, 1987; Mirsoleimani et al., 2014; Monselise et al., 1983; Smith, 1976). This would occur at the expense of the development of other tree organs and fruit quality (Lenz, 1967).

In comparative studies on the end-of-season leaf mineral nutrient contents of heavy- and low-fruiting mandarin trees, significantly lower levels of leaf total N were reported for heavy-fruiting ‘Michal’, ‘Murcott’ and ‘Wilking’ mandarins (Golomb and Goldschmidt, 1987; Monselise et al., 1983; Smith, 1976). For leaf P and K, a similar trend was reported in all these cultivars, as well as for ‘Kinnow’ mandarin (Mirsoleimani et al., 2014). In low-fruiting trees, mineral nutrients accumulated in the permanent structural tree organs and were

available for growth in the subsequent season, but in heavy-fruiting trees, 32%, 44% and 58% of the total tree N, P and K dry-matter, respectively, resided in the fruit and was removed at subsequent harvest (Golomb and Goldschmidt, 1987; Smith, 1976). In 'Murcott' mandarin, Smith (1976) reported a subsequent collapse of heavy-fruiting trees, with collapsed trees showing severe deficiencies of leaf N, P and K. However, mineral nutrient depletion of vegetative tissues was reported not to be the cause of the collapse, but rather a response, since lavish fertilisation did not prevent the occurrence (Smith, 1976). Furthermore, root starvation and malfunction due to carbohydrate consumption by fruit was proposed as the main cause of this phenomenon, since a reduction in fruit load in "on" trees prevented tree decline and resulted in recovery of leaf mineral nutrient contents (Smith, 1976). A similar reduction in root growth was reported in heavy-fruiting trees compared to low-fruiting trees (Lenz, 2000), but reduced root growth did not negatively affect nutrient uptake from the soil. In fact, Lenz (2000) reported that nutrient uptake and allocation of N, P and K by roots to fruit was higher in heavy-fruited trees, as well as the uptake and allocation of calcium (Ca) to leaves due to an apparent increased transpiration rate in the presence of a heavy crop.

Nevertheless, in general, the presence of a heavy fruit load and subsequent excessive demand is the confirmed perpetuator of altered mineral nutrient distribution across tree organs and not necessarily altered tree efficiency for mineral nutrient uptake. As opposed to high levels of leaf mineral nutrient contents in low-fruiting trees, the majority of mineral nutrients in heavy-fruiting trees accumulates in fruit and are consumed and removed by fruit at harvest (Golomb and Goldschmidt, 1987).

If nutrient applications to citrus trees are not increased under high fruit load conditions, a reduction in dry matter allocation to vegetative tissues and reduced leaf mineral nutrient content and vegetative growth can occur (Lenz, 1966, 2000). In 'Redblush' grapefruit, fruit formed dominant competitive sinks for N and accounted for between 40% and 70% of the

total N assimilation (Lea-Cox et al., 2001). Fruit assimilated up to 20% of the total N at the expense of spring vegetative shoot development and to the detriment of the overall tree N status (Lea-Cox et al., 2001). Cary (1970) reported a large response in vegetative growth in 'Washington Navel' sweet orange when N supply was increased, but fruit yield increased only slightly and fruit quality deteriorated. In 'Moncada' mandarin, Martínez-Alcántara et al. (2015) recently reported that although fruit accumulate large amounts of N, vegetative shoot development was not compromised by the presence or absence of fruit and the competence of fruit for N assimilation was not a decisive factor determining intensity or vigour of vegetative growth (Martínez-Alcántara et al., 2015). These reports are contrasting, and it is therefore not yet conclusive to what extent mineral nutrient availability in leaves can determine vegetative responses, and if leaf mineral nutrient content in heavy-fruiting trees is a cause or effect of poor vegetative shoot development.

In terms of regulating buds on a shoots' transition from a vegetative to reproductive state, availability of N and specifically ammonia-N in citrus buds during flower induction, have been shown to be a critical determinant of the level of cell division and initiation of the subsequent flowering response (Lovatt et al., 1988). During flower inductive conditions, ammonia-N accumulates in buds and initiates increased biosynthesis of arginine and several polyamines that lead to a subsequent increased potential for cell division after the release of stress conditions (Lovatt et al., 1988). Consequently, when suboptimal flower induction conditions were replaced with a foliar application of low-biuret urea, trees subjected to less than 8 weeks of low temperature or moderate water deficit stress exhibited increased leaf ammonia-N concentration and double the flowering intensity compared to non-N-treated trees (Lovatt et al., 1988). In heavy-fruiting trees and/or under conditions of tree carbohydrate depletion, N accumulated in the nitrate-N form due to fruit-load induced reduced activity of nitrate reductase, which requires energy (Golomb and Goldschmidt, 1987;

Monselise et al., 1983). Cultural practices supplementing heavy-fruiting trees with N therefore make use of applications of the reduced forms of N fertilizers, such as the various forms of ureas, as under these conditions the use of nitrate-N is generally less effective (Golomb and Goldschmidt, 1987).

It is clear that the intensity of fruiting is a strong determinant of the eventual mineral nutrient status of citrus trees. Other than the role for the level and form of available N in facilitating cell division following stress (Lovatt et al., 1988), very few reports provide evidence to support a direct regulative role of mineral nutrients in facilitating a meristem's transition from a vegetative to reproductive state under conditions of alternate bearing. Smith (1976) acknowledged this and concluded that plant mineral nutrient status is considered a result rather than a cause of severe alternate bearing and "tree collapse" in 'Murcott' mandarin. Also, the level to which fruit consume mineral nutrients at the expense of initiation and maintenance of growth of other tree organs that support citrus reproductive development, such as new vegetative shoot and root growth, are not yet elucidated. Martínez-Alcántara et al. (2015) reported that for alternate bearing 'Moncada' mandarin, fruit limited new vegetative shoot flush and therefore the subsequent flowering sites, but the response was not attributed to N limitation by fruit.

3.2. The hormonal theory of alternate bearing

3.2.1. Auxins (IAA)

The main endogenous auxin in citrus plant tissue is 1 *H*-indole-3-acetic acid (IAA) (Goldschmidt, 1976; Koshita et al., 1999; Verreynne, 2005; Yuan et al., 2003). 1 *H*-indole-3-acetic acid is synthesised in actively growing shoot apical meristems in lemon and sweet orange (Goldschmidt, 1976), in ovaries (Goldschmidt and Leshman, 1971) and petals of 'Satsuma' mandarin flowers (Takahashi et al., 1975), and in young fruit of 'Valencia' sweet

orange (Yuan et al., 2003) and 'Pixie' mandarin (Verreynne, 2005). 1 *H*-indole-3-acetic acid is phloem-transported from the area of synthesis to neighboring fruit (Yuan et al., 2003), leaves (Koshita et al., 1999) and roots (Muday and DeLong, 2001) along its pathway of basipetal, polar transport (Muday and DeLong, 2001) (Fig. 4). Although IAA is not commonly detected in citrus roots (Nehela et al., 2016), auxin transport in plant roots is complex and exhibits two distinct polarities: IAA moves acropetally towards the root apex through the central cylinder, and basipetally from the root apex through the outer layers of root cells (Muday and DeLong, 2001).

While IAA has been shown to inhibit and delay in vivo sprouting of 'Shamouti' sweet orange buds (Altman and Goren, 1974), Yuan et al. (2003) provided convincing evidence for the movement of IAA between different organs of a citrus tree under field conditions. In two mandarin cultivars, viz. 'Pixie' (Verreynne and Lovatt, 2009) and 'Satsuma' (Ehara et al., 1981; García-Luís et al., 1995b), as well as in 'Washington Navel' (Lenz, 1967) and 'Valencia' (Plummer et al., 1989) sweet oranges, fruit were shown to inhibit budbreak and the sprouting of new vegetative shoots during vegetative shoot flush in summer. Verreynne (2005) proved that the basipetal phloem-transport of IAA from young fruitlets to buds during summer was responsible for perpetuating the lack of flowering of "on" trees in 'Pixie' mandarin, as high endogenous IAA inhibited vegetative shoot development from lateral buds in a mechanism similar to the correlative inhibition of a terminal shoot tip on lateral or axillary bud development, called apical dominance (Bangerth, 1989; Cline, 1991; Dun et al., 2006).

In the classical apical dominance theory (Dun et al., 2006), IAA that is loaded into the shoot by the terminal bud or fruit at the shoot apical meristem establishes an IAA transport stream that is necessary to manifest the bud's competence as a carbohydrate sink (Cline, 1991; Dun et al., 2006). Once loaded in the phloem the IAA transport stream from the

terminal bud or fruit limits the outflow of IAA from axillary buds and prohibits the formation of the lateral bud's own IAA transport stream into the main stem (Bangerth, 1989; Li and Bangerth, 1999; Morris, 1977). Another hypothesis states that high IAA concentration affects new vegetative shoot development from lateral buds by directly regulating the concentration of other phyto-hormones, such as cytokinins (Bangerth, 1989, Morris, 1977; Nordstrom et al., 2004). Proof of this was provided by the increased cytokinin concentration measured in the xylem sap upon removal of the shoot apical meristem, and removal of the IAA supply to buds (Bangerth, 1994; Li et al., 1995; Tanaka et al., 2006; Turnbull et al., 1997). Some reports also suggest that during apical dominance, IAA might directly inhibit cytokinin synthesis in lateral buds, which causes inhibition of lateral bud sprouting (Nordstrom et al., 2004).

Apart from IAA's role during periods of vegetative shoot flush, there are also suggestions, albeit contrasting, of a molecular role for IAA in the up- or down-regulation of a meristem's transition from a vegetative to a reproductive state (Bangerth, 2009; Koshita et al., 1999; Shalom et al., 2012, 2014). 1 *H*-indole-3-acetic acid concentration increased in leaves of "off" 'Satsuma' mandarin shoots in response to a girdling treatment during flower induction, and shoots produced increased leafless inflorescences and a higher number of flowers in the subsequent spring (Koshita et al., 1999). In contrast, down-regulation of the expression of flowering-related genes during flower induction in "on" shoots of 'Murcott' mandarin was proposed to be a result of higher IAA concentration in the buds of fruiting shoots (Shalom et al., 2012, 2014). Bangerth (2006) proposed that IAA could act as a secondary messenger of floral inhibition to GA during flower induction, because IAA is the only plant hormone with a strictly polar and regulated transport pathway (Muday and DeLong, 2001), and independent of sink- or transpiration driven transport (Bangerth, 2009).

In citrus, however, export of IAA from fruit as the source declines during fruit development. In developing 'Satsuma' mandarin fruit, auxin activity peaked at 10 days after full bloom whereafter the concentration of IAA in fruitlets rapidly declined to undetectable levels 40 days after full bloom (Takahashi et al., 1975). This is in agreement with Gustafson (1939), who detected high IAA concentration in ovaries of sweet orange flowers during anthesis and in young fruit during the fruit set period, with ovaries of seedless lemon cultivars, interestingly, containing a higher concentration of IAA than cultivars that are seeded (Gustafson, 1939). Nevertheless, in agreement with Gustafson (1939), Takahashi et al. (1975) reported a rapid reduction in the concentration of IAA in fruitlets shortly after the fruit set period. Higher concentration of IAA in young fruit compared with mature fruit was also reported for 'Valencia' sweet orange fruit. In addition, a higher IAA was exported from young fruit (Yuan et al., 2003); while Koshita et al. (1999) found no differences in the IAA concentration in leaves on fruiting and non-fruiting 'Satsuma' mandarin shoots when fruit were fully mature.

Since the majority of studies indicate that export of IAA from fruit as its source declines as the fruit matures (Gustafson, 1939; Takahashi et al., 1975; Yuan et al., 2003), it is difficult to expect any significant role for IAA in altering meristematic flowering response at this late stage of fruit development. The role of fruit as a source of GAs and ABA, as well as the fruit's influence on carbohydrate availability and plant nutritional status might play a more prominent role at this late development stage. It is evident that young fruit are sources of high concentrations of IAA and fruit impose a hormonal inhibition on return bloom early in their development, by restricting the formation of new potential flowering positions in the form of new spring and summer vegetative shoots.

3.2.2. Cytokinins

The most commonly detected forms of cytokinins in citrus tissues are zeatin, dihydrozeatin (dhZ) and ribosyl-zeatin, as well as the zeatin precursor isopentenyladenine (2iP) (Davenport, 1990, Erner et al., 1976; Hendry et al., 1982b; Hernandez Miñana et al., 1989). Zeatin is a highly active cytokinin base and can have a cis- or trans-configuration (Van Staden and Drewes, 1991). According to Van Staden and Drewes (1991), cis-zeatin has much lower cytokinin activity in plants than the zeatin trans-isomer due to the existence of a cis-trans-isomerase enzyme that rapidly converts cis-zeatin to trans-zeatin. Cytokinin ribosides are intermediates in the biosynthesis of active cytokinin bases and are also the major transport forms of zeatin, 2iP and the reversible dhz, a zeatin metabolite (Sakakibara, 2006). Trans-zeatin riboside (t-ZR) is used for long-distance transport of cytokinins from roots to shoots in the xylem and cis-zeatin riboside (c-ZR) and isopentenyl adenosine (iPA) are used for cytokinin transport and signaling in the phloem (Hirose et al., 2008; Sakakibara, 2006).

Zeatin can be conjugated to O-glucosylated forms, viz. t-ZOG and c-ZOG which are both non-active forms of storage cytokinins (Bassil et al, 1993; Mok et al. 2000). During glycosylation, the addition of a sugar molecule modifies the parent zeatin molecule to be successfully stored or transported (Sakakibara, 2006). Active cytokinin bases are however detected at extremely low quantities in plant tissue relative to the storage or transportable cytokinins. The conjugated forms are usually physiologically inactive and can accumulate at very high concentrations in cell vacuoles (Bassil et al, 1993; Mok et al. 2000). Hydrolysis of zeatin conjugates by β-glucosidases can rapidly restore the levels of bioactive zeatin, and the process requires much less energy compared to the complete new de novo biosynthesis of cytokinin bases.

In isolation of other phyto-hormones, high cytokinin concentration in plant tissues promotes cell division and stimulates adventitious budbreak (Skoog and Armstrong, 1970; Letham and Palni, 1983). Exogenous applications of cytokinins and cytokinin derivatives enable rapid organogenesis in tissue-culture (Takahashi et al., 1975), and can stimulate vegetative growth (Nauer et al., 1979; Nauer and Boswell, 1981) and increase fruit set and fruit size in perennial fruit trees (Ferrer et al., 2017).

Although cytokinin biosynthesis in citrus can occur in actively growing tissues such as seeds and young fruitlets, and in leaves and buds (Van Staden and Davey, 1979), the main source of cytokinin synthesis is the apex of actively growing roots, from where they are exported to shoots via the transpiration stream in the xylem (Bangerth, 1994; Dixon et al., 1988; Saidha et al., 1983; Van Staden and Davey, 1981) (Fig. 4). Maintenance of a healthy tree canopy is therefore highly dependent on root growth (Bevington and Castle, 1985; Hendry et al., 1982a; Van Staden and Davey, 1979). In peach, for example, reduced shoot growth was caused by reduced xylem transported, root-supplied promotive growth substances (Cutting and Lyne, 1993) and in litchi, root growth and subsequent root-produced cytokinins were shown to strongly influence budbreak and vegetative shoot development (O'Hare and Turnbull, 2004). In studies in citrus, inhibition of bud sprouting during summer corresponded with lower cytokinin levels in buds of "on" 'Pixie' mandarin trees compared with "off" trees (Verreynne, 2005; Verreynne and Lovatt, 2009), but treatment of "on" shoots with 2iP failed to induce lateral bud sprouting in "on" shoots (Verreynne, 2005). Davenport (1990), however, pointed out that the vegetative response to exogenously-applied cytokinins might be cultivar and time-dependent, as well as on the specific type of synthetic cytokinin. Application of N6-benzyladenine and 6-(benzylamino)-9-(2-tetrahydropyranyl)-9H-purine for example, successfully induced lateral bud sprouting in sweet orange (Nauer et al., 1979;

Nauer and Boswell, 1981) and in 'Tahiti' lime (*C. aurantifolia* Christm.) (Davenport, 1990), but kinetin and 2iP treatments were ineffective.

No direct evidence exists that supports a direct involvement of cytokinins in alternate bearing in citrus. However, the known interaction of fruit load and root growth in citrus, might influence the subsequent production of the necessary levels of endogenous cytokinin required to stimulate the development of new vegetative shoots and adequate flowering sites during return bloom, especially considering the well-documented interaction between roots and vegetative shoot growth (Bevington and Castle, 1985).

3.2.3. Abscisic acid (ABA)

In citrus, ABA can be synthesised from β,β-carotenoids in fruit rinds and seeds (Goldschmidt, 1976), in mature leaves (Manzi et al., 2015) and in roots (Davies and Zhang, 1991). Abscisic acid regulates, among others, organ abscission, leaf stomatal conductance and state of dormancy of various plant tissues, especially in water-stressed plants (Manzi et al., 2016; Tardieu et al., 1996; Yuan et al., 2003) (Fig. 4).

To maintain a constant concentration of bioactive ABA in a particular plant tissue, excess ABA can be catabolised to downstream metabolites 7'-hydroxy-abscisic acid, phaseic acid and dihydrophaseic acid (Seiler et al., 2011). The abscisic acid glucose ester, ABA-GE is a conjugated form of ABA, generally considered as an inactive storage form of the bioactive ABA (Goodger and Schactman, 2010; Priest et al., 2006). The glucose ester of ABA can however be hydrolysed and converted to bioactive ABA as suggested by Manzi et al. (2015).

In addition to water-stressed plants, ABA can also be synthesised in different tissues of well-watered and fruiting citrus plants, and its content can be affected by factors that are unrelated to soil water status (Monselise and Goldschmidt, 1981). In well-watered and

alternate bearing 'Valencia' sweet orange trees, for example, Jones et al. (1976) reported a higher concentration of the ABA precursor 2-trans-4-trans ABA in buds of heavy-fruiting trees compared with low-fruiting trees. In agreement, Shalom et al. (2014) reported significantly lower concentrations of ABA and its catabolites in buds from well-watered "off" and de-fruited 'Murcott' trees compared with "on" trees. In well-watered alternate bearing 'Wilking' mandarin trees Goldschmidt (1984) reported significantly higher ABA concentration in leaves and dormant buds of "on" trees. In fact, "on" tree leaves contained higher ABA concentration than that of typically water-stressed plants, and the upregulated ABA biosynthesis was attributed to an unknown, but special type of stress-related response to excessive fruiting that is unique to alternate bearing citrus trees (Monselise and Goldschmidt, 1981).

Besides roots and leaves, alternative sources of synthesis of bioactive ABA in citrus, and the transport thereof to neigbouring organs in alternate bearing trees have not been conclusively documented. It could be possible that ABA is produced by fruit rinds and seeds (Goldschmidt, 1976; Jones et al., 1976) and transported to leaves via the phloem. Phloem transport of ABA from fruit as the source is supported by Koshita et al. (1999) who reported that ABA accumulated in leaves of girdled and fruiting 'Satsuma' mandarin branches. In agreement, Shalom et al. (2014) reported reduced concentrations of ABA and its catabolites in de-fruited 'Murcott' mandarin buds, and attributed the response to the absence of fruit. It is, however, difficult to consider fruit as the source of excess ABA in leaves, since ABA transport occurs in a passive manner and mass-flow in the phloem under heavy-fruiting conditions would generally be directed towards and not away from fruit. For example, Yuan et al. (2003) reported that ABA levels in leaves were not affected by fruit removal, thereby supporting the notion that fruit are unlikely the main source of high ABA concentration in leaves.

The physiological response of both well-watered and water-stressed plants to high ABA concentration nevertheless appears to be consistent. However, higher ABA concentration in tissues of well-watered and heavy-fruited citrus trees rules out a role for water deficit stress on altered ABA biosynthesis as reported for in non-fruiting and water-stressed citrus plants (Manzi et al., 2015). The mechanism of ABA homeostasis under alternate bearing and sufficiently-irrigated conditions might therefore be different. It might be possible that a lack of root growth in "on" trees could result in de novo ABA biosynthesis and signaling from carbohydrate-stressed roots to leaves (Bower et al., 1990), which contributes to an inhibition of, or a general reduction in bud sprouting in "on" trees.

3.2.4. Gibberellins (GAs)

Fruit rinds and developing seeds, as well as vegetative plant tissues and growing roots are major sources of endogenous GA synthesis in citrus (Erner et al., 1976; Koshita et al., 1999; Kawarada and Sumiki, 1959; Talon et al., 1990b; Wallerstein et al., 1973) (Fig. 4). Some of the first isolations of GA from plant tissues were that of GA_1 from leaves in vegetative shoots of 'Satsuma' mandarin (Kawarada and Sumiki, 1959). Seasonal determinations of naturally occurring GAs in bark and shoots of 'Shamouti' sweet orange revealed a large increase in the concentration of compounds with GA-like activity towards spring, with the sources of synthesis believed to most likely be that of shoot meristems and roots (Wallerstein et al., 1973). More recent isolations of endogenous GAs from vegetative tissues revealed the most common forms of GAs in buds of 'Salustiana' sweet orange to be that of GA_1, GA_{19}, GA_{20} and GA_{29} (Talon et al., 1990b), and GA_1 and GA_3 in leaves of 'Satsuma' mandarin (Koshita et al., 1999).

The earliest reports of fruit as the source of GA synthesis in citrus were those of Wiltbank and Krezdorn (1969) and Goldschmidt and Galily (1974), whereafter Monselise

and Goren (1978) isolated GA_1 and GA_9 from young 'Washington Navel' sweet orange and 'Eureka' lemon fruit. In citrus fruit, GAs are synthesised in the rind (Monselise and Goren, 1978), more specifically in the flavedo (Erner et al., 1976), while high concentrations of GAs are also detectable in ovaries (Talon et al., 1990a, 1990b) and seeds (Monselise and Goldschmidt, 1982). The main forms of GAs in citrus ovaries of seeded and strongly parthenocarpic cultivars are GA_1, GA_3, GA_8, GA_{20} and GA_{29}, however, in weakly parthenocarpic and/or seedless cultivars only low levels of GA_3 could be detected (Talon et al., 1990a, 1990b).

The effects of exogenous GAs on flower development in citrus are well-documented. The most familiar of these is a lack of inflorescences and return bloom following application of GA_3 under conditions favorable for flower induction (Goldschmidt et al., 1985; Guardiola et al., 1982; Monselise and Halevy, 1964). Winter GA treatments reduced leafless inflorescences, promoted leafy inflorescences and increased vegetative shoots in 'Satsuma' and 'Clementine' mandarins (Guardiola et al., 1982). In 'Orri' mandarin the same treatments reduced both leafless and leafy inflorescences and increased vegetative shoots (Goldberg-Moeller et al., 2013); whereas a total reduction in flower numbers, irrespective of inflorescence types, was obtained in 'Shamouti' and 'Salustiana' sweet oranges (Monselise and Halevy, 1964; Muñoz-Fambuena et al., 2012).

Recently the mode of action of inhibition by GA_3 application during flower induction was shown to be a reduction in the expression of *CiFT* in the leaves and buds of 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012). A similar response of inhibition of *CiFT* expression to foliar GA_3 treatments was obtained in 'Orri' mandarin (Goldberg-Moeller et al., 2013). Although elevated levels of naturally occurring endogenous GA_1 and GA_3 during flower induction in leaves of fruiting shoots of 'Satsuma' mandarin correlated strongly with

reduced flowering response (Koshita et al., 1999), it is not yet clear if the translocation of endogenous GAs from fruit and roots fulfills a similar role to exogenous GA_3.

The bi-directional translocation and inhibiting effect of endogenous GAs on flowering response across long distances has not yet been elucidated (Bangerth, 2009), but the known direct regulatory role of GAs on *CiFT* expression inhibition might conform to the evolutionary adaptation of perennial plants that restricts saturation of buds by flowers, and allows for sprouting of vegetative shoots to maintain plant longevity beyond two seasons (Bangerth, 2009; Thomas et al., 2000). The current role for GAs in the hormonal theory of alternate bearing in citrus suggests that high endogenous GAs, most likely synthesised in the fruit rind and seeds, are translocated to leaves and/or buds where it directly inhibits expression of the *CiFT* gene. The response manifests as a lack of inflorescences and poor return bloom.

4. Concluding perspectives: alternate bearing in citrus

The causes of alternate bearing in citrus appear to be of a complex and combinative nature. There seems to be conspicuous causal factors such as seediness and time of harvest that are unique to certain species, but, as a whole, discrepancies exist between these and their direct roles in initiating and maintaining alternate bearing in citrus. Alternate bearing in most citrus species seems to manifest as an effect of either or both of the following two primary causal factors: a limitation in the development of new potential flowering sites; and/or the inhibition of expression of the primary citrus flowering gene *CiFT*.

4.1. Inhibition of summer vegetative shoot development

Endogenous IAA transmitted from fruit, inhibits sprouting of lateral buds and subsequent summer vegetative shoot development – the potential sites for return bloom

flowers (Verreynne and Lovatt, 2009). However, according to Martínez-Alcántara et al. (2015), the presence of fruit during summer also restricts carbohydrate allocation to, and subsequent sprouting of buds. In both schools of thought, fruit remain the principal inhibitor of summer vegetative shoot development and is therefore the primary determinant of return bloom flowering. In the absence of fruit, IAA treatment of vegetative shoots with similar carbohydrate status resulted in the same sprouting inhibition response as that obtained in the presence of fruit (Verreynne, 2005). 1 *H*-indole-3-acetic acid therefore appears to override the role of carbohydrates in this regard. Three questions regarding the role of IAA and other hormones still remain to be answered, and would provide significant further insight to their roles in alternate bearing: (1) Do endogenous hormones have a carry-over effect after the removal of their source of synthesis? There appears to be uncertainty as to whether or not vegetative tissues carry 'memory' of endogenous hormones. Some evidence suggests this, with the detection of higher IAA concentration in buds from "on" shoots compared to "off" shoots during very late periods of fruit development, when fruit are not synthesizing IAA anymore (Shalom et al., 2012; 2014). On the other hand, fruit removal and subsequently also the IAA-transporting source results in immediate vegetative sprouting of lateral buds on parent shoots (Verreynne, 2005), and would suggest the contrary. (2) To what degree are citrus shoots and branches autonomous and how is autonomy initiated and maintained? Considering that fruit-transmitted IAA is only transported in a polar basipetal direction, the influence of fruit-transmitted IAA and other endogenous hormones on neighboring shoots, branches and/or tree limbs, are not yet understood. There appears to be a certain level of maintenance of a structural autonomy within citrus trees – the well-documented alternate fruiting habit on a shoot and branch level (Monselise and Goldschmidt, 1982; Monselise et al., 1983), and results from experiments tracing supplemented labelled compounds confirm this (Yuan et al., 2003). Experiments on this question are difficult due to the unavoidable

sharing of a mutual root system, and the determination of how an autonomous growth habit manifests and to what extent it reaches, still needs to be addressed. (3) Can cytokinin overcome the inhibition of IAA on bud sprouting, i.e. is inhibition of bud-sprouting a factor of high endogenous IAA concentration, or also high concentration of other inhibitors such as ABA, which would support a 'hormonal balance' concept that was suggested by Goldschmidt (2015). Verreynne (2005) and Verreynne and Lovatt (2009) reported a lower ratio of the cytokinin to IAA concentration in buds of "on" trees compared to "off" trees as a result of the polar basipetal transport of IAA from fruit, which manifested in an inhibition of bud sprouting, reduced summer vegetative shoot flush and poor return bloom (Verreynne, 2005). Treatment of "on" shoots with a synthetic cytokinin 2iP, however, failed to induce lateral bud sprouting in "on" shoots (Verreynne, 2005). Davenport (1990) reported that the vegetative response to exogenously-applied cytokinin might be cultivar and time-dependent, and on the specific type of synthetic cytokinin used. Alternatively, an inhibition of summer vegetative shoot development in citrus may also possibly result from the synthesis and translocation of an alternative inhibiting substance such as ABA (Bower et al., 1990; Goldschmidt, 1984).

4.2. Inhibition of floral gene expression

Both low temperature and water deficit stress induce flower induction (Chica and Albrigo, 2013; Valiente and Albrigo, 2004) and the subsequent expression of citrus floral genes in vegetative tissues (Nishikawa et al., 2007; Tang, 2017). Both environmental conditions require the cessation of active shoot and root growth in late autumn. Under conditions of uninterrupted root and shoot growth, *CiFT* expression is restricted or even completely inhibited (Nishikawa, 2013). The only endogenous substance(s) proven as an inhibitor of the expression of floral genes in citrus are GAs (Goldberg-Moeller et al., 2012; Muñoz-Fambuena et al., 2012). A lack of flower development in the absence of fruit, but

under conditions of continuous growth, such as obtained under tropical conditions, could therefore be a result of *CiFT* inhibition by an uninterrupted GA signal from actively growing roots and/or shoot tips. In a non-fruiting shoot, the temporary interruption of phloem transport by girdling normally increases flowering above the girdle (Cohen, 1981) and would conform to the concept that the GA responsible for floral inhibition in the absence of fruit is transported from actively growing roots. However, the exclusion of actively growing roots as the suspected source of GAs, by girdling or water deficit stress in a heavy fruiting scenario, are generally less effective, even if carbohydrate levels in the source tissues are high (García-Luís et al., 1995b; Goldschmidt et al., 1985; Koshita et al., 1999). It therefore seems plausible that the reduced flowering intensity during return bloom following an "on" year is a result of high endogenous GAs that inhibited floral bud development in the presence of fruit, but that the GAs responsible for this effect seems to be mainly produced and transported from fruit, especially considering that both shoot and root growth are generally absent in heavily-fruiting trees and in winter, and would not be a source of GA synthesis during the normal period of flower induction. Although this seems to be the most likely scenario, it is not yet conclusive. For GA to be transported away from a terminal fruit to lateral buds and leaves, the hormone would have to be actively transported, since mass-flow in the phloem would be directed towards and not away from fruit. An active method of GA transport has, however, not yet been established (Bangerth, 2009).

As opposed to endogenous inhibitors of *CiFT*, a line of thought not yet considered is that of a possible endogenous stimulator(s) or initiator(s) of *CiFT* expression. A hypothesis could be that an endogenous substance(s) is produced by non-growing shoot and root tips, and transmitted via the xylem to buds and leaves where it directly stimulates or initiates *CiFT* expression. However, currently under optimal flower induction conditions, and in a fruiting scenario, endogenous GAs, supposedly from the fruit, still seem to override this signal

(García-Luís et al., 1995b; Goldschmidt et al., 1985; Koshita et al., 1999; Shalom et al., 2012, 2014).

4.3. Practical considerations

Altogether, fruit load remains the most important determinant of return bloom and intensity of alternate bearing in citrus, due to its influence on the formation of new bearing shoots and the inhibition of flower development. Fruit load is primarily determined by the ability of a citrus tree to produce flowers, but can also be a factor of the level of fruit set as influenced by spells of environmental extremities such as heat, water stress, hail and frost, and a cultivar's natural ability to set fruit as influenced by sexual fertilisation and subsequent seed development. However, fruit set can be manipulated through the implementation of specific cultural practices such as foliar application of GA_3 (Rivas et al., 2006; Schaffer et al., 1985) and trunk girdling (Rabe and Van Rensburg, 1996), or by chemical (Agustí et al., 2002) or manual (Stander and Cronjé, 2016) fruit thinning.

Except for pre-bloom, foliar-applied low-biuret urea to increase flowering potential under suboptimal flower induction conditions (Lovatt et al., 1988; Lovatt, 2013), the majority of current commercial practices to manage alternate bearing are based on increasing flowering potential by reducing fruit load in an "on" year, or by optimising fruit set during an "off" year. Currently, in citrus, no commercial practice can directly increase flowering and eliminate alternate bearing subsequent to an "on"-year. Winter girdling of "off" trees can increase the numbers of flowers and eventual fruit (Cohen, 1981; Schaffer et al., 1985), but since fruit are also major sources of phloem-transported hormones (Erner et al., 1976; Talon et al., 1990b; Verreynne, 2005), the flowering response to winter girdling is typically weak or absent when applied to "on" trees (García-Luís et al., 1995b; Goldschmidt et al., 1985; Koshita et al., 1999).

With the roles of IAA and GA identified in alternate bearing in citrus there is scope for testing compounds with anti-IAA and anti-GA activity during summer and winter, respectively, with the goal of optimising return bloom following an "on" year. Paclobutrazol has been reported to inhibit GA biosynthesis in evergreens such as mango (Blaikie et al., 2004) and in deciduous fruit trees such as pear (Asín et al., 2007). In citrus, a foliar spray with paclobutrazol during floral bud development in 'Satsuma' mandarin reduced the activities of GA_{20} and GA_{19}, both intermediates in the synthesis of active GAs such as GA_1, GA_3 and GA_7 (Yamaguchi, 2008) in leaves, and increased the number of flowers (Ogata et al., 1996). Muñoz-Fambuena et al. (2012) reported that citrus trees treated with paclobutrazol increased the expression of flowering genes and conversion of apical meristems to flowers. However, because the effect of triazoles on flowering is reversible by endogenous GA (Martínez-Fuentes et al., 2013), a variation in effectivity in flowering promotion has been reported in sweet orange (Delgado et al., 1986a; Martínez-Fuentes et al., 2004; Moss, 1970), mandarin (Delgado et al., 1986b; Martínez-Fuentes et al., 2004), lime (Davenport, 1983) and in kumquat (*Fortunella crassifolia* Swingle × *F. margarita* Swingle) (Iwahori and Tominaga, 1986).

Foliar or root applications of cytokinin and ABA could also provide novel insights into alternate bearing and the related physiological processes, and provide significant commercial solutions to citrus growers, especially considering the well-documented interaction of fruit load with roots and vegetative shoot growth (Bevington and Castle, 1985). However, in citrus, studies with cytokinins have mostly been conducted in tissue-culture or in potted and non-fruiting citrus trees (Hendry et al., 1982a, 1982b; Van Staden and Davey, 1979), and the role of ABA is yet to be demonstrated in alternate bearing (Goldschmidt; 1984; Jones et al., 1976; Shalom et al., 2014).

Table 1. A summary of studies conducted on alternate bearing in *Citrus* spp. post the review by Monselise and Goldschmidt (1982).

Cultivar	Time of harvest (Southern hemisphere)	Principle finding	Reference
‘Satsuma’ mandarin (seedless)	March to April	Fruit inhibitted expression of the gene *FLOWERING LOCUS T* (*CiFT*) in late autumn, which resulted in poor return bloom;	Nishikawa et al. (2016)
		Carbohydrates did not limit flowering, but played an important role in flowering intensity.	García-Luís et al. (1995b)
		Some undetermined role for leaf ABA content during flower initiation in strongly alternate bearing trees in an “on” cycle.	Okuda (2000)
‘Pixie’ mandarin (low-seeded)	June to August	Fruit as source of IAA inhibitted summer vegetative shoot flush and return bloom flowering.	Verreynne (2005); Verreynne and Lovatt (2009)
‘Kinnow’ mandarin (seeded)	July to September	Low K and P in “on” trees correlated significantly with poor return bloom.	Mirsoleimani et al. (2014)
‘Nadorcott’ mandarin (low-seeded)	July to August	Leaf starch levels in late autumn negatively correlated with fruit yield and positively with return bloom.	Van der Merwe (2012)
		Summer fruit thinning eliminated alternate bearing.	Stander and Cronjé (2016)
‘Murcott’ mandarin	July to	Fruit load affected bud fate by altering expression of flowering control genes in buds; buds from de-fruited and/or “off” trees had	Shalom et al. (2012, 2014)

(seeded)	September	significantly lower IAA and ABA during critical stages of flower development as opposed to “on” buds;	
		‘Murcott’ achieved excessive levels of fruit set (poor self-thinning ability) which led to over-bearing in an “on” year, tree collapse (‘Murcott collapse’), and a subsequent poor return bloom and an “off” year.	Schaffer et al. (1985)
		Fruit inhibitted shoot growth by limiting carbohydrate partitioning to, and utilization by new summer vegetative growth.	Martínez-Alcántara et al. (2015)
		Fruit inhibitted flowering by suppressing *CiFT* and *SOC1* gene expression in leaves.	Muñoz-Fambuena et al. (2011, 2012)
‘Moncada’ mandarin (seeded)	October to December	The largest groups of proteins up-expressed in “off” buds during flower induction were those for proteins involved in carbohydrate and amino acid metabolism, while the largest group of proteins up-expressed in “on” buds was related to primary metabolism, oxidative stress and defense responses.	Muñoz-Fambuena et al. (2013a)
		Primary metabolism was more active in “off” leaves during flower induction, while genes up-expressed in “on” leaves were more related to proteins involved in oxidoreductase activity.	Muñoz-Fambuena et al. (2013b)
‘Salustiana’ sweet orange	June to August	Fruit inhibits return bloom. Flowering and CO_2 assimilation are	Monerri et al. (2011)

(low-seeded)		not limited by carbohydrates.	
		Sucrose concentration remains constant in leaves and roots of "on" and "off" trees, but starch accumulates in "off" trees due to fruit altering expression of genes related to starch metabolism.	Nebauer et al. (2014)
		GA_3 inhibits flowering by suppressing *CiFT* gene expression in leaves.	Muñoz-Fambuena et al. (2012)
		Carbohydrates play no role in flower inhibition. Roots contribute significantly to the carbohydrate balance and metabolism of a tree.	Dovis et al. (2014)
'Valencia' sweet orange (seeded)	September to November	Fruit inhibit flowering from the time they complete their growth.	
		Fruit inhibit leafless inflorescences, have no effect on leafy inflorescences and increase vegetative shoot flush.	Martínez-Fuentes et al. (2010)

Table 2. The origin of vegetative shoot flushes of flower-bearing shoots in various *Citrus* spp. cultivars in different production regions.

Cultivar	Time of the vegetative shoot flush that becomes a typical flower-bearing shoot	Region
'Clementine' mandarin	Summer flush (Krajewski and Rabe, 1995b)	Stellenbosch, South Africa
'Satsuma' mandarin	Spring flush (Ehara et al., 1981)	Ogi-gun, Japan
'Pixie' mandarin	Summer flush (Verreynne and Lovatt, 2009)	California, USA
'Washington Navel' sweet orange	Spring flush (Lovatt et al., 1984)	California, USA
	Summer flush (Guardiola et al., 1982)	Valencia, Spain
'Shamouti' sweet orange	Spring flush (personal correspondence; Avi Sadka)	Jaffa, Israel
'Hamlin' sweet orange	Summer flush (Valiente and Albrigo, 2004)	Florida, USA
'Valencia' sweet orange	Summer flush (Valiente and Albrigo, 2004)	Florida, USA

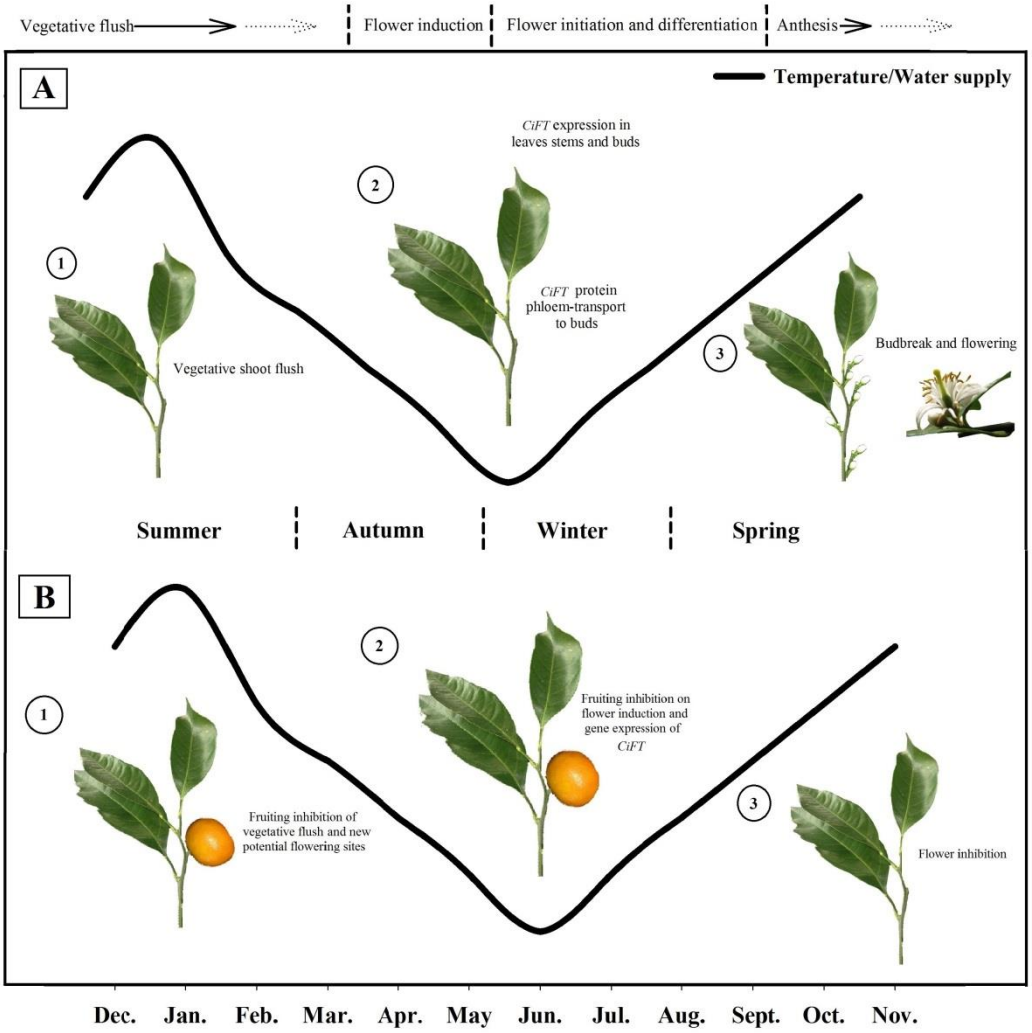

Fig. 1. A conceptual model summarising the interactions of environmental conditions and plant endogenous factors, and influence on flower developmental events in the vegetative (A) and reproductive (B) phases in *Citrus* spp. under subtropical conditions in the Southern hemisphere. A: Vegetative growth flushes in spring, summer and autumn provide the positions for flowers in the subsequent spring (1). Low temperature or water deficit stress initiate flower induction and the expression of the gene citrus *FLOWERING LOCUS T* (*CiFT*) in newly formed shoots, and the transport of *CiFT* protein to buds (2). After flower initiation and differentiation, flowers develop at the onset of spring (3). B: Fruit inhibit sprouting of new vegetative shoots and potential for optimal flower-site development (1). Fruit inhibit flower induction and the expression of *CiFT* (2). The result is a lack of flowers and fruiting potential in the subsequent spring (3).

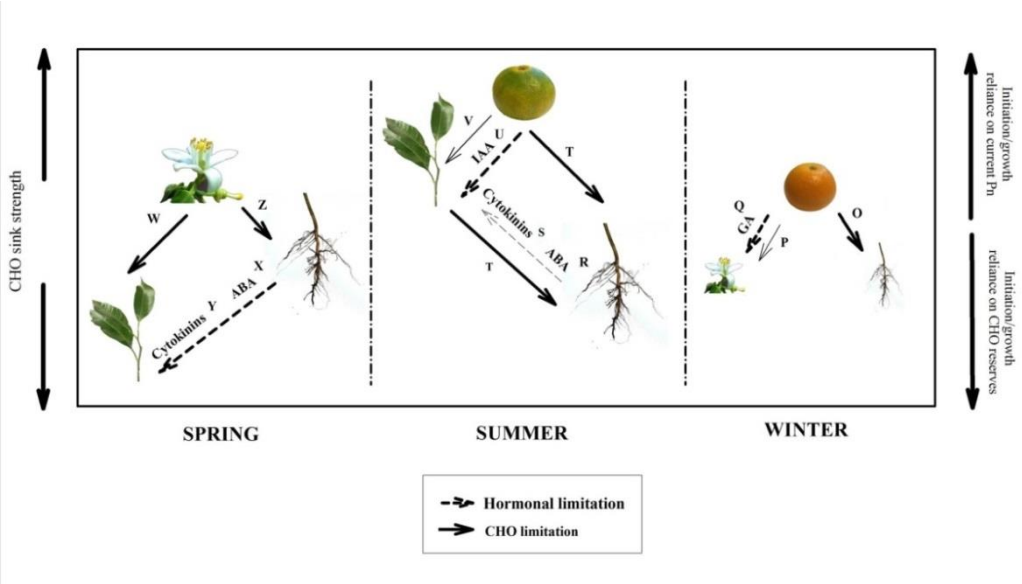

Fig. 2. An integrated model for alternate bearing in *Citrus* spp. which considers the roles of hormones, abscisic acid (ABA), cytokinins, gibberellins (GAs) and 1 *H*-indole-3-acetic acid (IAA), and carbohydrates (CHO). In spring, flowers are the major carbohydrate sink, and rely on photosynthesis (Pn) and CHO reserves. Profuse flowering and fruit set inhibits root growth and vegetative shoot flush in "on" trees. In summer, fruit are the major CHO sinks and rely strongly on CHOs from Pn for growth. Fruit inhibit sprouting of new vegetative shoots as well as root CHOs. With CHO requirement being low in winter, fruit rely mostly on current Pn and restrict flower developmental and reserve CHO accumulation in roots.

[Z] Bustan and Goldschmidt (1996); Dovis et al. (2014); Duncan and Eissenstat (1993)

[Y] Davenport (1990); Mataa and Tominaga (1998);

[X] Goldschmidt (1984)

[W] Reed and MacDougal (1938); Nebauer et al. (2014); Sanz et al. (1987)

[V] Lenz (1967); Martínez-Alcántara et al. (2015)

[U] Ehara et al. (1981); Plummer et al. (1989); Verreynne and Lovatt (2009)

[T] Goldschmidt and Golomb (1982); Goldschmidt (1997); Smith (1976)

[S] Bevington and Castle (1985); Cossmann (1939); Marloth (1949); Reed and MacDougal (1938)

[R] Goldschmidt (1984)

[Q] Koshita et al. (1999); Muñoz-Fambuena et al. (2011)

[P] Cohen (1981); García-Luís et al. (1995b); Goldschmidt et al. (1985)

[O] Dovis et al. (2014); Monerri et al. (2011).

Fig. 3. In *Citrus* spp., leaves and buds can generate a flowering signal and not necessarily only either of the two. Citrus trees can therefore have a hysteranthous flowering response, i.e. they can sprout flowers in the absence of leaves, as is the case for these 'Fairchild' mandarin (*C. reticulata*) trees grown in Citrusdal in South Africa. Photographed in Sept. 2015.

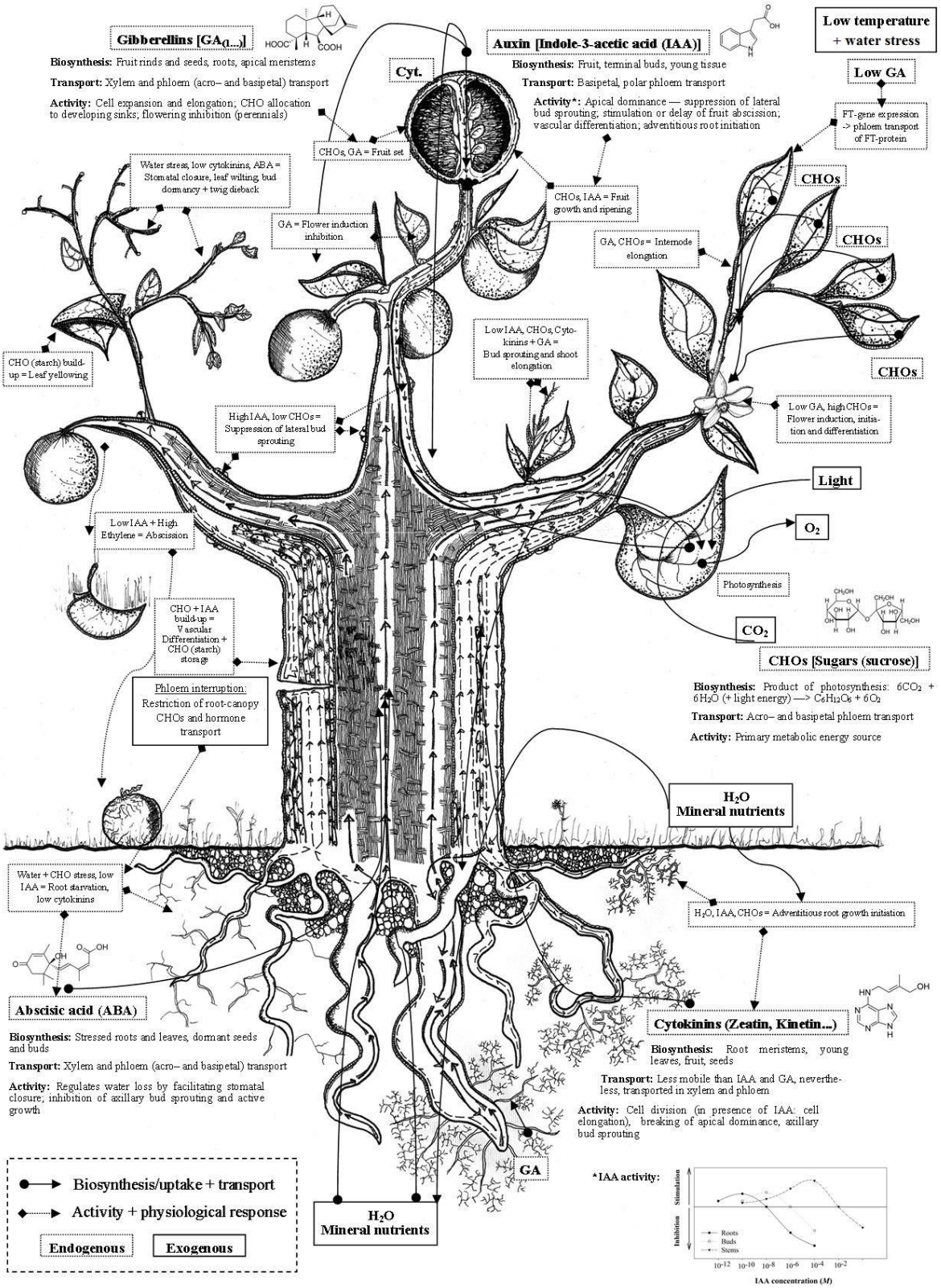

Fig. 4. A generalised diagram summarising the interaction of plant endogenous hormones and carbohydrates (CHOs), on the reproductive physiology of *Citrus* spp. as influenced by fruiting status, plant stress, and mineral nutrient and water supply.

Chapter 2: The role of carbohydrates in the nutritional theory of alternate bearing in *Citrus* spp.

Abstract. The objective of this study was to investigate the role of carbohydrates in the nutritional theory of alternate bearing in *Citrus* spp. by determining if any relationships exist between seasonal leaf and root carbohydrate concentrations, and selected phenological responses at the shoot-, branch- and tree-level over two seasons in an alternate bearing model cultivar, 'Nadorcott' mandarin (*C. reticultata* Blanco). Fruit load and the number of newly developed vegetative shoots were the most important determinants of return bloom. Sprouting of a higher number of new vegetative shoots from "off" shoots, "off" branches and "off" trees was unrelated to leaf carbohydrate concentration. Fruit load and root sugar concentration provided the best correlations with the intensity of vegetative shoot flushes. Root sugar concentration peaked during full bloom and higher root growth activity was observed prior to a higher number of new vegetative shoots developing in "off" trees during summer. The root sugar concentration early in the season was lower and root and shoot growth were absent, or lower in "on" trees. These results concur with previous research and confirm that lack of vegetative shoot development is a contributing cause of poor flowering and unrelated to leaf carbohydrates. These results indicate that fruit are the major carbohydrate sink and probably disturb the balance between vegetative shoot development and root growth by limiting carbohydrate allocation to roots. The study emphasizes that lack of vegetative shoot development in "on" trees cannot be interpreted independently from the effects of excessive fruiting on roots, and that the role of carbohydrates in the nutritional theory of alternate bearing in citrus is probably of secondary importance.

1. Introduction

Alternate or biennial bearing in perennial fruit trees is the synchronised tendency to flower profusely and produce an excess amount of fruit in one season followed by a sparse number of flowers and fruit in the following season (Monselise and Goldschmidt, 1982). Thereafter, the alternate bearing cycle continues in subsequent seasons – seasons of heavy fruiting are referred to as "on" years, whereas seasons of low fruit numbers are called "off" years. Irregular bearing is when a tree produces flowers and fruit in an irregular pattern of seasonal intensity, with more than one season of low fruit yields following an "on" year, or vice versa (Monselise and Goldschmidt, 1982). Alternate bearing in *Citrus* spp., however, is more commonly reported than irregular bearing and can manifest at the shoot-level, on individual branches or trees, or across entire production regions (Monselise and Goldschmidt, 1982). Alternate bearing occurs in deciduous fruit and nut trees such as apple [*Malus* × *sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] (Guitton et al., 2012), pear (*Pyris communis* L.) (Jonkers, 1979), pecan [*Carya illinoinensis* (Wangenh.) C. Koch] (Wood et al., 2004), pistachio (*Pistachia vera* L.) (Rosecrance et al., 1998) and prune (*Prunus domestica* L.) (Davis, 1931), but is more common in evergreen fruit trees, e.g. avocado (*Persea americana* Mill.) (Garner and Lovatt, 2008), citrus (Monselise and Goldschmidt, 1982), coffee (*Caffea arabica* L.) (Vaast et al., 2005), litchi (*Litchi chinensis* Sonn.) (Menzel, 1983), mango (*Mangifera indica* L.) (Souza et al., 2004) and olive (*Olea europaea* L.) (Bustan et al., 2011).

Factors responsible for the initiation and maintenance of alternate bearing appear to be complex and of a combinative nature, and the fundamental cause(s) is an enigma (Bangerth, 2009). In certain citrus cultivars with a high tendency for alternate bearing, the phenomenon first seemed to have conspicuous causal factors, viz. the level of seediness and time of harvest (Monselise and Goldschmidt, 1982). However, strong discrepancies have since been

reported for these factors to be accepted as a rule, since in other cultivars with the same attributes, alternate bearing can be non-prolific or totally non-prevalent (Sanderson and Treeby, 2014). Alternate bearing has also been reported in some seedless e.g. 'Shamouti' sweet orange [*C. sinensis* L. (Osb.)] (Schaffer et al., 1985) and early-maturing, e.g. 'Satsuma' mandarin (*C. unshiu* Marc.) (Iwasaki and Owada, 1960; Okuda, 2000) citrus cultivars.

In contrast to the cause of alternate bearing, the mechanism perpetuating alternate bearing appears to be similar for different fruit crops, with the subsequent flowering response determined by the intensity of fruiting. Although fruiting coincides with important phenological events in the majority of alternate bearing crops (Monselise and Goldschmidt, 1982), the alternate bearing habit in citrus is sustained by a lack of flowering in the spring following an "on" year (Davenport, 2000; Goldschmidt and Golomb, 1982; Hield and Hilgeman, 1969), and not due to a negative effect of fruit on fruit set despite adequate flowering (Goldschmidt and Golomb, 1982). Furthermore, fruit impose a flowering inhibition on vegetative buds either on the sprouting of new and potential flowering sites (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009), or during the period of flower induction (Krajewski and Rabe, 1995a; Koshita et al., 1999; Muñoz-Fambuena et al., 2011). Fruit are therefore limiting the number of new vegetative shoots with the potential to undergo flower induction.

Studies on how fruit regulate their inhibitive effect on flowering have produced two generalised theories of alternate bearing – the hormonal theory and the nutritional theory (Bangerth, 2009; Barnett and Mielke, 1981; Davenport, 2000; Goldschmidt, 1999). The hormonal theory of alternate bearing suggests that phyto-hormones such as abscisic acid, gibberellins and 1 *H*-indole-3-acetic acid inhibit either the formation of new vegetative shoots and therefore newly available flowering positions during summer (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009), and/or the expression of citrus flowering genes during

flower induction (Koshita et al., 1999; Muñoz-Fambuena et al., 2011). The nutritional theory of alternate bearing, on the other hand, suggests that the flowering response is dependent on available plant metabolic energy as determined by fruit load, viz. carbohydrates. In the absence of fruit, carbohydrates accumulate in the leaves, bark and roots of a tree and are available for bud sprouting and flower development in the subsequent spring (Dovis et al., 2014, Goldschmidt and Golomb, 1982; Monerri et al., 2011). In heavy flowering and fruiting situations, fruit limit carbohydrate allocation to developing and competing sinks, e.g. vegetative shoots (Martínez-Alcántara et al., 2015) and roots (Smith, 1976), which can negatively impact on tree condition (Smith, 1976), subsequent reproductive development (Dovis et al., 2014) and consistent production of fruit in the long-term.

The objective of this study was to determine whether any relationship exists between leaf CO_2 assimilation, leaf and root carbohydrate concentrations and tree phenological responses in alternate bearing 'Nadorcott' mandarin (*C. reticulata* Blanco) trees. To address this question, parameters of leaf gas exchange, monthly leaf and root carbohydrate concentrations and phenological responses, viz. flowering, vegetative shoot flush, root growth and fruit load were measured at both the tree- and shoot-level in trees of contrasting fruit loads, over a period of two seasons. To test these findings, leaf carbohydrate concentration and phenological events were critically evaluated in response to source/sink manipulations in a time-course study.

2. Materials and methods

2.1. Plant material and experimental site

Ten-year-old 'Nadorcott' mandarin trees grown under commercial conditions and budded onto 'Carrizo' citrange [*C. sinensis* L. (Osb.) × *Poncirus trifoliata* (L.) Raf.]

rootstock were selected from an orchard with a history of alternate bearing in De Doorns (lat. 33°51'S, long. 19°52'E) in the Western Cape Province of South Africa.

Trees were spaced at 5 × 2 m (1000 trees per ha) in a sandy soil with $pH_{(KCl)}$ 4.4. The Western Cape Province of South Africa experiences Mediterranean-type climatic conditions; summer typically occurs from December to February; autumn from March to May; winter from June to August, and spring from September to November. The region receives an annual rainfall of between 400 and 600 mm, with the majority occurring from May to August. The orchards were cultivated, pruned, and sprayed according to good agricultural practices and the trees were watered using a drip irrigation system with four emitters per tree. The total amount of water applied to each tree amounted to ≈4000 L per annum. The fertilizer rate (kg per ha) was based on annual leaf mineral nutrient analysis and potential yield (kg fruit per ha). Nitrogen (N) was annually applied at a rate of 240 kg N per ha, with 25% applied as foliar, 20% as a soil application and 55% was dissolved in the irrigation solution (fertigation) and split uniformly into applications from September to April. The majority of phosphorous (P) and potassium (K) were annually applied at a rate of 12 kg P and 265 kg K per ha, respectively, with the majority applied via fertigation and a small fraction applied by foliar sprays.

2.2. Treatments and experimental design

The experiments were set up as a two-factorial completely randomised design using whole trees (factor 1) and shoots (factor 2) as experimental units (n=10). Heavy- ("on") and low-fruiting ("off") trees were selected based on their contrasting fruit loads. To ensure that trees were uniformly selected, trunk circumferences of individual trees were measured and canopy volumes determined at the beginning of the experiment by measuring tree height,

canopy height and canopy radius in the N, S, E and W directions of each tree. The canopy volume [V (m^3)] was calculated according to the following formula (Burger et al., 1970):

$V = r^2(\pi h - 1.046r)$

r = canopy radius;

h = height of the fruit bearing canopy.

The same trees were used in both seasons. The alternate bearing index (I) of the two treatments with contrasting fruit loads was calculated using the following formula (Gur et al., 1969):

$$I = \frac{1}{(n-1)}\left[\frac{(a2-a1)}{(a2+a1)} + \frac{(a3-a2)}{(a3+a2)} + \cdots\right]$$

n = number of seasons;

a = fruit yield in the corresponding season.

Branch experiments were set up in a randomised complete block design, in which a tree represented a block and a single branch represented a replicate (n=8). Due to a generally strong autonomous phenological growth habit of branches in mandarin trees, branches can be used to extrapolate results to alternate bearing in whole-tree scenarios (Monselise et al., 1983). All branches were located on the outside of the western side of the tree canopy at a height of ≈1.5 m above the orchard floor and had a fruit-to-leaf ratio of approximately one fruit per ten leaves and an average branch circumference of 55 mm. The following treatments were applied to single branches in moderate bearing trees on 20 Nov. 2014 in summer and 22 Apr. 2015 in autumn: 1) complete de-fruiting of branches; 2) de-fruiting and girdling of branches; 3) girdling of fruiting branches; and 4) fruiting branches left intact. For the girdling treatments a ring of bark approximately 3 mm in width was removed from around the branch by using a sharp knife. The branch treatments were repeated during the following season on the same dates, but on different branches.

2.3. Data collection

2.3.1. Tree and shoot phenology

The number of flowers per tree was estimated by counting the number of flowers within the limits of a 0.5 × 0.5 × 0.5 m frame during full bloom in October. The tree canopy was divided into an East and West sector and an upper- and lower height. Four flower counts were performed in each tree, one in each quadrant. The total number of flowers was estimated by extrapolating the mean number of flowers per frame to the total tree volume. The same procedure was used to estimate the number of new vegetative shoots after cessation of periods of vegetative shoot flush in November, February and April.

The phenological pattern of different shoot types in "on" and "off" trees was followed by randomly selecting five vegetative ("off") and five reproductive ("on") shoots from each tree during full bloom in Oct. 2014. All shoots were approximately 12 months of age and had triangular internodes, a length of ≈15 cm and were located on the outside of the tree canopy at a height of ≈1.5 m above the orchard floor. On each shoot the number of nodes, the number of vegetative shoots and total number of flowers were counted in addition to the classification of inflorescence type. Inflorescences were classified as leafy, i.e. buds sprouting both flowers and leaves, or leafless, i.e. buds sprouting flowers only. In February and March the numbers of persistent fruit and new vegetative shoots that developed during the subsequent vegetative shoot flushes were recorded for each shoot, and return bloom and vegetative response were determined on the same shoots during the subsequent season.

For the branch experiments, the number of new vegetative shoots and the total number of flowers were counted on branch replicates subsequent to the cessation of the summer vegetative shoot flush in February and during full bloom in October.

For root growth observations, acrylic minirhizotron tubes were installed prior to winter in 2015. The tubes were installed parallel to the row direction at an inclined angle of 45° with the soil vertical for approximately 90 cm, thus exploring a vertical soil depth of approximately 60 cm. The bottoms of the tubes were sealed with a plug and the tops that protruded from the soil were capped with a white cap to reflect as much sunlight as possible and prevent water from entering. Two tubes, one on the Eastern side and one on the Western side of the tree canopy were installed below the canopy of each of one representative "on" and "off" tree. The top of the tube was located about 50 cm from the trunk and near the canopy dripline. Digital images were captured in each tube with a root scanner (CI-600 In-Situ Root Imager, CID-BioScience Inc., Camas, WA, USA). Three incremental vertical colour images of 21.6 cm × 19.6 cm were captured down each minirhizotron tube and the number of new roots counted at monthly intervals. To confirm observations of the first season, additional tubes were installed in four separate "on" and "off" tree replicates prior to winter in 2015. Root growth evaluations started at the end of Aug. 2016 and continued at monthly intervals.

Soil and ambient temperatures were logged throughout the study using a soil probe and air temperature logger (TinyTag®, Plus 2, Gemini Data Loggers, Chichester, UK).

2.3.2. Yield

Commercial harvest of fruit commenced in the middle of August after fruit quality standards complied with specifications established by fruit export markets, and was completed by the end of August. To determine the total fruit yield, in kg per tree, all fruit were harvested from individual trees on the same day prior to the start of commercial harvest. A sample of 100 randomly collected fruit per tree was collected from each tree and the diameter of each fruit was measured with an electronic calliper. Each fruit was assigned to a

fruit size category of which the average fruit weight was determined in order to estimate the total number of fruit per tree.

2.3.3. Leaf gas exchange

In each of the five "on" and "off" shoots in eight "on" and "off" tree replicates, one leaf was tagged for repeated measurements of different parameters of leaf gas exchange. The measurements in each of the five "on" and "off" shoots were pooled to represent each treatment replicate (n=8). The rates of leaf CO_2 assimilation (A_c, expressed as µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), leaf stomatal conductance (g_s, expressed as $mmol \cdot m^{-2} \cdot s^{-1}$) and leaf transpiration (E, expressed as mmol $H_2O \cdot m^{-2} \cdot s^{-1}$) were measured at monthly intervals on selected cloudless days using a portable infra-red gas analyzer (Li-6400, LI-COR, Lincoln, NE, USA). Data collection started at ≈8:00 AM and was completed between 11:00 AM and 12:00 PM on each measurement date. Measurements were conducted using a closed chamber. The airflow rate was set at 300 $\mu mol \cdot s^{-1}$, photosynthetic photon flux of 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the block temperature at 25 ºC, with controlled CO_2 concentration of 380 ppm.

2.3.4. Leaf and root carbohydrates

A sample consisting of eight leaves was collected from each treatment replicate between 9:00 and 10:00 AM. The eight leaves consisted of two leaves sampled from each of four vegetative shoots. Only mature leaves were sampled from the third to fifth position on fully hardened, non-fruiting and purely vegetative shoots. All shoots had triangular internodes, a length of ≈15 cm and were located on the outside of the tree canopy at a height of ≈1.5 m above the orchard floor. The spring leaf samples were collected from vegetative shoots that developed during the previous season's vegetative shoot flushes, the summer leaf samples were collected from vegetative shoots that developed during the current season's

spring vegetative shoot flush, and the autumn and winter leaf samples were collected from vegetative shoots that developed during the current season's summer vegetative shoot flush. A sample of fibrous roots (<0.5 mm in diameter) was collected from representative, pooled root tissues that were sampled from four different areas around the trunk of each tree. The root and leaf samples were washed with distilled water, frozen at –80 °C and freeze-dried (Christ Beta 1–8 LD Freeze Dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), before being ground to a fine powder with an analytical grinder (Yellow line, A10, IKA-Werke, Staufen, Germany).

Total sugars were extracted from 100 mg of each dried leaf and root sample with 5 mL 80% (v/v) ethanol at 80 °C for 1 h. The extraction process was repeated twice following the first extraction and the respective supernatants pooled. The pellets were then extracted three times with 5 mL de-ionized water at 80 °C for 24 h for the determination of total water-soluble polysaccharides. Total starch was determined from the remaining pellet by quantifying the glucose released following an enzymatic digestion of the residue for 17 h at 60 °C, with the amyloglucosidase enzyme (AMG) [Sigma Aldrich (Pty) Ltd, Aston Manor, South Africa].

The 80% ethanol, water and AMG enzyme extracts were analyzed for total soluble sugars using the phenol–sulphuric acid assay (Brummer and Cui, 2005). Briefly, a volume of 20 µL of each of the respective extracts was added to 180 µL de-ionized water, 200 µL phenol (5 $mL \cdot L^{-1}$) and 1000 µL concentrated sulphuric acid. Absorbance was determined on a spectrophotometer (Cary 50 Series, Varian, Mulgrave, Australia) at 490 nm, precisely after 30 min against a blank prepared for the standard. A standard curve for glucose concentrations was prepared by diluting 0, 50, 100, 150 and 200 µL glucose stock solution (0.10 $mg \cdot mL^{-1}$) with de-ionized water to a final volume of 200 µL. The sugar concentrations were expressed as milligrams per gram leaf or root dry weight and are respectively referred

to as sugar concentration, polysaccharide concentration and starch concentration. The sum values of the three components collectively contribute to the total carbohydrate concentration.

2.4. Statistical analysis

STATISTICA data analysis software (Dell Inc. 2015, Round Rock, TX, USA) was used to analyse the data. Analysis of variance (ANOVA) or repeated-measures ANOVA was performed when responses were repeated on the same respondent. Mean separations were carried out using Fisher's least significant difference test, where applicable, at $P \leq 0.05$. Relationships between two continuous variables were analysed by regression analysis and the strength of the relationship indicated by Spearman's correlation coefficient. The percentage variation explained is $100*R^2$ % which is indicated as $(-)R^2$ if the correlation was negative.

3. Results

3.1. Tree phenology

The first period of vegetative shoot flush, i.e. the spring flush, which coincided with the sprouting of inflorescences, started about 2 weeks after harvest at the end of August and continued for 8 weeks until full bloom during the third week of October (Fig. 1A). The second period of vegetative shoot flush, i.e. the summer flush, started in the middle of January and continued until the end of February, with "off" trees sprouting significantly more summer vegetative shoots than "on" trees (Table 1; Fig. 1D). A third, short period of vegetative shoot flush occurred at the end of March, i.e. the autumn flush (Fig. 1F), but these flush measurements were combined with those of the summer flush, as they contributed very little to the total number of newly developed vegetative shoots, and no significant differences were recorded between treatments (data not shown). Nevertheless, the number of total new

vegetative shoots that developed in “off” trees was almost double that in “on” trees in both seasons (“off” = 863 and 1439 vs. “on” = 306 and 766) (Table 1).

Intensity of return bloom flowering negatively correlated with fruit yield in both seasons (Table 1). The flowering response of the ten “off” trees of 2014 (season 1) was significantly and 1.7-fold higher than the “on” trees (“off” = 51 097 flowers per tree vs. “on” = 30 034 flowers per tree); however in season 2, return bloom of the “off” trees was ≈230-fold higher compared with that of the “on” trees (“off” = 37 712 flowers per tree vs. “on” = 165 flowers per tree).

The monitoring of the root zones of five “on” and “off” trees at monthly intervals indicated one major period of root growth during summer and a shorter period of root growth during early autumn (Figs. 1C, 1E; 2 and 3). Vegetative shoot flush and root growth showed alternating growth patterns in “off” trees and the first period of root flush started during early summer in November, after cessation of the first vegetative shoot flush in spring (Fig. 1C). This root flush peaked in December and ceased at the end of January, with “off” trees showing a more intense root flush compared to “on” trees, in which new root growth was almost completely absent (Figs. 2 and 3). A second period of root flush started at the end of March, but from May onwards, no new root growth was observed (Figs. 1E, 2 and 3). Interestingly, root growth on the western side of the tree was more pronounced compared to the eastern side of the tree, for both “on” and “off” trees (Fig. 2).

3.2. Shoot phenology

In both “on” and “off” trees, “on” shoots sprouted significantly fewer new vegetative shoots during spring compared to “off” shoots in both seasons (Tables 2 and 3). However, tree fruiting status (“on”/off”) influenced the number of new vegetative shoots that sprouted on individual shoots during spring of season 1, with “off” shoots in “off” trees sprouting

significantly more new vegetative shoots during spring, compared to “off” shoots in “on” trees (Table 2).

There were no significant differences in the number of leafy inflorescences that sprouted on individual shoots in “on” and “off” trees in both seasons (Tables 2 and 3). The number of leafless inflorescences and total number of flowers that developed from different shoot types, however, were significantly different, and were influenced by the tree fruiting status (Tables 2 and 3). In season 1, “on” shoots from both “on” and “off” trees sprouted significantly more leafless inflorescences and total number of flowers compared to “off” shoots; however, the number of leafless inflorescences and total number of flowers were significantly higher in “on” shoots from “on” trees, compared to “on” shoots from “off” trees.

There were no significant differences in the percentage (%) fruit set on different shoot types in season 1 (Table 2), but in season 2, fruit set (%) was higher in “off” shoots (Table 3).

Tree fruit load significantly affected the number of new vegetative shoots that sprouted from different shoot types during summer. “Off” shoots sprouted significantly more vegetative shoots during summer compared to “on” shoots, and “off” shoots from “off” trees sprouted significantly more summer vegetative shoots compared to “off” shoots from “on” trees (Table 2).

The higher number of total new vegetative shoots that developed on “off” shoots in “off” trees during season 1 significantly affected the number of nodes and potential flowering positions during the subsequent season (Table 3). “Off” shoots from “off” trees had more available nodes and potential sites for flower development compared to “off” shoots from “on” trees, and therefore produced significantly more flowers during the subsequent spring compared to “off” shoots from “on” trees, and “on” shoots from “off” trees (Table 3).

3.3. Yield

The ten selected "off"-trees during 2014 (season 1) showed a strong alternate bearing habit (I=0.77) (Table 4) and their fruit yields fluctuated severely between seasons (Table 1). "On"-trees selected in 2014, however, did not show a clear alternate bearing habit (I=0.08 and 0.15) (Table 4), and their fruit yields decreased over the following two seasons (Table 1). The fruit yields of the two treatments did however differ significantly in each season and provided a sufficient contrast to evaluate any significance between treatments in each season. Fruit yield of the ten "off" trees of 2014 (season 1) was significantly and 7.3-fold lower compared with the "on" trees ("off" = 126 fruit per tree vs. "on" = 918 fruit per tree); however in season 2, fruit yield of the "off" trees was only 1.9-fold lower compared with that of the "on" trees ("off" = 657 fruit per tree vs. "on" = 1225 fruit per tree) (Table 1).

3.4. Leaf gas exchange

Apart from some anomalies, leaf Ac, gs and E, was always higher in "on" shoots than in "off" shoots (Tables 5 and 6). With the exception of December in season 1, leaf A_c was significantly higher in "on" trees compared with "off" trees throughout spring and summer in both seasons, as well as in October and December of season 1, for "on" shoots compared with "off" shoots (Tables 5 and 6). In December of season 1, however, A_c of leaves representing different shoot types was significantly influenced by tree fruiting status – there were no significant differences in A_c between leaves from different shoot types in "off" trees, and "on" shoots in "on" trees; however, A_c of leaves from "off" shoots in "on" trees was significantly lower compared with other shoot types (Table 5).

During spring of season 1, and spring and summer of season 2, leaf g_s was significantly higher in "on" trees compared with "off" trees, and significantly higher in "on" shoots compared with "off" shoots in October of season 1 (Tables 5 and 6). In December of season

1, however, g_s of leaves representing different shoot types was significantly influenced by tree fruiting status – there were no significant differences in g_s between leaves from different shoot types in "off" trees, and "on" shoots in "on" trees; however, g_s of leaves from "off" shoots in "on" trees were significantly lower compared with other shoot types (Table 5).

During spring (October and November) of season 1, and during spring and summer of season 2, leaf *E* was significantly higher in "on" trees compared with "off" trees, and significantly higher in "on" shoots compared with "off" shoots in October of season 1 (Tables 5 and 6). During early summer (December) of season 1, leaf *E* was significantly higher in "off" trees compared with "on" trees, and similar for different treatments in January (Table 5).

3.5. Leaf and root carbohydrates

Over the two seasons, leaf sugar concentration showed different seasonal patterns of accumulation – leaf sugar concentration peaked during spring of season 1, but in season 2 it only started to increase in winter (Fig. 4A). In both seasons, leaf sugar concentration was similar for "on" and "off" trees in September and October, but leaf sugar concentration was significantly higher in "off" trees from November to June in season 1 (Fig. 4A). In season 2, there were no significant differences in leaf sugar concentration between "on" and "off" trees at any time (Fig. 4A).

In both seasons leaf polysaccharide concentration peaked during spring and was significantly higher in "off" trees in October of season 1, and September of season 2 (Fig. 4B). Except for "off" trees having significantly higher leaf polysaccharide concentration during March and June of season 2, the leaf polysaccharide concentration of "on" and "off" trees remained similar throughout the rest of the season (Fig. 4B).

Leaf starch concentration of both treatments peaked during September in both seasons and decreased towards winter (Fig. 4C). In both seasons, leaf starch concentration in September was higher for "on" trees, however, from October to June, leaf starch concentration in "off" trees was significantly higher than "on" trees (Fig. 4C).

Throughout season 1, the major carbohydrate component in roots of "off" trees was sugars, whereas the major carbohydrate component in roots of "on" trees was starch (Fig. 5). Root sugar concentration during spring was significantly higher in roots of "off" trees (Fig. 4E). Root sugar concentration in "off" trees increased substantially from September to October (full bloom) and after full bloom, decreased rapidly and was significantly lower compared to "on" trees by November (Fig. 4E). Throughout the rest of summer and in winter (June), the root sugar concentration in "off" trees was significantly higher compared with "on" trees (Fig. 4E). During October of season 2, the root sugar concentration in "off" trees was again significantly higher compared with "on" trees and showed a distinct increase from September to October, followed by a rapid decrease in November (Fig. 4E). For the rest of season 2, a similar pattern of root sugar concentration was evident compared with season 1, but differences were not consistently significant between treatments (Fig. 4E).

In season 1, root polysaccharide concentration remained relatively stable in "off" trees; however, in "on" trees root polysaccharide concentration increased in November, peaked in December and remained significantly higher until winter in June (Fig. 4F). A similar pattern was evident in season 2, except for a non-significant difference in root polysaccharide concentration between "on" and "off" trees in June (Fig. 4F).

Root starch concentration was similar for "on" and "off" trees during September of season 1, but from October to March, root starch concentration in "on" trees was significantly higher than root starch concentration in "off" trees (Fig. 4G). From June to September, root starch concentration was significantly higher in "off" trees, but for the rest of season 2, root

starch concentration in “on” and “off” trees was similar and any significant differences were erratic (Fig. 4G).

3.6. Branch manipulations

The summer vegetative response to de-fruiting and girdling and de-fruiting was significantly higher compared to the fruiting and girdling, and the fruiting treatments (Table 7). During summer, leaf sugar concentration decreased in response to de-fruiting and girdling and remained significantly lower compared to the other treatments throughout the experiment, except for December (Table 8). There were no significant differences in leaf sugar concentration between girdling, de-fruiting, and fruiting treatments throughout the experiment. Polysaccharide and starch concentrations in the leaves increased in response to de-fruiting and girdling, and remained significantly higher than the other treatments throughout the experiment (Table 8).

The flowering response to the combination of de-fruiting and girdling was significantly higher compared with the other treatments (Table 7). During winter, leaf sugar concentration 2 and 6 weeks after treatment was significantly lower in the combined de-fruiting and girdling treatment compared with the other treatments (Table 8). There were no significant differences between leaf sugar concentration of the girdling, de-fruiting, and fruiting treatments, 2, 4 and 6 weeks after treatments (Table 8). Polysaccharide and starch concentrations in the leaves increased significantly in response to de-fruiting and girdling, and remained significantly higher compared to the other treatments from week 4 to 6 weeks after treatment (Table 8). There were no significant differences in leaf sugar concentration between girdling, de-fruiting, and fruiting treatments throughout the experiment, except for 6 weeks after treatment, when the fruiting treatment had a significantly lower leaf sugar concentration compared with girdling and de-fruiting treatments (Table 8).

4. Discussion

Fruit load in 'Nadorcott' mandarin trees was the central factor in determining the number of flowers in the subsequent season [R^2=(-)0.80 and R^2=(-)0.73 in seasons 1 and 2, respectively; (P<0.001)]. The number of flowers and fruit load also had a strong inverse relationship with the number of new vegetative shoots in spring [R^2=(-)0.80 and R^2=(-)0.79 in seasons 1 and 2, respectively; (P<0.001)], summer [R^2=(-)0.81 and R^2=(-)0.78 in seasons 1 and 2, respectively; P<0.001] and with total new vegetative shoots [R^2=(-)0.79 and R^2=(-0.85 in seasons 1 and 2, respectively; P<0.001]. The number of new vegetative shoots that developed in "off" trees was 2- to 3-fold higher in spring and summer, and the number of total new vegetative shoots that developed in "off" trees was almost double that in "on" trees ("off" = 863 and 1439 vs. "on" = 306 and 766). Fewer new vegetative shoots developed when fruit load was high, i.e. in "on" trees, than when fruit load was low, i.e. in "off" trees. These results concur with those from previous studies in citrus (García-Luís et al., 1995b; Krajewski and Rabe, 1995b; Lenz, 1967; Martínez-Alcántara et al., 2015; Monselise and Goldschmidt, 1982; Southwick and Davenport, 1987; Verreynne and Lovatt, 2009), as well as with studies in other alternate bearing evergreens, e.g. in olive (Dag et al., 2010). The higher number of new vegetative shoots in "off" trees affected flowering in the subsequent spring; "off" trees had more nodes and more potential sites available from which a flower could develop. Hence, tree flower number was 1.7-fold higher in "off" trees in spring of season 1 ("off" = 51 097 flowers per tree vs. "on" = 30 034 flowers per tree), and ≈230-fold higher in spring of season 2 ("off" = 37 712 flowers per tree vs. "on" = 165 flowers per tree).

The inhibition of the vegetative shoot flush and return bloom by fruit load was unrelated to parameters of leaf gas exchange, or to leaf carbohydrate concentration. Apart from some anomalies, photosynthesis, stomatal conductance and transpiration rates during

spring and summer were always higher in leaves in "on" shoots and "on" trees, from which fewer new vegetative shoots developed, than in "off" shoots in "off" trees, which was unsurprising (Syverstsen et al., 2003). The relationship between leaf sugar concentration and the number of new spring and summer vegetative shoots, however, was non-significant and very weak. Due to a higher starch concentration in leaves in "off" trees, than in "on" trees [season 1: 98 vs. 72 $mg \cdot g^{-1}$ leaf dry weight DW; $P<0.0001$; season 2: 53 vs. 42 $mg \cdot g^{-1}$ leaf dry weight DW; $P<0.0001$], leaf starch concentration and the number of new vegetative shoots had a stronger relationship in summer (season 1: $R^2=0.53$, $P=0.040$; season 2: $R^2=0.71$, $P<0.001$). However, when testing the significance of the apparent relationship using branch experiments, results failed to provide confirmation of the tree-level results. When fruiting branches were girdled, leaf carbohydrate concentration increased ≈3-fold compared to non-fruiting branches (298 vs. 112 $mg \cdot g^{-1}$ leaf DW; $P<0.0001$), but very few new vegetative shoots sprouted per branch compared to non-fruiting branches (1.6 vs 8.6; $P=0.0021$). The study furthermore showed that although high leaf starch concentration correlated with the number of new vegetative shoots, leaf starch concentration did not contribute to new vegetative shoot growth in "off" trees, but accumulated to near-toxic levels in the palisade mesophyll parenchyma cells, the spongy mesophyll parenchyma cells and in the phloem cells of the leaf vein (see Chapter 3).

Furthermore, if carbohydrates were the reason for development of a higher number of summer vegetative shoots in "off" trees, shoot flushes would have occurred in a continuum and would not have been interrupted. The overall inhibition of summer vegetative shoot development in "on" trees appears to rather be regulated by the presence of fruit and an endogenous regulator other than carbohydrates (Malik et al., 2015; Verreynne, 2005; Verreynne and Lovatt, 2009).

Besides more flowering positions, flowering intensity was consistently higher in "off" shoots in "off" trees, than in "off" shoots in "on" trees. From "off" shoots in "off" trees ≈50% more nodes developed than from "off" shoots in "on" trees and, additionally, flower intensity in "off" shoots in "off" trees, i.e. the number of flowers that sprouted from a single node in an individual shoot, was ≈5-fold that of "off" shoots in "on" trees. "Off" shoots in "off" trees had higher leaf carbohydrate concentrations throughout flower induction period and sprouted a higher number of flowers compared to "off" shoots in "on" trees, where fruit were also absent, but fruit load of the tree was higher, and leaf carbohydrate concentration lower. Winter leaf starch concentration was therefore negatively correlated with fruit yield in both seasons and positively with return bloom (R^2=0.68 and R^2=0.82 in seasons 1 and 2, respectively; P<0.001). This flowering response to high leaf starch concentration during winter may be purely coincidental, but similarly, at the branch-level, a ≈2-fold elevation of leaf carbohydrate concentration by girdling during the corresponding period increased return bloom flowering ≈2-fold in the absence of fruit compared with branches, where fruit was also absent, but leaf carbohydrate concentrations were lower. This effect of winter leaf carbohydrate concentration on flowering appears to manifest independent of the number of newly available shoots and a localised effect of fruit presence on a shoot or branch, but by the effects an "on" crop has on plant available energy.

Apart from fruit load, the number of newly developed vegetative shoots – the most important determinant of return bloom flowering and subsequent fruit load in this study – correlated with the level of root growth activity. In "on" trees, root growth was almost completely absent and the development of new vegetative shoots was halved. In contrast, two distinct peaks of root growth and three vegetative shoot flushes occurred in a synchronised pattern in "off" trees. The lack of root growth in "on" trees could be explained

by a source-limitation of carbohydrates due to excessive flowering and fruiting, and would be consistent with the competence theory concerning carbohydrate sinks (Goldschmidt and Golomb, 1982). In this theory, the presence of a powerful carbohydrate sink such as developing flowers and fruit reduces carbohydrate availability to other sinks such as roots. Since roots cannot synthesise their own source of energy, root growth is entirely dependent on carbohydrates from leaves (Pregitzer et al., 2000). Restriction of the phloem transport pathway by girdling, for example, can cause an accumulation of soluble sugar and starch in leaves and result in a reduction in soluble sugar and starch concentration in roots (Li et al., 2003). Excessive fruiting results in a similar effect as that of girdling, but instead of accumulating in leaves, carbohydrates are consumed by flowers and fruit, i.e. under heavy flowering and fruiting conditions, reproductive sinks compete with roots for carbohydrates and generally win (Goldschmidt and Golomb, 1982). In other fruiting plants, a heavy fruit load forced vines (*Vitis vinifera* L.) to accumulate less sugar in roots (Morinaga et al., 2003); in heavy-fruiting apple, Palmer (1992) reported reduced dry-matter partitioning to roots; and in persimmon (*Diospyros kaki* Thunb.), root sugar concentration increased 1.5-fold when 80% of fruit were thinned, and 2.2-fold when 100% of fruit were thinned (Choi et al., 2005). In other alternate bearing-prone evergreens, high root mortality was associated with low root carbohydrate concentration and a heavy fruit load in coffee (Nutman, 1933); and severe chemical thinning of olive fruit increased carbohydrate concentration in roots by 84% (Bustan et al., 2011). In citrus, allocation of dry matter and root growth were reduced by fruit in ‘Washington Navel’ sweet orange (Cary, 1970), while on the other hand, fruit removal in ‘Valencia’ sweet orange and ‘Satsuma’ mandarin trees during spring increased carbohydrate concentration in roots (Duncan and Eissenstadt, 1993) and resulted in a significant increase in root mass density during summer (Duncan and Eissenstadt, 1993; Okuda, 2000).

Dovis et al. (2014) and Monerri et al. (2011) recently showed that heavy-flowering citrus trees require up to four times more photo-assimilates than low-flowering trees, and therefore, under conditions of alternate bearing, root growth could be restricted even more. Indeed, excessive fruiting in 'Kinnow' and 'Murcott' mandarins was reported to completely deplete carbohydrate reserves in roots (Jones et al., 1975), and in a severe case resulted in death of feeder roots (Smith, 1976). In this study, the profuse number of flowers and fruit in "on" 'Nadorcott' mandarin trees appeared to have consumed the majority of sugars during spring and early summer, limited carbohydrate allocation to roots and resulted in a subsequent lack of root growth activity during summer. Root sugar concentration during spring was negatively correlated with total flowers during full bloom [R^2=(-)0.89 and R^2=(-)0.69 in seasons 1 and 2, respectively; P<0.001] and with fruit yield in summer [season 1: R^2=(-)0.62, P<0.010; season 2: R^2=(-)0.86, P<0.001]. The low flower intensity in "off" trees therefore explains the ≈3-fold increase in root sugar concentration during full bloom, as the lack of reproductive sinks during this period apparently allowed for the distribution of photosynthetically-fixed sugars and readily-available carbohydrates from leaves to roots. Higher root sugar concentration in "off" trees probably explains the subsequent spike in early summer root growth, after cessation of the spring vegetative shoot flush and prior to initiation of the summer vegetative shoot flush, as more carbohydrates were readily-available to initiate and maintain their growth.

Overall, this study confirmed that the lack of vegetative shoot development is a major cause of poor flowering following an "on" year (García-Luís et al., 1995; Lenz, 1967; Martínez-Alcántara et al., 2015; Monselise and Goldschmidt, 1982), but leaf carbohydrate concentration does not limit summer vegetative shoot development (Verreynne, 2005). Furthermore, under field-conditions, the important inter-dependent relationship between root growth and vegetative shoot flushes in citrus was illustrated by Bevington and Castle (1985)

and others (Eissenstat and Duncan, 1992; Mataa et al., 1996) and the results in this study for the first time indicate a similar relationship in alternate bearing citrus trees. The interaction of fruit, roots and vegetative shoots, and the synthesis and accumulation in these tissue types of phyto-hormone substances such as ABA and cytokinin should be investigated in further studies on alternate bearing in citrus, as well as how it relates to the well-documented role of IAA as reported by Verreynne and Lovatt (2009) (see Chapter 5).

Furthermore, leaf carbohydrate concentration during winter does not appear to be a limiting factor for flowering, but does appear to influence the intensity of the subsequent return bloom in "off" crop scenarios only. How it regulates this effect, i.e. by directly upregulating expression of flowering genes, or facilitating floral organogenesis as a source of energy, is not clear. In an "on" crop scenario and during winter, the influence of carbohydrates on flowering is not critical, which concurs with numerous other reports (Cohen, 1981; García-Luís et al., 1995a; Goldschmidt et al., 1985; Jones et al., 1974). The lack of flowering response in an "on" crop scenario cannot be explained by factors other than probable fruit-produced phyto-hormones, which have been shown to directly determine flowering response in an upstream event by limiting the expression of citrus flowering genes (García-Luís et al., 1995a; Goldberg-Moeller et al., 2013; Goldschmidt et al., 1985; Koshita et al., 1999; Muñoz-Fambuena et al., 2011).

5. Conclusions

This study investigated the possible role of carbohydrates in the nutritional theory of alternate bearing of 'Nadorcott' mandarin and evaluated the relative importance of the contribution of leaf and root carbohydrates to the lack of or excessive development of flowers and fruit at the shoot-, branch- and tree-level. The results concur with previous studies and confirm that the lack of vegetative shoot development in "on" shoots, "on" branches and "on"

trees plays a central role in poor return bloom and in perpetuating the alternate bearing cycle in citrus. The lack of new vegetative shoot development limits the number of new available flowering sites in the subsequent spring. Vegetative shoot development during spring and summer was unrelated to leaf gas exchange parameters and leaf carbohydrate concentration, but rather to the presence of fruit, and indicates a possible role for an endogenous regulator other than carbohydrates. Leaf carbohydrate concentration during winter does not seem to be a limiting factor for flowering, but appears to increase the intensity of the subsequent return bloom in "off" crop scenarios only. In an "on" crop scenario, the influence of carbohydrates during winter does not appear to be important, concurring with previous reports. Fruit load and root sugar concentration provided the best correlations with the intensity of vegetative shoot flushes. Root sugar concentration peaked during full bloom and higher root growth activity was observed prior to periods of increased vegetative shoot development in "off" trees. The early-season root sugar concentration was lower and root and shoot growth were absent, or lower in "on" trees. The study provides new insights into how fruit load influences vegetative shoot development in alternate bearing citrus trees. These results affirm that fruit are the major carbohydrate sink and most probably disturb the balance between root growth and vegetative shoot development.

Table 1. Total fruit yield, vegetative response and return bloom of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees for three seasons.

Tree fruiting status	Fruit yield in the current year (kg per tree)	Fruit per tree in the current year (no.)	Return bloom and vegetative response in the following year (no. per tree)			
			Total flowers	Total new spring shoots	Total new summer shoots	Total new shoots
Season 1						
B[z]: "Off"	14 b[y]	126 b	51 097 a	163 b	144 b	306 b
W: "On"	84 a	918 a	30 034 b	493 a	369 a	863 a
P value	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Season 2						
B: "On"	110 a	1225 a	165 b	1018 a	420 a	1439 a
W: "Off"	71 b	657 b	32 712 a	598 b	167 b	766 b
P value	0.0005	<0.0001	0.0004	0.0007	<0.0001	<0.0001
Season 3						
B: "Off"	16 b	144 b				
W: "On"	52 a	621 a				
P value	<0.0001	<0.0001				

[z] For easier interpretation of results over three seasons, treatments were assigned colours blue (B) and white (W).

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=10).

Table 2. The phenological pattern of different shoot types ("on" or "off") in "on" and "off" treatments of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during season 1. Values are expressed as number per shoot or in percentage (%) for fruit set measurements.

Treatments	Nodes	New spring vegetative shoots	Dormant buds	Leafy inflorescences	Leafless inflorescences	Flowers	Fruit	Fruit set %	New summer vegetative shoots
Tree									
B[z]:"On"	11.1 ns[y]	-	6.4 ns	1.5 ns	-	-	0.9 ns	22.4 ns	-
W:"Off"	10.6	-	6.7	1.5	-	-	0.7	29.4	-
Shoot									
"On" shoots	11.4 ns	-	5.6 b	2.5 a	-	-	1.5 a	21.8 ns	-
"Off" shoots	10.7	-	7.5 a	0.5 b	-	-	0.1 b	30.1	-
Tree × Shoot									
B:"On" × "on" shoots		0.1 c[x]			3.4 a	12.7 a			0.3 c
B:"On" × "off" shoots		1.9 b			0.1 c	0.7 c			1.2 b
W:"Off" × "on" shoots		0.7 c			1.3 b	6.4 b			0.5 c
W:"Off" × "off" shoots		3.2 a			0.00 c	0.5 c			3.5 a
P value									
Tree	0.0705	0.0411	0.4531	0.9751	0.0012	0.0111	0.3748	0.4902	0.0478

Shoot	0.4895	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.4211	<0.0001
Tree × Shoot	0.7381	0.0186	0.3141	0.5100	0.0022	0.0162	0.4295	0.6198	0.0237

[z] For easier interpretation of results from two seasons, treatments were assigned colours blue (B) and white (W).

[y] No significant difference.

[x] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's least significant difference test; n=10).

Table 3. The phenological pattern of different shoot types ("on" or "off") in "on" and "off" treatments of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during season 2. Values are expressed as number per shoot or in percentage (%) for fruit set measurements.

Treatments	Nodes	New spring vegetative shoots	Dormant buds	Leafy inflorescences	Leafless inflorescences	Flowers	Fruit	Fruit set %
Tree								
B[z] :"Off"	-	3.3 ns[x]	7.1 ns	1.4 ns	4.4 ns	-	1.4 ns	26.8 ns
W:"On"	-	2.6	8.0	1.5	4.8	-	1.4	22.2
Shoot								
"On" shoots	-	4.3 a	6.9 ns	0.7 b	1.7 b	-	0.9 b	36.0 a
"Off" shoots	-	1.6 b	8.1	2.2 a	7.5 a	-	2.0 a	13.0 b
Tree × Shoot								
B:"Off" × "on" shoots	11.4 c[y]					1.92 c		
B:"Off" × "off" shoots	18.2 b					5.70 b		
W:"On" × "on" shoots	12.3 c					1.32 c		
W:"On" × "off" shoots	27.6 a					24.43 a		
P value								
Tree	0.0348	0.2991	0.1614	0.8332	0.7925	0.0187	0.9058	0.4589
Shoot	0.0002	<0.0001	0.0598	0.0005	<0.0001	<0.0001	0.0058	0.0015

Tree × Shoot	0.0251	0.2915	0.8032	0.2105	0.9533	0.0162	0.0714	0.7148

[z] For easier interpretation of results over two seasons, treatments were assigned colours blue (B) and white (W)

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=10).

[x] No significant difference.

Table 4. Total fruit yield and alternate bearing index (*I*) of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees.

Treatments	Tree fruit yield (kg per tree)			Alternate bearing index	
	2014	2015	2016	2014 to 2015	2015 to 2016
Tree B[z]	14 b[y]	110 a	16 b	0.77 a	0.77 a
Tree W	84 a	71 b	52 a	0.08 b	0.15 b
P value	0.0002	0.0005	<0.0001	0.0007	0.0021

[z] For easier interpretation of results over two seasons, treatments were assigned colours blue (B) and white (W).

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=10).

Table 5. The rates of photosynthesis, stomatal conductance and transpiration of leaves in different shoot types ("on" or "off") in "on" and "off" treatments of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during summer of season 1.

Treatments	Leaf photosynthesis (A_c) µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$				Leaf stomatal conductance (g_s) mmol $\cdot m^{-2} \cdot s^{-1}$				Leaf transpiration (E) mmol $H_2O \cdot m^{-2} \cdot s^{-1}$			
	Oct.	Nov.	Dec.	Jan.	Oct.	Nov.	Dec.	Jan.	Oct.	Nov.	Dec.	Jan.
Tree												
B[z]:"On"	6.11 a[y]	3.66 a	-	6.51 a	0.09 a	0.06 a	-	0.11ns	1.83a	1.18a	1.13b	2.18ns
W:"Off"	3.22 b	3.28 b	-	5.88 b	0.07b	0.04 b	-	0.11	1.16b	0.94b	1.60a	2.12
Shoot												
"On" shoots	5.36 a	3.62 ns[x]	-	6.24 ns	0.09 a	0.05 ns	-	0.11ns	1.63a	1.06ns	1.50a	2.18ns
"Off" shoots	3.97 b	3.32	-	6.15	0.07b	0.05	-	0.11	1.36b	1.05	1.24b	2.13
Tree × Shoot												
B:"On" × "on" shoots			3.76a				0.06 a					
B:"On" × "off" shoots			2.72b				0.04 b					
W:"Off" × "on" shoots			3.45a				0.06 a					
W:"Off" × "off" shoots			3.52a				0.06 a					
P value												

Tree	<0.0001	0.0234	0.1858	0.0252	0.0002	0.0003	0.0072	0.4321	<0.0001	0.0002	<0.0001	0.4532
Shoot	0.0004	0.0675	0.0110	0.7178	0.0071	0.8699	0.0041	0.7031	0.0108	0.9209	0.0024	0.6503
Tree × Shoot	0.2501	0.2804	0.0022	0.9874	0.1947	0.6272	0.0238	0.9213	0.2759	0.8124	0.0847	0.8607

[z] For easier interpretation of results from two seasons, treatments were assigned colours blue (B) and white (W)

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=8).

[x] No significant difference.

Table 6. The rates of photosynthesis, stomatal conductance and transpiration of leaves in different shoot types ("on" or "off") in "on" and "off" treatments of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during summer of season 2.

Treatments	Leaf photosynthesis (A_c) µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$				Leaf stomatal conductance (g_s) mmol $\cdot m^{-2} \cdot s^{-1}$				Leaf transpiration (E) mmol $H_2O \cdot m^{-2} \cdot s^{-1}$			
	Oct.	Nov.	Dec.	Jan.	Oct.	Nov.	Dec.	Jan.	Oct.	Nov.	Dec.	Jan.
Tree												
B[z] :"Off"	3.10 b[y]	1.15 b	1.28 b	2.34 b	0.03b	0.02 b	0.03 b	0.03 b	-	-	0.73 b	0.73 b
W:"On"	5.08 a	1.52 a	2.02 a	4.80 a	0.05a	0.03 a	0.02 a	0.07 a	-	-	0.99 a	1.37 a
Shoot												
"On" shoots	3.99 ns[x]	1.43 ns	1.68 ns	3.26 ns	0.04ns	0.02 ns	0.02 ns	0.05 ns	-	-	0.88 ns	0.98ns
"Off" shoots	4.20	1.24	1.62	3.89	0.05	0.02	0.02	0.06	-	-	0.85	1.13
Tree × Shoot												
B:"Off" × "on" shoots									0.94 b	0.72 c		
B:"Off" × "off" shoots									0.83 b	0.84 bc		
W:"On" × "on" shoots									1.09 b	1.10 a		
W:"On" × "off" shoots									1.39 a	0.93 ab		
P value												

Tree	<0.0001	0.0461	<0.0001	<0.0001	0.0061	0.0045	0.0083	<0.0001	0.0010	0.0032	0.0047	<0.0001
Shoot	0.5495	0.2955	0.6557	0.1152	0.3203	0.5027	0.5469	0.1062	0.3223	0.7656	0.6742	0.2180
Tree × Shoot	0.1470	0.2440	0.7150	0.5700	0.0810	0.0880	0.2710	0.2090	0.0472	0.0024	0.3370	0.1120

[z] For easier interpretation of results from two seasons, treatments were assigned colourrs blue (B) and white (W).

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=8).

[x] No significant difference.

Table 7. The vegetative and reproductive responses to summer and winter branch treatments in 'Nadorcott' mandarin (*C. reticulata*).

Treatments	Vegetative response (summer experiment) (no. shoots per branch)	Flowering response (winter experiment) (no. flowers per branch)
Defruited and girdled	6.1 a[z]	79 a
Fruiting and girdled	1.6 b	7 c
Defruited	8.6 a	34 b
Fruiting	0.5 b	14 bc
P value	0.0021	0.0010

[z] Different letters in the same column denote significant differences between values, ($P<0.05$; Fisher's LSD test; n=8).

Table 8. The effects of branch source/sink alterations in moderate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during summer, at the end of Nov. 2014, and winter, at the end of Apr. 2015, on the concentrations of leaf sugars, leaf polysaccharides, leaf starch and leaf total carbohydrates.

	Leaf sugars				Leaf polysaccharides				Leaf starch			
	mg·g^{-1} leaf DW[z]											
	Summer experiment											
Treatments	22 Nov.	4 Dec.	18 Dec.	7 Jan.	22 Nov.	4 Dec.	18 Dec.	7 Jan.	22 Nov.	4 Dec.	18 Dec.	7 Jan.
Defruited and girdled	121.7 ns[y]	71.7 b[x]	85.3 ns	92.2 b	60.5 ns	128.4 a	122.4 a	111.2 a	73.4ns	150.6 a	155.6 a	144.9 a
Fruiting and girdled	111.4	84.6 a	92.6	105.9 a	68.6	141.6 a	117.4 a	106.0a	96.6	151.8 a	138.8 a	128.8 a
Defruited	112.4	89.3 a	91.6	104.4 a	55.5	98.4 b	59.6 b	53.7 b	78.5	110.6 b	61.8 b	51.2 b
Fruiting	120.4	92.1 a	91.8	105.7 a	63.8	103.0 b	65.1 b	51.1 b	80.5	106.0 b	64.3 b	53.7 b
P value	0.4757	<0.0001	0.1903	0.0056	0.1950	<0.0001	<0.0001	<0.0001	0.1672	0.0038	<0.0001	<0.0001
	Winter experiment											
Treatments	22 Apr.	5 May	19 May	4 Jun.	22 Apr.	5 May	19 May	4 Jun.	22 Apr.	5 May	19 May	4 Jun.
Defruited and girdled	120.1 ns	111.1 b	120.3 b	119.8 b	40.3 ns	41.3ns	55.5 a	56.6 a	34.9 ns	81.4 a	133.0 a	165.9 a
Fruiting and girdled	119.4	130.9 a	134.7 a	134.7 a	31.5	37.9	29.0 b	27.3 b	38.1	5.2 c	16.3 c	27.3 b
Defruited	121.6	124.5 a	127.0 ab	127.1 a	34.6	35.2	29.5 b	22.5 b	32.9	31.1 b	42.7 b	24.7 b

Fruiting	121.7	122.6 ab	132.6 a	119.8 b	35.9	35.6	29.6 b	24.0 b	33.0	32.6 b	24.5 c	21.5 b
P value	0.1009	0.0056	0.0425	0.0316	0.1949	0.3815	<0.0001	<0.0001	0.5483	<0.0001	<0.0001	<0.0001

[z] Dry weight.

[y] No significant difference.

[x] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=8).

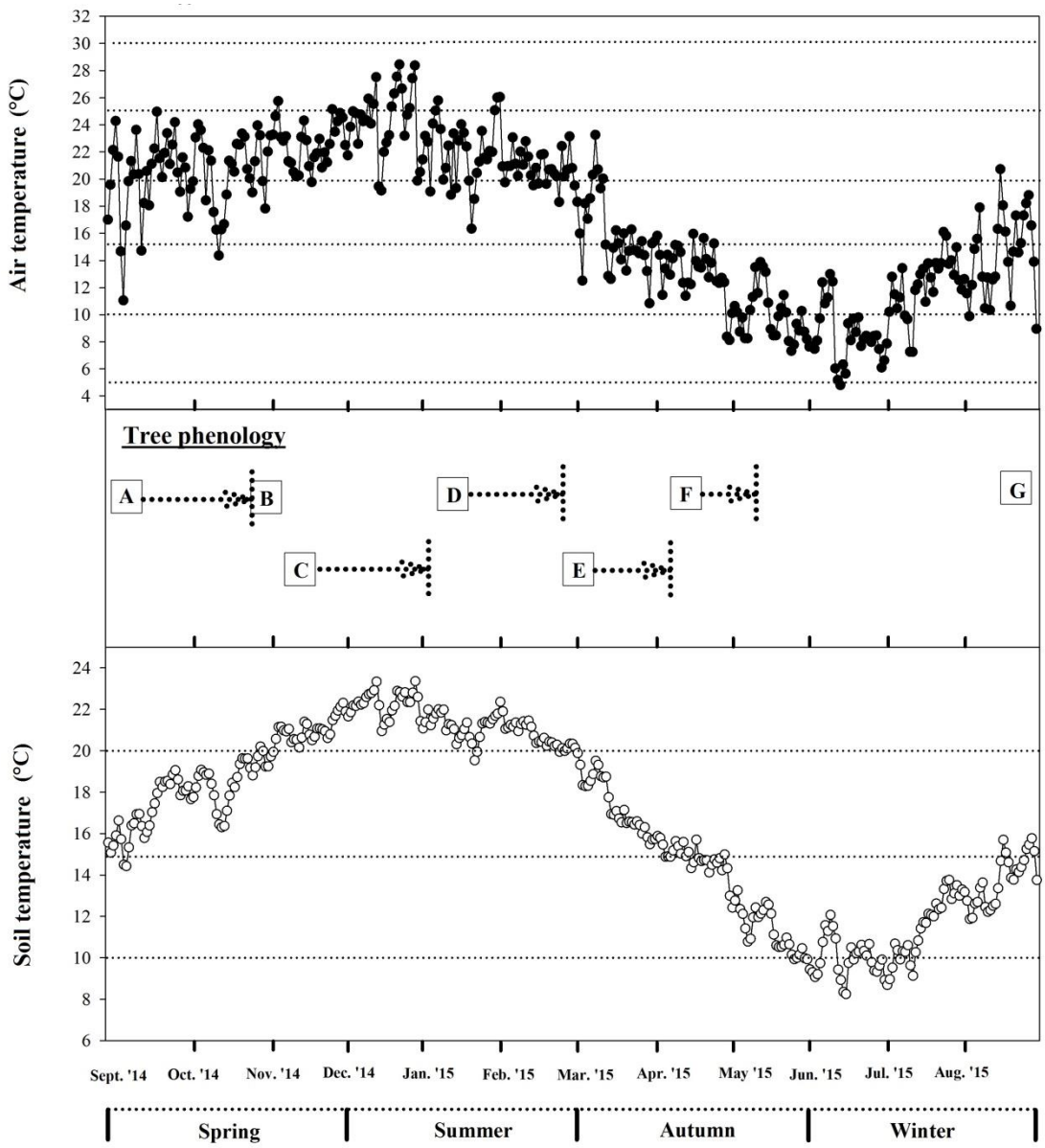

Fig. 1. Seasonal average air temperature, tree phenological events and soil temperature in the experimental 'Nadorcott' mandarin (*C. reticulata*) orchard in De Doorns, South Africa. Tree phenology: A) First vegetative shoot flush in spring; B) Full bloom; C) First root flush; D) Second vegetative shoot flush in summer; E) Second root flush; F) Third vegetative shoot flush in autumun; and G) Harvest. Temperatures were logged throughout the study using a soil probe and air temperature logger (TinyTag®, Plus 2, Gemini Data Loggers, Chichester, UK).

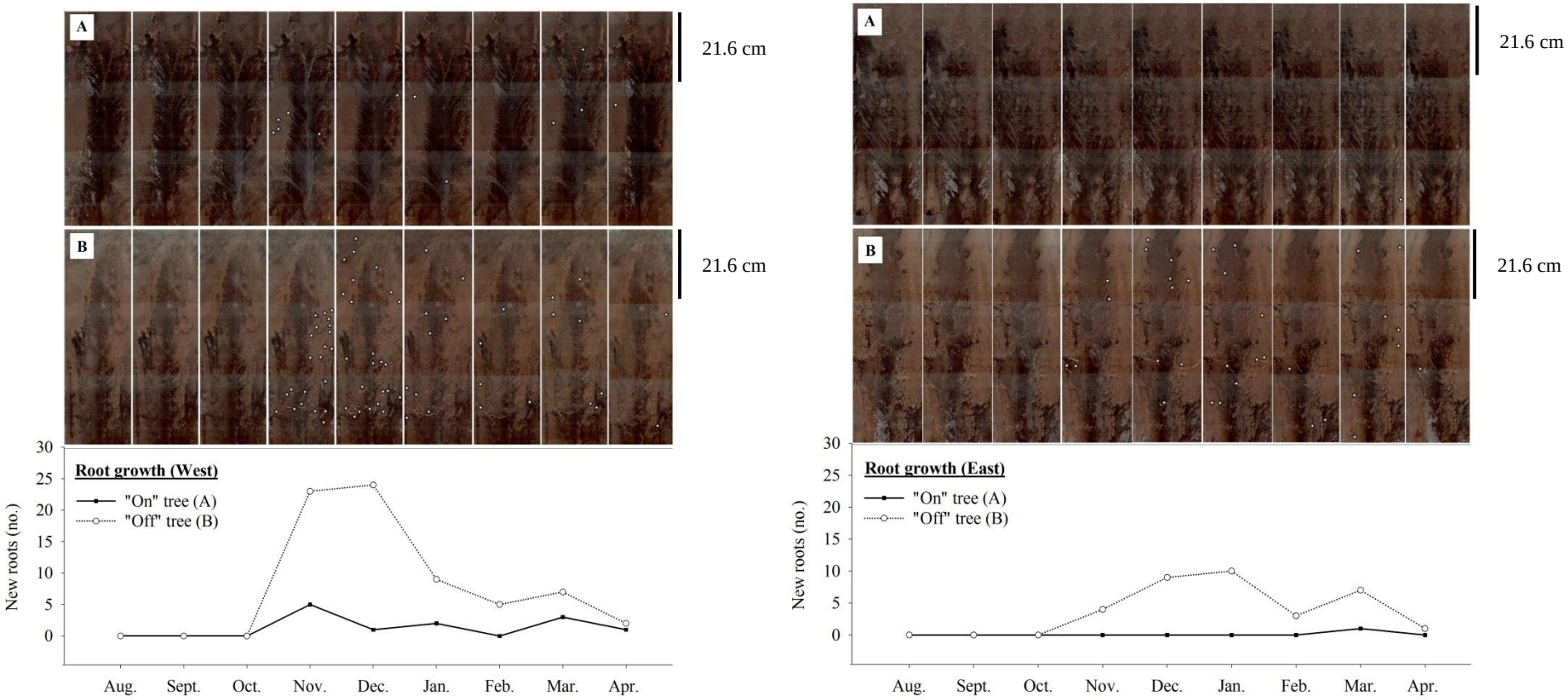

Fig. 2. The pattern of root growth activity on the Western (left) and Eastern (right) side of the tree canopy, respectively, of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees with contrasting fruit loads (A: "on" tree, B: "off" tree). Minirhizotron tubes were installed prior to winter in 2015 with evaluations starting in Aug. 2016, and continued at monthly intervals. Digital images were captured in each tube with a root imager (CI-600 In Situ Root Scanner, CID-BioScience Inc., Camas, WA, USA). Three incremental, vertical images were captured down each minirhizotron tube and new roots were counted at monthly intervals. A star indicates a new root within each observation.

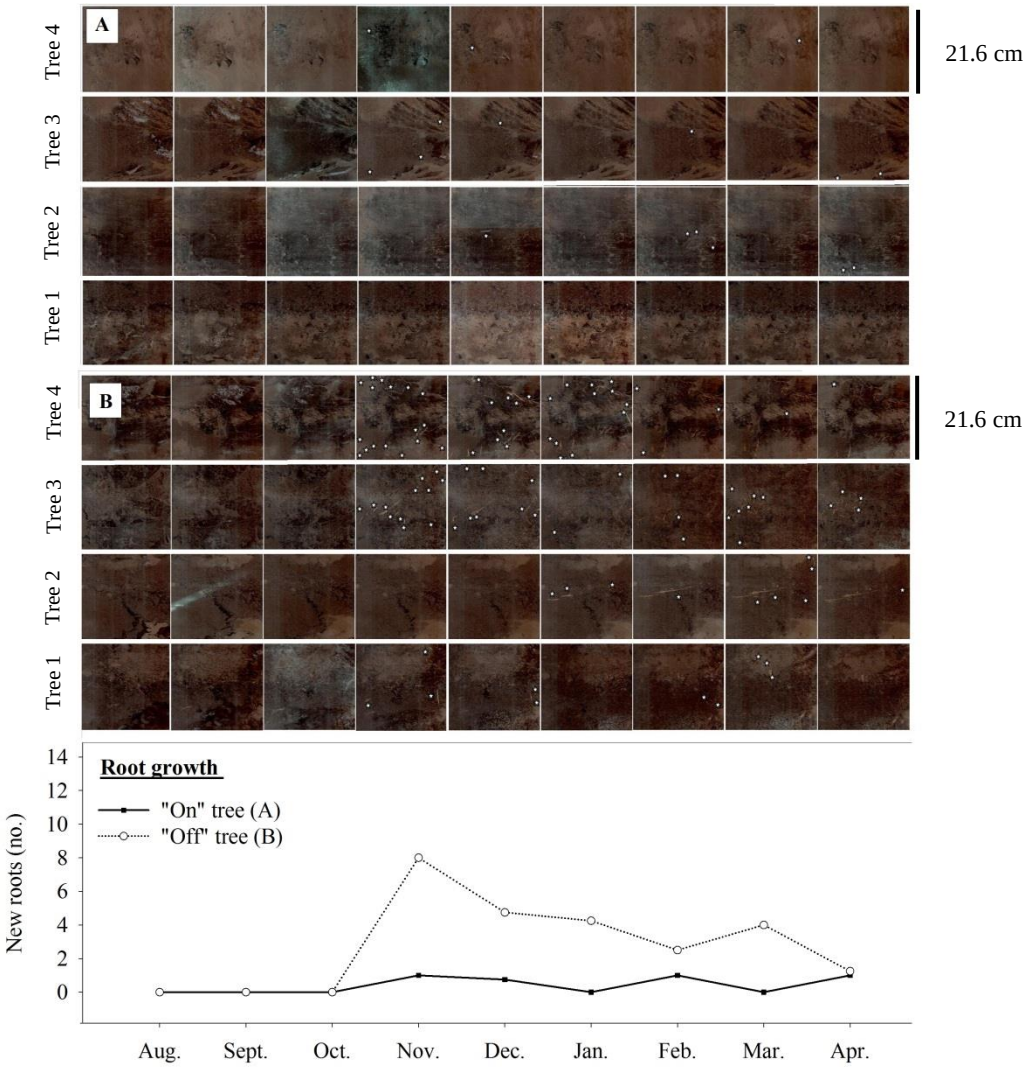

Fig. 3. The root growth pattern of four ten-year-old "on" (A) and "off" (B) 'Nadorcott' mandarin (*C. reticulata*) trees. Minirhizotron tubes were installed prior to winter in 2016 and evaluations started in Aug. 2016 and were continued at monthly intervals. Digital images were captured in each tube with a root imager (CI-600 In Situ Root Scanner, CID-BioScience Inc., Camas, WA, USA). Vertical images were captured down each minirhizotron tube and new roots counted at monthly intervals. A star indicates a new root within each observation.

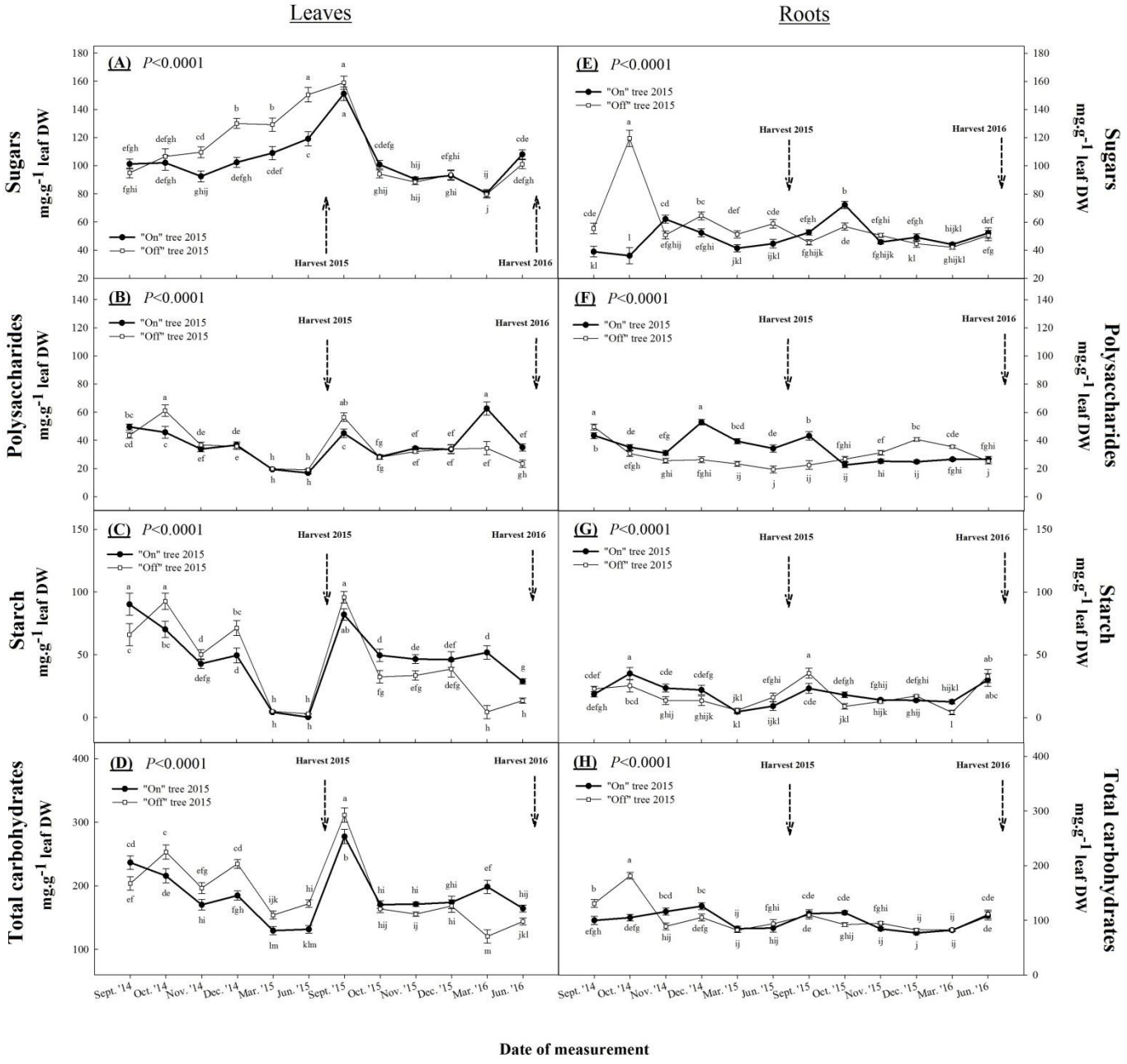

Fig. 4. The seasonal concentrations of different carbohydrate components in leaves and roots of ten-year-old "on" and "off" 'Nadorcott' mandarin (*C. reticulata*) trees: A and E) sugars; B and F) polysaccharides; C and G) starch; and D and H) total carbohydrates. The arrows indicate the time of harvest for each season. Bars denote standard errors of the means, and different letters, significant differences between values (P<0.05; Fisher's LSD test; n=10). DW = dry weight.

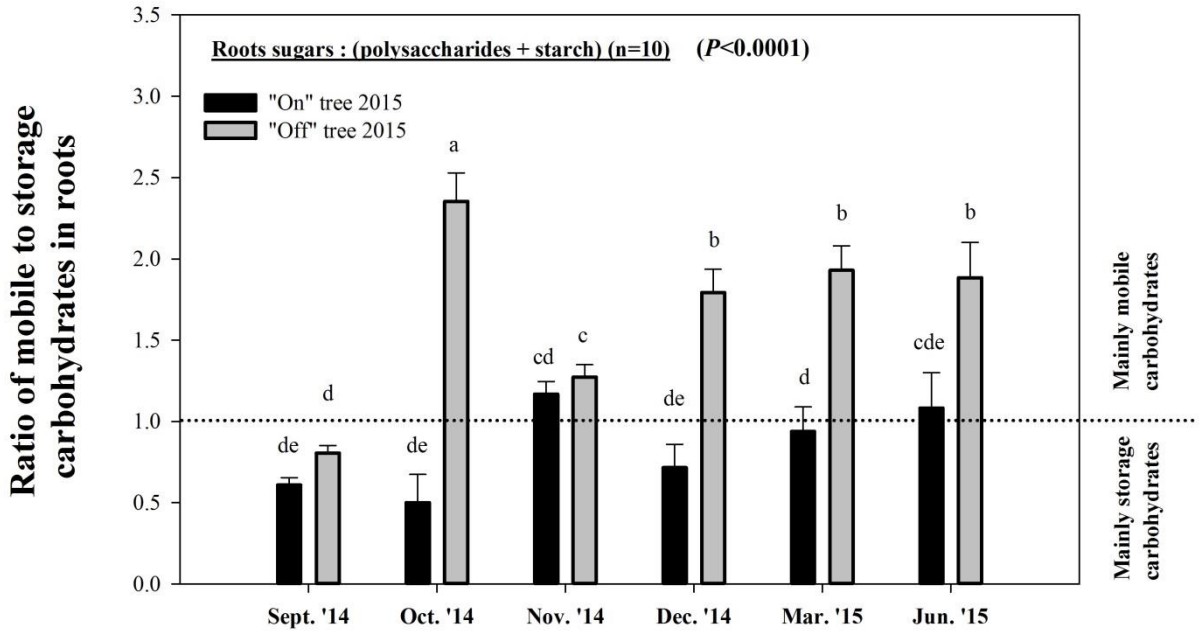

Fig. 5. The ratio of the concentration of sugars to storage carbohydrates, i.e. the sum of polysaccharides and starch, in roots of ten-year-old "on" and "off" 'Nadorcott' mandarin (*C. reticulata*) trees. Bars denote standard errors of the means and different letters significant differences between values ($P<0.05$; Fisher's LSD test; n=10).

Chapter 3: Fruit-load-induced starch accumulation causes leaf chlorosis in "off" 'Nadorcott' mandarin trees

Abstract. Leaf chlorosis often develops in low-fruiting ("off") trees of healthy, severely alternate bearing 'Nadorcott' mandarin (*Citrus reticulata* Blanco) trees, whereas leaves of heavy-fruiting ("on") trees maintain a typical dark, green colour. This study investigated the role of fruit load and leaf carbohydrate concentration on the development of leaf chlorosis in "off" trees under a broad spectrum of source/sink relationships. Leaf chlorosis in "off" trees was mediated by low sink activity and leaf total chlorophyll concentration strongly correlated with leaf starch concentration [R^2 = (-)0.74; P<0.001]. A decrease in leaf total chlorophyll concentration in "off" trees coincided with a peak in leaf starch concentration during winter, after activity of all the major sinks reached a minimum. In "on" trees, leaf starch concentration was lower compared to "off" trees, the major carbohydrate component was sugars, and leaves maintained a healthy dark green colour. Summer de-fruiting of "on" trees resulted in accumulation of leaf starch, but loss in leaf total chlorophyll concentration was delayed until autumn. Lower leaf nitrogen (N) and lower leaf sugar concentration do not appear to be the cause of leaf chlorosis in "off" alternate bearing trees, but rather a response to the disruptive effect of fruit-load-induced starch accumulation on the leaf chloroplasts. Results can be used to prevent unnecessary winter N applications in "off" 'Nadorcott' mandarin trees. This phenomenon was previously artificially induced at the branch-level, but this is the first report of fruit-load-induced leaf chlorosis on a whole-tree level in citrus.

1. Introduction

'Nadorcott' (*Citrus reticultata* Blanco), also known as 'W. Murcott', is a late-maturing mandarin cultivar developed from a seed of the highly-seeded 'Murcott' mandarin (Nadori,

2006). 'Murcott' is of unknown parentage, but is believed to be a tangor: a mandarin hybrid between a mandarin and a sweet orange [*C. reticulata* × *C. sinensis* (Osbeck)]. Both 'Nadorcott' and 'Murcott' are prone to alternate bearing (Smith, 1976; Stander and Cronjé, 2016). Alternate bearing is the synchronised tendency of a perennial fruit tree to flower profusely and produce an excess amount of fruit in one season ("on" year), followed by a sparse number of flowers and fruit in the following season ("off" year) (Monselise and Goldschmidt, 1982).

In alternate bearing citrus (*Citrus* spp.) trees, leaf chlorosis often develops during an "off" year, or subsequent to an "on" year. The phenomenon is closely associated with an altered leaf nutritional status in response to extreme seasonal fluctuations in tree fruit load, and has been reported in 'Murcott' (Schaffer et al., 1986; Smith, 1976) and 'Wilking' (Goldschmidt and Golomb, 1982) mandarins. In severely alternate bearing trees of commercial 'Nadorcott' mandarin orchards in South Africa, leaf chlorosis during the winter of an "off" year is a common occurrence (personal observations). Classic leaf chlorosis symptoms in healthy, perennial evergreen fruit trees are most commonly associated with, but not limited to, a deficiency of mineral nutrients (Lavon et al., 1999). However, in comparative studies in absolute "on" and "off" trees, higher levels of mineral nutrients are generally reported in leaves of "off" trees, as opposed to "on" trees (Golomb and Goldschmidt, 1987; Mirsoleimani et al., 2014; Smith, 1976). In "off" trees, the majority of mineral nutrients reside in the leaves, whereas in "on" trees, the majority is consumed by fruit (Golomb and Goldschmidt, 1987; Mirsoleimani et al., 2014; Smith, 1976).

Alternatively, numerous studies have implicated a role for carbohydrates in the development of leaf chlorosis, with specific reference to starch. Excessive starch accumulation was identified as a cause of chlorosis in leaves of cucumber (*Cucumis sativus* L.) (Schaffer et al., 1991), tobacco (*Nicotiana tabacum* L.) (Herold and McNeill, 1979) and

'Cherry' tomato (*Lycopersicon esculentum* Mill.) (Lebsky and Poghosyan, 2007); as well as in apple [*Malus* × *sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] (Shecter and Proctor, 1994) and citrus (Schaffer et al., 1986). Starch is the main form of stored carbohydrates and can accumulate in leaves whenever the carbohydrate supply exceeds the carbohydrate demand (Dovis et al., 2014; Goldschmidt and Golomb, 1982; Loescher et al., 1990; Monerri et al., 2011; Nebauer et al., 2014). Starch can also accumulate in response to structural interference of the sugar transport pathway by girdling (Cohen, 1981; Schaffer et al., 1986) or bacterial infection (Etxeberria et al., 2009; Gonzalez et al., 2012; Koh et al., 2012) of the phloem. In citrus, the combination of girdling and fruit removal can cause excessive starch grain accumulation in the leaf chloroplast, rupturing and disintegration of the thylakoid membrane and the subsequent development of leaf chlorosis (Cohen, 1981; Li et al., 2003; Schaffer et al., 1986).

In other fruit trees, excess starch accumulation has been reported to cause leaf chlorosis in trees with extremely low fruit loads. Snyder-Leiby and Wang (2008) observed abnormally high leaf starch concentration, disintegrated chloroplast membrane structure and leaf yellowing in 'Honeycrisp' apple in response to excessive thinning. Schupp et al. (2001) reported that the de-blossoming of 'Golden Delicious' apple trees led to accumulation of numerous large starch granules in the leaf chloroplasts, which disrupted the chloroplast membranes. While accumulation of starch in leaves of low-fruiting citrus trees is a well-documented physiological occurrence (Dovis et al., 2014; Goldschmidt and Golomb, 1982; Monerri et al., 2011; Nebauer et al., 2011, 2014), a subsequent response of leaf chlorosis has not yet been reported.

This study investigated the possibility that development of leaf chlorosis as observed in natural alternate bearing 'Nadorcott' mandarin trees in an "off" phase is related to starch build-up in response to a lack of sink activity. To address this, leaf carbohydrate status and

leaf chlorosis response of natural “on” and “off” ‘Nadorcott’ mandarin trees were monitored. In addition, the response of leaf carbohydrate concentration and leaf colour were compared under a broad spectrum of source/sink relationships.

2. Materials and methods

2.1. Plant material and experimental site

Ten year-old ‘Nadorcott’ mandarin trees grown under field conditions and budded on ‘Carrizo’ citrange [*C. sinensis* × *Poncirus trifoliata* (L.) Raf.] rootstock were selected from orchards with a history of alternate bearing in De Doorns (lat. 33°51’S, long. 19°52’E) and Citrusdal (lat. 32°81’S, long. 19°01’E) in the Western Cape Province of South Africa. Trees were spaced at 5 × 2 m in a sandy soil with $pH_{(KCl)}$ 4.4. The Western Cape Province of South Africa experiences Mediterranean-type climatic conditions; summer typically occurs from December to February; autumn from March to May; winter from June to August and spring from September to November. The region receives an annual rainfall of between 400 and 600 mm, with the majority occurring from May to August. The orchards were cultivated, pruned, and sprayed according to good agricultural practices: trees were watered using a drip irrigation system with four emitters per tree, and the amount of water applied to each tree amounted to ≈4000 L per annum. The fertilizer rate [kg per hectare (ha)] was based on annual leaf mineral nutrient analysis and potential yield (kg fruit per ha). Nitrogen (N) was annually supplied at a rate of 240 kg N per ha, with 25% applied foliar, 20% as a soil application, and 55% dissolved in the irrigation solution (fertigation) and split uniformly into applications from September to April.

2.2. Treatments and experimental design

The whole-tree experiments were set up in a completely randomised design (n=10). Heavy ("on") and low-fruiting ("off") trees were randomly selected based on contrasting fruit loads. To ensure that replicate trees were uniformly selected, trunk circumferences were measured and canopy volumes determined at the beginning of the experiment by measuring the tree height, canopy height and canopy radius (average radius in the N, S, E and W directions) of each tree replicate. The canopy volume (m^3) was calculated according to the following formula (Burger et al., 1970):

$V = r^2(\pi h - 1.046r)$

r = canopy radius;

h = height of the fruit bearing canopy.

At time of commercial harvest in August, the total yield (kg) of each replicate tree was determined. "On" trees yielded an average of 84 kg fruit per tree, whereas "off" trees yielded an average of 14 kg fruit per tree.

To perform the de-fruiting experiments, the "on" cycles of 12 trees were desynchronised at two different timings by removing all fruit of six trees in Jan. 2016 for the summer treatment and six trees in Apr. 2016 for the winter treatment (n=6).

The branch experiment was set up in a randomised complete block design. A tree represented a block and a single branch represented a replicate (n=8). All branches were located on the outside of the western side of the tree canopy at a height of ≈1.5 m above the orchard floor and had a fruit-to-leaf ratio of ≈1 fruit per 10 leaves and an average branch circumference of 55 mm.

The following treatments were applied to single-replicate branches on 20 Nov. 2014 and 22 Apr. 2015, respectively: 1) complete de-fruiting of branches; 2) de-fruiting and girdling of branches; 3) girdling of fruiting branches; and 4) fruiting branches left intact. For

the girdling treatments a ring of bark approximately 3 mm in width was removed around the branch by using a sharp knife. The branch treatments were repeated during the following season on the same dates. Leaf samples were collected from branch replicates at 2-week intervals, starting on day of treatments and continued until 6 weeks after treatments.

2.3. Data collection

2.3.1. Leaf carbohydrates

A sample consisting of eight leaves was collected from each treatment replicate between 9:00 and 10:00 AM. The eight leaves consisted of two leaves from each of four vegetative shoots of the previous spring's vegetative shoot flush (a leaf age younger than 12 months). The leaf samples were washed with distilled water, frozen at –80 °C and freeze-dried (Christ Beta 1–8 LD Freeze Dryer; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) before being ground to a fine powder with an analytical grinder (Yellow line, A10; IKA-Werke, Staufen, Germany).

Total sugars were extracted from 100 mg dried leaf powder with 5 mL 80% (v/v) ethanol at 80 °C for 1 h. The extraction process was repeated twice following the first extraction and the supernatant pooled. Total starch was determined from the pellet by quantifying the glucose released following an enzymatic digestion of the residue for 17 h at 60 °C, with the amyloglucosidase enzyme (AMG) [Sigma Aldrich (Pty) Ltd, Aston Manor, South Africa].

The 80% ethanol extracts and AMG enzyme extracts were analysed for total soluble sugars using the phenol–sulphuric acid assay (Brummer and Cui, 2005). Briefly, a volume of 20 µL of each of the respective extracts was added to 180 µL de-ionized water, 200 µL phenol (5 $mL \cdot L^{-1}$) and 1000 µL concentrated sulphuric acid. Absorbances were determined on a spectrophotometer (Cary 50 Series, Varian; Mulgrave, Australia) at 490 nm, precisely

after 30 min against a blank prepared for the standard. A standard curve for glucose concentrations was prepared by diluting 0, 50, 100, 150 and 200 µL glucose stock solution (0.10 $mg \cdot mL^{-1}$) with de-ionized water to a final volume of 200 µL. The sugar concentrations were expressed as $mg \cdot g^{-1}$ leaf dry weight (DW) and are respectively referred to as leaf sugar concentration and leaf starch concentration. The sum values of the two components contributed to the total leaf carbohydrate concentration.

2.3.2. Leaf gas exchange

Two leaves were tagged on each of five of the fruiting and the completely de-fruited branch treatment replicates (n=5) for repeated measurements of different parameters of leaf gas exchange. The values of the measurements in the two leaves were pooled to represent the average value for a single treatment replicate (n=5). Measurements started on the day of treatment (day 0) and were repeated on the same leaves 1 (day 1), 5 (day 5) and 14 (day 14) days after treatment. On each date, measurements started at 8:00 AM, continued at 1-h intervals and were completed by 15:00 AM. The rates of leaf CO_2 assimilation (*A*c, expressed as µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$), leaf stomatal conductance (*g*s, expressed as $mmol \cdot m^{-2} \cdot s^{-1}$) and leaf transpiration (*E*, expressed as mmol $H_2O \cdot m^{-2} \cdot s^{-1}$) were measured using a portable Infra-red gas analyser (Li-6400, LI-COR, Lincoln, NE, USA). Measurements were conducted using a closed chamber. The airflow rate was set at 300 $\mu mol \cdot s^{-1}$, photosynthetic photon flux of 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the block temperature set at 25 ºC with controlled CO_2 concentration of 380 ppm.

2.3.3. Iodine staining and microscopy

Chlorotic “off”, and dark, green “on” leaves and petioles were cut perpendicular to the long-axis with a sharp razor blade and immersed for 2 min at room temperature in a 2% (v/v)

iodine solution. Tissue samples were rinsed in water, mounted on a stand and immediately observed under a stereo microscope (Carl Zeiss ERc5s; Göttingen, Germany). Images were captured with a Canon PowerShot S3 IS equipped with MM99 adapter (Martin Microscope Co.).

2.3.4. Leaf colour and total chlorophyll concentration

Leaf colour was measured with a portable electronic leaf relative total chlorophyll concentration meter (SPAD meter, CCM-200, Opti-Sciences; Tyngsboro, Mass.). Measurements were taken on the sampled leaves for carbohydrate measurements and each replicate represented an average of eight leaf readings per sample. A standard curve for SPAD meter readings against chlorophyll concentration was created by extracting the total leaf chlorophyll from 100 mg dried leaf powder of each of ten leaf samples ranging from a yellow to dark green leaf colour in 5 mL acetone for 24 h at 4 °C in the dark. The extraction process was repeated twice following the first extraction and pooled. The decanted extracts were combined, filtered through a 0.45 µm filter (Millex-HV; Millipore Coroporation, Milford, Mass., USA) and absorptions measured with a spectrophotometer at 470, 645 and 662 nm. The extinction coefficients of Lichtenthaler (1987) were used to calculate total leaf chlorophyll concentrations which are expressed as $mg \cdot g^{-1}$ leaf DW.

2.3.5. Leaf N

Analysis of leaf samples for determination of total leaf N concentration was carried out using inductively-coupled plasma-emission spectroscopy at an analytical laboratory [Bemlab (Pty) Ltd., Strand, South Africa]. Leaf N determination was done from the same leaf samples for carbohydrate and total chlorophyll concentration analyses. Total leaf N concentration is expressed as $mg \cdot g^{-1}$ leaf DW.

2.4. Statistical analysis

STATISTICA data analysis software version 13 (Dell Inc. 2015, Round Rock, TX, USA) was used to analyse the data. Analysis of variance (ANOVA) or repeated-measures ANOVA was performed when responses were repeated on the same respondent. Mean separations were carried out using Fisher's least significant difference test where applicable, at $P \leq 0.05$. Relationships between leaf starch concentration and total leaf chlorophyll concentration were analysed with regression analysis and the strength of the relationship indicated by Spearman's correlation coefficient. The percentage variation explained is $100*R^2$ % which is indicated as $(-)R^2$ if the correlation was negative.

3. Results and discussion

The results confirm previous findings, yet at the same time, provide new insights into citrus leaf chlorosis. Leaf chlorosis in "off" 'Nadorcott' mandarin trees was associated with an altered source/sink relationship and the subsequent leaf starch concentration. In whole-tree experiments, total leaf chlorophyll concentration was negatively correlated with leaf starch concentration [R^2=(-)0.74; P<0.001] (Fig. 1), and leaf chlorosis manifested in response to high leaf starch concentration and in the absence of, or low activity of both the fruit and root sinks.

A decrease in total leaf chlorophyll concentration in "off" and summer de-fruited "on" trees manifested during winter and coincided with a peak in leaf starch concentration once the activity of all the major sinks reached a minimum (Table 1 and Fig. 2; see Chapter 2, Figs. 2 and 3). Accumulated starch granules were visible in the palisade mesophyll parenchyma cells, the spongy mesophyll parenchyma cells and in the phloem cells of leaf veins in chlorotic leaves (Figs. 2 and 4).

In “on” and winter de-fruited “on” trees, leaves maintained a healthy, dark green colour (Table 1 and Fig. 5), leaf starch concentration remained relatively low, and very few starch granules were visible in the leaf mesophyll cells, or starch in the xylem and phloem of the leaf petiole (Figs. 2 and 5). In concurrence with whole-tree experiments, leaf starch accumulated and total leaf chlorophyll concentration decreased in response to the elimination of both the fruit and root sinks in the summer and winter branch experiments (Fig. 6). Complete de-fruiting of branches alone did not alter the leaf starch concentration or reduce total leaf chlorophyll concentration, but shortly after eliminating the root sink by girdling, starch accumulated and leaf total chlorophyll concentration decreased rapidly (Fig. 6). Whenever fruit were absent, but the root sink prevailed, instead of accumulating in the leaf as starch, photo-assimilates appeared to be transported to the roots, little accumulation of starch occurred in the leaf and no symptoms of leaf chlorosis developed (Fig. 6).

The starch-induced leaf chlorosis response as reported here is distinct to chlorosis symptoms typically associated with nutrient deficiency and concurs with other studies. The disorder is characterised by intervascular chlorosis that spread gradually to form a complete yellow leaf blade, and eventual chlorotic leaves are thick and rolled (Fig. 2). Leaf chlorosis associated with natural leaf senescence after two or more growing seasons (Schneider, 1968) is generally not associated with high leaf starch concentration, since carbohydrate reserves in old leaves are mobilised and exported from the leaf prior to leaf drop (Ruan, 1993). Also, mineral nutrient deficiency symptoms generally manifest in either old or young leaves. In contrast, leaf yellowing as reported here appeared prematurely on all generations of mature source leaves, and was indeed accompanied by high leaf starch concentration and no leaf drop. Nitrogen concentration during summer and early autumn was generally similar in leaves in trees of contrasting fruit loads and up until mid-winter, no significant difference in leaf N concentration occurred between natural “on” and “off” trees (Fig. 5C). Although leaf

N concentration in summer de-fruited "on" trees was lower subsequent to the de-fruiting treatment in January compared to the other treatments, the leaf N concentration still remained within the acceptable commercial upper and lower threshold values for leaf N concentration (22 to 26 $mg \cdot g^{-1}$ leaf DW) (Raveh, 2013). Throughout the experiment, leaf chlorosis appeared to manifest independent of leaf N concentration and therefore suggests that a decrease in total leaf chlorophyll concentration in "off" trees as reported here, is not a reaction to reduced leaf N, but rather related to fruit-load-induced starch accumulation. This is supported by Schaffer et al. (1986), who reported little role for leaf N concentration in the development of leaf chlorosis in sink-less 'Murcott' mandarin trees, but a similar modest decrease in leaf N concentration was attributed to a decrease in protein levels as a result of chloroplast plastid disintegration in response to de-fruiting and girdling. Lower leaf N concentration in chlorotic leaves was suggested as a response to, rather than a cause of starch-induced leaf chloroplast disintegration and this notion is supported by results from the current study.

The reduction in total leaf chlorophyll concentration in reaction to high leaf starch concentration concur with previous research in citrus and other crops. Excessive accumulation of starch can cause leaf chlorosis in cucumber (Schaffer et al., 1991), tobacco (Herold and McNeill, 1979) and tomato (Lebsky and Poghosyan, 2007). In cucumber, a low-night/high-day temperature regime and short winter days were proposed as possible environmental cues inducing the disorder (Robbins and Pharr, 1987; Chatterton and Silvius, 1979), but Schaffer et al. (1991) suggested reduced winter root sink activity as a more probable cause, similar to what occurred in this study (see chapter 2, Figs. 2 and 3). In citrus, phloem blockages by girdling or bacterial infection of the phloem can create a carbohydrate back-log which results in starch accumulating in the leaf and the disintegration of the chloroplast thylakoid system (Etxeberria et al., 2009; Schaffer et al., 1986). Alternatively to

phloem blockages, low fruit load appears to play a major role in exacerbating the phenomenon in perennial fruit trees, and although starch accumulation and resulting reduction in leaf total chlorophyll concentration in response to low fruit load have been reported in the deciduous apple tree (Snyder-Leiby and Wang, 2008; Schupp et al., 2001), this is the first study to report on the whole-tree phenomenon in the evergreen citrus tree. In this study starch accumulated in leaves in "off" trees as early as summer, but leaf chlorosis symptoms developed in autumn and winter when root growth activity came to a hold (see Chapter 2, Figs. 2 and 3)

Starch is a product of photosynthesis and exists in two forms – small soluble, linear-chain amyloses and branched, highly insoluble amylopectins (Wang et al., 1998). Starch accumulates during day-light and is mobilised at night or other times of low photosynthesis to maintain a constant carbon supply to carbohydrate sinks (Smith et al., 1987). When photosynthesis rates exceed that of amylase degradation, starch can build up in the leaf chloroplast (Li et al., 2003; Nebauer et al., 2014). Normally, carbohydrate accumulation is known to lead to feedback inhibition of photosynthesis (Syverstsen et al., 2003) and a decrease in sugar production (Barth et al., 2003; Eckardt, 2003; Kuhn et al., 1997). However, in "off" and eventual chlorotic 'Nadorcott' mandarin leaves from the current study, both sugars and starch built up early in the season and accumulated towards the onset of autumn. If feedback inhibition of photosynthesis occurred as a result of starch accumulation, sugar production would have decreased, however, photosynthesis was not affected (Fig. 3) and leaf sugar concentration only decreased at the onset of winter (Fig. 5). Our results concur with Nebauer et al. (2011) and Schaffer et al. (1986) and suggest that feedback inhibition did not initially play a role in this cultivar, at least not until autumn and mid-winter.

The development of leaf chlorosis in "off" 'Nadorcott' mandarin trees in an alternate bearing cycle appears to have a physiological cause related to an excess accumulation of leaf

sugars due to the absence of fruit and reduced root sink activity, either as a result of girdling, or in response to low winter temperatures. Fruit regulate the control of expression of various genes associated with mobilisation and storage of carbohydrates. In citrus leaves from "off" trees, the expression of important genes of enzymes involved in starch synthesis are up-regulated, viz. plastidic starch phosphorylase, ADP-glucose pyrophosphorylase, the plastidic ATP/ADP translocator, cytoplasmic phosphoglucomutase and sucrose synthase (Li et al., 2003; Nebauer et al., 2011, 2014), while gene expression of enzymes responsible for the degradation and mobilisation of starch from the leaf chloroplast, a-amylase and sucrose synthase (Lloyd et al., 2005; Smith et al., 2004; Zeeman et al., 1998) are down-regulated (Nebauer et al., 2014). In addition, an abnormal increase in leaf starch concentration correlates with low expression of genes for sucrose transporters (Nebauer et al. 2011, 2014) which are responsible for plasma-membrane export of sucrose in source tissues (Eckardt, 2003). Expression of these transporters is enhanced under high photo-assimilate availability and demand, however, low demand in "off" trees induce increased starch synthesis via the down-regulation of these sucrose transporters (Nebauer et al., 2014). Changes in content of specific sugars have been suggested to signal the up- or down-regulation of these genes (Li et al., 2003), but Nebauer et al. (2014) suggested that altered gene expression responsible for excessive starch accumulation in source tissues of "off" trees are mediated by the lack of a hormonal signal transmitted by fruit (Nebauer et al., 2014). The phyto-hormones 1 *H*-indole-3-acetic acid and gibberellic acid are responsible for phloem loading and export of photo-assimilates from source tissues (Daie et al., 1986). In this study, lack of fruit in "off" trees may have been responsible for potential down-regulation or dysfunctional enzyme activity in the source tissue that did not mobilize starch effectively, while the subsequent dysfunctional phloem loading thereof due to alternating phyto-hormone levels in the absence of fruit could also have played a role.

4. Conclusions

Leaf chlorosis in "off" 'Nadorcott' mandarin trees correlated negatively with seasonal leaf starch concentration, which was mediated by low fruit and root sink activity. During winter, a decrease in total leaf chlorophyll concentration in "off" trees coincided with a peak in leaf starch concentration once the activity of all the major sinks reached a minimum. In "on" trees, leaf starch concentration was lower compared to "off" trees, the major carbohydrate component was sugars and leaves maintained a healthy, dark green colour. Previous and current studies provide sufficient evidence to propose that in "off" 'Nadorcott' mandarin trees accumulated starch granules eventually ruptured the leaf chloroplast – the photosynthetic apparatus and main source of sugars which subsequently led to a disintegration of the chloroplast thylakoid membrane, a decrease in sugar production and the manifestation of leaf chlorosis. Lower leaf N concentration and lower leaf sugar concentration do not appear to be a cause of leaf chlorosis in "off" trees, but rather a response to starch accumulation and its well-documented disruptive effect on the photosynthetic apparatus. These results can be used to prevent unnecessary winter N applications in "off" 'Nadorcott' mandarin trees. Future research objectives should be aimed at determining a tree fruit load threshold for the initiation of fruit-load-induced leaf chlorosis in the form of a leaf-to-fruit ratio, as well as to determine and negate the likely negative impact of accumulated starch granules on the photosynthetic apparatus and overall tree performance during winter.

Table 1. The effects of fruit load on leaf colour and the concentration of various leaf carbohydrate components in alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during winter 2015.

Tree fruiting status	Tree fruit yield (kg per tree)	Leaf relative total chlorophyll content[z]	Leaf sugars (mg·g^{-1} leaf dry weight)	Leaf starch (mg·g^{-1} leaf dry weight)	Leaf total carbohydrates (mg·g^{-1} leaf dry weight)
"On"	84 a[y]	115.2 a	79.8 ns[x]	41.3 b	121.1 b
"Off"	16 b	68.6 b	80.5	118.6 a	199.1 a
P value	<0.0001	0.0015	0.0913	<0.0001	<0.0001

[z] Measured with a portable electronic leaf relative total chlorophyll content meter (SPAD meter, CCM-200, Opti-Sciences; Tyngsboro, Mass., USA).

[y] Means with a different letter within a column differ significantly at the 5% level (Fisher's LSD; n=10).

[x] No significant differences.

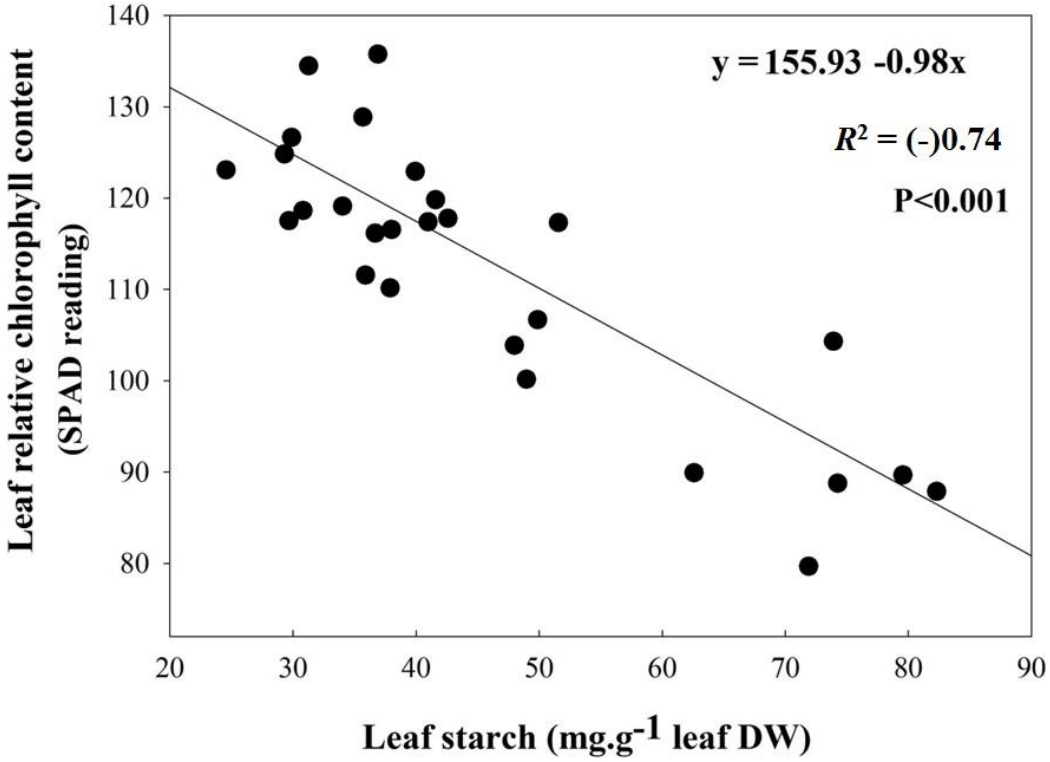

Fig. 1. The correlation between seasonal leaf starch concentration and leaf relative total chlorophyll concentration in whole-tree source/sink alteration experiments in 'Nadorcott' mandarin (*C. reticulata*) trees. DW = dry weight.

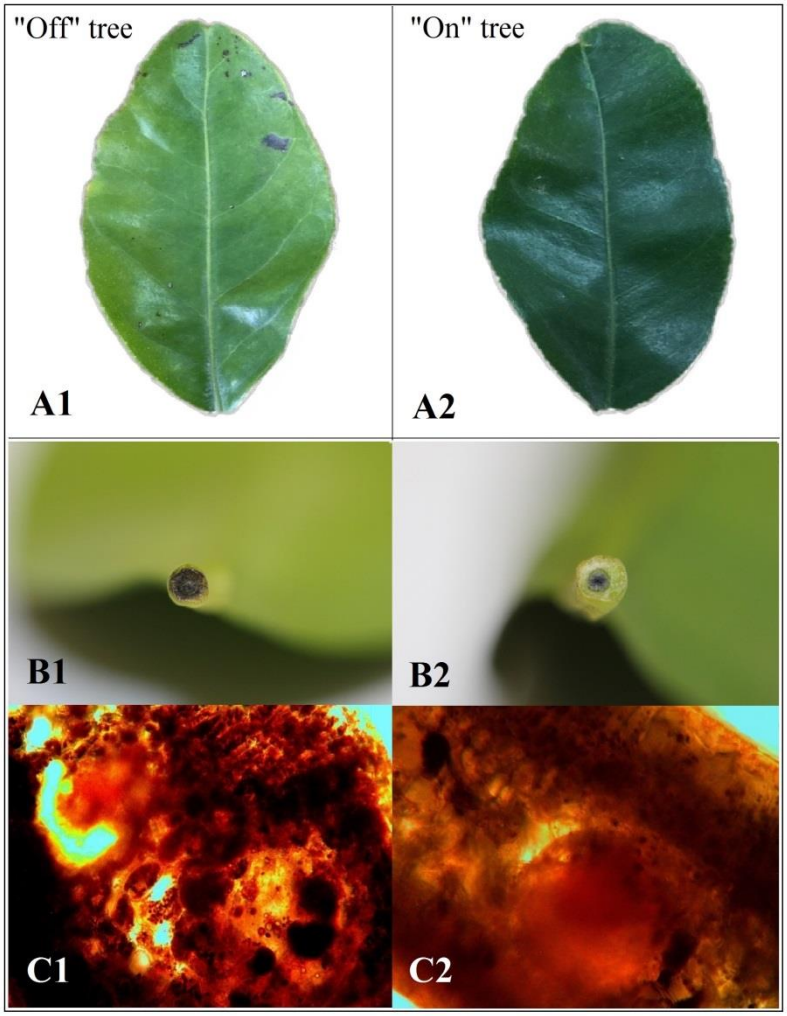

Fig. 2. The effects of fruit load ("off" = low fruiting; "on" = heavy fruiting) on leaf colour (A1 and A2) and the distribution of starch granules in the leaf petiole (B1 and B2) and the leaf blade (C1 and C2) of 'Nadorcott' mandarin (*C. reticulata*) trees during winter in 2015. Leaves were dissected, stained with a 2% (v/v) iodine solution and examined using microscopic (Carl Zeiss ERc5s; Göttingen, Germany) photographic comparisons.

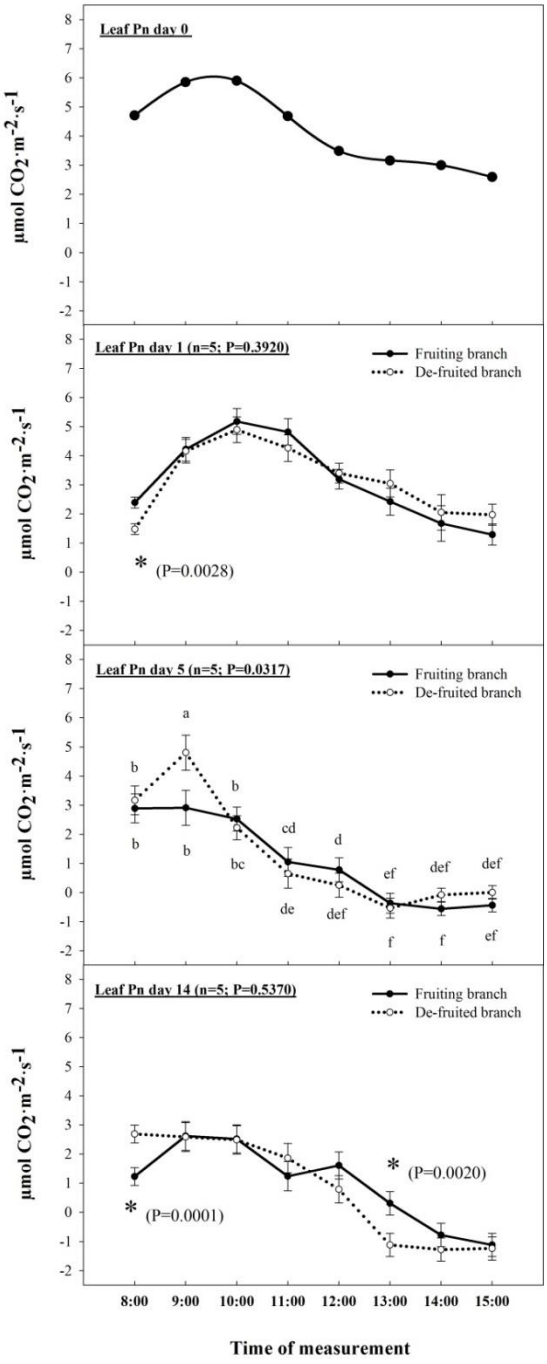

Fig. 3. The effect of de-fruiting on rate of leaf photosynthesis in ten-year-old 'Nadorcott' mandarin (*C. reticulata*) trees during summer in 2015. Bars denote standard errors of the means and different letters significant differences between values ($P<0.05$; Fisher's LSD test; n=5).

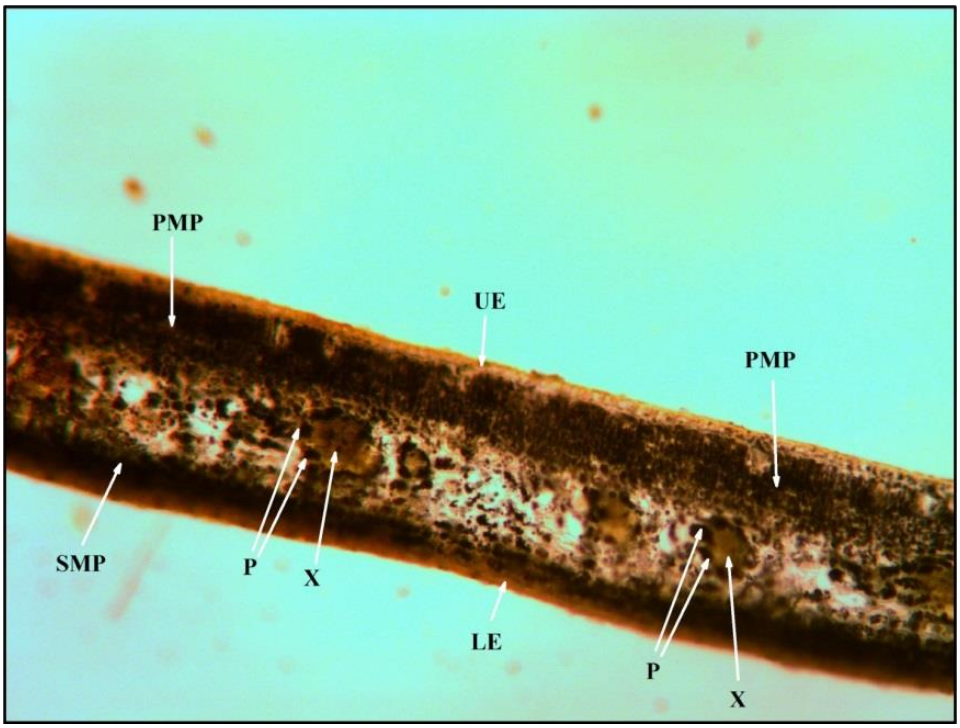

Fig. 4. Disks of chlorotic leaf blades from "off" 'Nadorcott' mandarin (*C. reticulata*) trees were dissected, stained with a 2% (v/v) iodine solution and examined for distribution of accumulated starch granules using microscopic (Carl Zeiss ERc5s; Göttingen, Germany) photograph comparisons. Starch stains dark-brown with iodine and were subsequently observed in the palisade mesophyll parenchyma cells (PMP), the spongy mesophyll parenchyma cells (SMP) and in the phloem cells (P) of the leaf vein. Little to no starch was observed in the xylem (X) and upper- (UE) and lower leaf epidermis (LE).

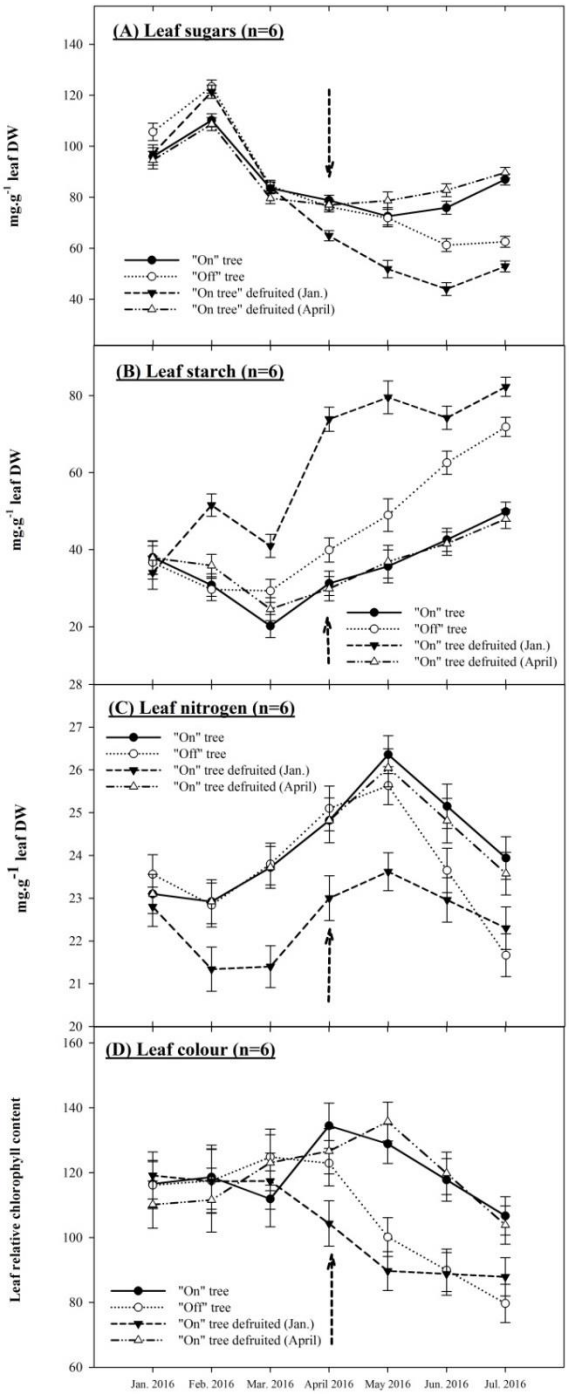

Fig. 5. The effects of fruit load ("on" = heavy fruiting; "off" = low fruiting) and the complete de-fruiting of "on" 'Nadorcott' mandarin (*C. reticulata*) trees during summer in Jan. 2016, and autumn in Apr. 2016 (dotted arrow indicates timing of application for the April treatment) on the concentrations of: A) leaf sugars, B) leaf starch, C) leaf nitrogen and D) leaf relative total chlorophyll content. Bars denote standard errors of the means (n=6). DW = dry weight.

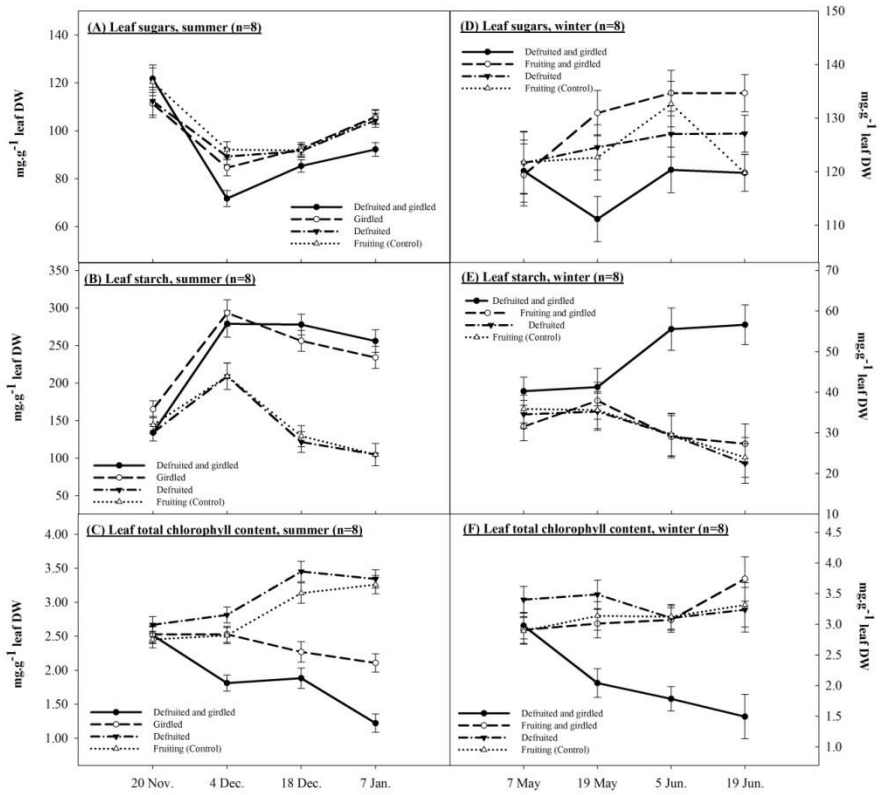

Fig. 6. The effects of branch source/sink alterations in 'Nadorcott' mandarin (*C. reticulata*) trees at the end of Nov. 2014 in summer, and at the end of Apr. 2015 in winter on the concentrations of: A and D) leaf sugars, B and E) leaf starch and C and F) leaf total chlorophyll concentration. Bars denote standard errors of the means (n=8). DW = dry weight.

Chapter 4: An assessment of the role of mineral nutrients in alternate bearing 'Nadorcott' mandarin trees

Abstract. The relationship between fruit load and the concentration of macro-nutrients in leaves was studied in alternate bearing 'Nadorcott' mandarin (*Citrus reticulata* Blanco) trees. Fruit load affected the leaf mineral nutrient concentrations, and the crop removal factor, i.e. the g mineral element removed per kg fruit per tree, was consistent in both seasons. The crop removal factor was higher for each mineral nutrient in "off" trees – one kg fruit removed 2.3 g N, 0.3 g P, 3.1 g K, 1 g Ca and 0.4 g Mg in "off" trees, compared to 1.3 g N, 0.2 g P, 1.7 g K, 0.6 g Ca and 0.2 g Mg per one kg fruit in "on" trees. Fuit loads of 84, 110 and 52 kg fruit per tree in "on" trees, however, removed 217 g N, 28 g P, 296 g K, 100 g Ca and 35 g Mg per tree, which averaged 1.5 to 6 times more than that of fruit loads of 14, 71 and 16 kg fruit in "off" trees. In "off" trees, N, P and K accumulated in leaves to between 20% and 30% higher concentrations compared to "on" trees. Although the concentrations of macro-nutrients were generally slightly higher in leaves of "off" trees than of "on" trees, the higher nutrient status did not manifest in, or consistently correlate, with intensity of summer vegetative shoot development and/or flowering response. Apart from some anomalies, the concentrations of macro-nutrients in leaves were unaffected by defruiting and foliar spray applications of mineral nutrients to "on" or "off" trees, and showed no consistent relationship with treatment effects on parameters of vegetative shoot flush and flowering. Leaf mineral nutrient concentration do not relate to any parameters of flowering or fruit load under conditions of alternate bearing in 'Nadorcott' mandarin and appears to be a consequence of fruit load, not a determinant thereof.

1. Introduction

'Nadorcott' (*Citrus reticulata* Blanco), also known as 'W. Murcott', is a late-maturing mandarin cultivar that developed from a seed of the highly-seeded and strongly alternate bearing 'Murcott' mandarin (Nadori, 2006). 'Murcott' is of unknown parentage, but is believed to be a tangor; a hybrid between a mandarin and a sweet orange (*C. reticulata* × *C. sinensis* (L.) Osbeck). 'Nadorcott' mandarin trees produce fruit with good quality attributes and can yield high crop loads, but this often makes the trees prone to alternate bearing.

Alternate bearing is a perpetuating and problematic phenomenon that occurs in certain fruit trees, and is characterised by trees flowering profusely and producing an excess amount of fruit in one season ("on" year), followed by the production of a sparse number of flowers and fruit in the following season ("off" year) (Monselise and Goldschmidt, 1982). In citrus (*Citrus* spp.), alternate bearing compromises the consistency of orchard management practices and leads to costly challenges in the production, harvesting, transport, packing and marketing of fruit. The "on" crop is generally characterised by a large number of small fruit, whereas the "off" crop is comprised of large unattractive fruit (Galliani et al., 1975; Hield and Hilgeman, 1969; Monselise and Goldschmidt, 1982; Moss et al., 1974).

It has not been elucidated how fruit load in alternate bearing citrus trees affects mineral nutrient uptake and allocation, as well as if and where it has a role in the nutritional theory of alternate bearing, as was shown for other alternate bearing fruit and nut trees, e.g. in olive (*Olea europaea* L.) (Fernández-Escobar et al., 1999) and pistachio (*Pistachia vera* L.) (Brown et al., 1995; Rosecrance et al., 1998). The impact of fruit load on mineral nutrient distribution was reported in comparative studies in heavy- and low-fruiting mandarin trees (Golomb and Goldschmidt, 1987; Monselise et al., 1983; Smith, 1976), but it is not clear if and how this effect impacts on the perpetuating habit of alternate bearing. The question remains whether mineral nutrient status is a contributing cause of alternate bearing and if so,

could good fertilisation practices overcome it. Alternatively, does mineral nutrient status simply reflect a response to the extremities of plant developmental events that accompanies this phenomenon, as suggested by Smith (1976).

A direct influence of certain mineral elements, such as nitrogen (N), on citrus reproductive development is known (Lovatt et al., 1988). It has also been shown that fruit can consume mineral nutrients at the expense of initiation and maintenance of growth of other tree organs that support citrus reproductive development, e.g. growth of new vegetative shoots (Martínez-Alcántara et al., 2015) and roots (Lenz, 2000; Smith, 1976). However, few of these experiments were conducted under field-conditions, and many of the interpretations were based on results from one season only, and confined to cultivars which are not prone to alternate bearing and that have unique phenologies, e.g. lemon (*C. limon* L.). In addition, although the effects of mineral nutrient availability on flowering and vegetative shoot and root growth in these studies have been researched independently, knowledge on how mineral nutrients interact with different organ types at various phenological stages, and how this affects citrus reproductive development under conditions of alternate bearing is lacking.

In citriculture, annual fertilisation practices aim to optimise plant growth and maximise fruit yield (Embleton et al., 1978; Koo et al., 1984). Fertiliser recommendations in South African citrus production make use of routine leaf analysis that is based on a combination of leaf mineral nutrient norms that were developed for sweet orange cultivars in the USA (Chapman, 1949; Embleton et al., 1973; Koo et al., 1984) and in sweet orange, grapefruit (*C. paradisi* Macf.) and lemon in South Africa (Du Plessis 1977; Du Plessis et al., 1992; Du Plessis and Koen, 1992). These leaf mineral nutrient norms are used to assess the tree nutritional status and to fertilise according to the plant's nutritional demand to achieve a target fruit load (Jones and Embleton, 1969). However, it is not clear if these norms are also applicable to relatively new mandarin cultivars (Raveh, 2013), and to what extent they apply

to managing the development of vegetative shoot flush, root growth and flowering in the context of alternate bearing.

The primary objective of this study was to investigate the role of the major mineral nutrients in alternate bearing 'Nadorcott' mandarin trees. To address this question, seasonal changes in concentrations of macro-nutrients, viz. N, phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg), were measured in leaves of "on" and "off" 'Nadorcott' mandarin trees and correlated with flowering, vegetative shoot flush, root growth and tree fruit load over two production seasons. Additionally, leaf mineral nutrient concentration and phenological events were measured in response to source/sink manipulations at different phenological stages, as well as in response to foliar applications of important mineral nutrients.

2. Materials and methods

2.1. Plant material and experimental site

Ten- to fifteen-year-old 'Nadorcott' mandarin trees budded on 'Carrizo' citrange [*C. sinensis* L. (Osb.) × *Poncirus trifoliata* (L.) Raf.] rootstock were selected from orchards with a history of alternate bearing in De Doorns (lat. 33°51'S, long. 19°52'E) for experiment 1, in Citrusdal (lat. 32°81'S, long. 19°01'E) for experiment 2 and in Riviersonderend (lat. 34°13'S, long. 19°89'E) for experiment 3. All the experimental sites are located in the Western Cape Province of South Africa. The Western Cape forms part of one of five climatically diverse citrus growing regions in Southern Africa and experiences Mediterranean-type climatic conditions: summer typically occurs from December to February; autumn from March to May; winter from June to August and spring from September to November. The region receives an annual rainfall of between 400 and 650 mm and the majority occurs from May to August.

The orchard in De Doorns was used in the main experiment. The orchard was cultivated, pruned and sprayed according to good agricultural practices: trees were spaced at 5 × 2 m (1000 trees per ha) in a sandy soil with $pH_{(KCl)}$ 4.4 and watered using a drip irrigation system with four emitters per tree and total water supply was ≈4000 L per tree per annum. All trees received consistent and standard fertiliser applications with the rate of application (kg per ha) based on annual leaf mineral nutrient analysis and a target fruit yield of 60 to 70 tons of fruit per ha. For N, a leaf concentration of between 22 and 26 $mg \cdot g^{-1}$ leaf dry weight (DW) was considered optimum by the citrus grower. Total annual N application amounted to 240 kg per ha, of which 25% was applied as foliar applications, 20% as soil applications and 55% was dissolved in the irrigation solution (fertigation) and split uniformly into eight applications from September to April. The rates of annual P and K applications were targeted to maintain optimum leaf P and K concentrations of between 1.1 and 1.5 $mg \cdot g^{-1}$ leaf DW for P, and 9 and 16 $mg \cdot g^{-1}$ leaf DW for K. The total annual P and K applications amounted to 12 kg P and 265 kg K per ha with the majority applied via fertigation and a small fraction applied by foliar sprays. The Ca and Mg applications were targeted to maintain optimum leaf concentrations of between 35 and 50 $mg \cdot g^{-1}$ leaf DW for Ca, and 3 and 5.5 $mg \cdot g^{-1}$ leaf DW for Mg and amounted to 60 and 22 kg per ha, respectively. Calcium was applied via fertigation from August to November, whereas Mg was applied as one foliar spray in August plus increasing portions from September to March via fertigation.

2.2. Treatments and experimental design

2.2.1. Experiment 1

In this experiment the pattern of concentrations of the five major macro-nutrients in leaves in “on” and “off” trees were followed as independent variables over a period of two seasons and the data were used in regression analyses with selected determinants of flowering

and fruit load as dependent variables. The selected trees were representative of heavy- or low-fruiting trees and subsequently included as single-tree replicates of "on" and "off" treatments in a completely randomised design (n=8). Most of the trees in the orchard bore similar fruit yields, but for the purpose of this experiment individual trees that showed an opposite and natural alternate bearing trend were selected prior to harvest in Aug. 2014.

2.2.2. Experiment 2

To validate the interpretation of the results from experiment 1, leaf N, P, K, Ca and Mg concentrations as well as vegetative shoot flush and flowering responses to complete fruit removal of heavy-fruiting trees were determined at two phenological stages. The "on" cycles of 12 separate trees were desynchronised by removing all fruit of six heavy-fruiting trees in Jan. 2016 for the summer treatment, and six heavy-fruiting trees in Apr. 2016 for the winter treatment. The experiment was set up in a completely randomised design, using whole trees for treatment replicates (n=6).

2.2.3. Experiment 3

Mineral nutrient foliar sprays were applied to heavy-fruiting whole-tree replicates ≈210 days after full bloom on 11 May 2016 between 08:00 and 10:00 AM. The foliar sprays consisted of the following treatments that were set up in a randomised complete block design (n=6): 1) an untreated control; 2) N [10 $g \cdot L^{-1}$ low biuret urea (Sasol, Sandton, South Africa) containing 460 $g \cdot kg^{-1}$ N]; and 3) K [30 $g \cdot L^{-1}$ fully soluble, crystalline formulation of potassium nitrate (KNO_3) (Sasol, Sandton, South Africa)]. Foliar sprays were applied until the point of runoff at a rate of ≈4 L spray solution per tree using a backpack mist-blow sprayer [Stihl SR430; Andreas Stihl (Pty) Ltd, Pietermaritzburg, South Africa] set at droplet size 2 (1 – fine droplet size, 5 – coarse droplet size). To avoid drift between different

treatments, buffer trees were left untreated between treated and control trees in the same row, as well as buffer rows between treated rows. The trees were selected for uniformity in tree condition based on a dark, green leaf colour. All trees were similar in canopy volume with a height of approximately 3.5 to 4.0 m and an across-row width of 2.5 to 3.5 m. The trees had a trunk circumference of ≈35 cm as measured above the bud union and a uniform and evenly distributed fruit load.

2.3. Data collection

2.3.1. Flowering and vegetative shoot and root growth

In experiment 1, the number of flowers per tree was estimated by counting the number of flowers within the limits of a 0.5 × 0.5 × 0.5 m frame during full bloom in October. The tree canopy was divided into an Eastern and Western sector and an upper- and lower-half. A flower count was performed in each of these four respective quadrants per tree. The total number of flowers was estimated by extrapolating the mean number of flowers per frame to the total tree volume. The same procedure was used to estimate the number of new vegetative shoots after cessation of periods of vegetative shoot flushes in November, February and April.

2.3.2. Yield

In all the experiments commercial harvest of fruit commenced in mid-July once fruit quality indices complied with specifications established by fruit export markets and was completed by the end of August. To determine the total fruit yield of the treatments, all fruit were harvested separately from individual trees on the same day prior to the start of commercial harvest. A sample of 100 fruit was randomly collected from each tree replicate and the transverse diameter (in mm) of each fruit was measured using an electronic fruit size

measuring calliper (CD-6" C; Mitutoyo Corp, Tokyo, Japan). Each fruit was assigned to a fruit size category of which the average fruit weight was determined and fruit size distribution from each treatment replicate was extrapolated for the total number of fruit per tree.

2.3.3. Leaf and fruit sampling for mineral nutrient analysis

The leaf sampling protocol for analysis of mineral nutrients is different for the major citrus producing countries. In USA [California (Chapman, 1968; Embleton et al., 1973) and Florida (Koo et al., 1984; Koo and Sites, 1956; Smith, 1966)] and Australia (Jorgensen and Price, 1978), leaves are sampled from four to 10 months-old, non-fruiting and purely vegetative shoots. In contrast, citrus growers in Israel (Raveh, 2013) and South Africa (Du Plessis 1977; Du Plessis et al., 1992; Du Plessis and Koen, 1992) sample leaves from four to six-months-old fruiting shoots. The use of fruiting shoots, however, may not be wholly reliable as the characteristics of fruit on different trees and shoots might be different and therefore also their respective potentials to assimilate mineral nutrients from neighboring leaves. Also, results from analyses of leaves sampled from fruiting shoots cannot necessarily be used to predict what will happen in the future as it is not guaranteed that fruit will remain on the tree during the period between sampling and timing of the next fertiliser application. More importantly, non-fruiting and purely vegetative shoots are those on which the majority of the next season flowers and fruit will develop from (Verreynne and Lovatt, 2009), which is of direct interest in this experiment since results from such leaf mineral nutrient analyses could be used in validating predictions of flowering and fruit load with regression analysis.

In this study only mature leaves were sampled from the third to fifth position on fully hardened, non-fruiting and purely vegetative shoots. All shoots had triangular internodes, a length of ≈15 cm and were located on the outside of the tree canopy at a height of ≈1.5 m

above the orchard floor. In experiment 1, the September and spring leaf samples were collected from vegetative shoots that developed during the previous season's vegetative shoot flushes, the December and summer leaf samples were collected from vegetative shoots that developed during the current season's spring vegetative shoot flush, and the March and autumn, and June and winter leaf samples were collected from vegetative shoots that developed during the current season's summer vegetative shoot flush. In experiment 2, leaf samples were collected from vegetative shoots that developed during the current season's summer vegetative shoot flush, starting on the day of treatment in January and continued at monthly intervals until commercial harvest commenced in July. In experiment 3, leaf samples were collected on the day of treatment and again 2 weeks thereafter.

One leaf sample consisted of eight leaves that were collected from each of four shoots from each treatment replicate between 8:00 and 10:00 AM on each sampling date. After sampling the leaves were kept cool and washed with distilled water before being frozen at –80 °C and freeze-dried (Christ Beta 1–8 LD Freeze Dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The leaves were ground to a fine powder with an analytical grinder (Yellow line, A10, IKA-Werke, Staufen, Germany) and stored at –80 °C until analysis.

In experiment 1, fruit samples were collected from different canopy positions at the time of commercial harvest to determine the mineral nutrient concentration of fruit from each of three distinct fruit size categories. For each sampled fruit, the transverse diameter was measured with an electronic calliper and weight (g) of each fruit determined with an electronic scale (W22 Series; UWE Co., Hsin Tien, Taiwan). Fruit were subsequently assigned to a fruit size category, viz. small (≈50 mm and 71 g), medium (≈63 mm and 131 g)

and large (≈76 mm and 203 g). From each fruit size category six fruit samples consisting of 12 fruit were analysed for mineral nutrient concentration.

The concentrations of the mineral nutrients in the fruit tissue (rind and pulp combined) were used to calculate the total weight of the respective elements removed by the total fruit loads in "on" and "off" trees by using the total fruit yield in kg fruit per tree and fruit size distribution data for each of the treatment replicates.

2.3.4. Analysis of mineral nutrient concentration

Mineral nutrient analyses of individual elements in leaf and fruit samples were conducted by an accredited commercial chemical and microbiology analytical laboratory [Bemlab (Pty) Ltd., Strand, South Africa] according to published protocols (Hou and Jones, 2000). Briefly, 1 g of fruit or dried leaf tissue was made up to a volume of 50 mL with a 50:50 hydrochloric acid (50%) solution for extraction through filter paper. The P, K, Ca and Mg concentrations were analysed using inductively-coupled plasma-emission spectroscopy (Varian PRX–OEX, Varian Inc., Palo Alto, CA, USA) against suitable standards and subsequent to a nitric-hydrochloric total acid digestion step. For analysis of total N, 0.15 g of each sample was combusted at 850 °C and analysed using a LECO N analyzer (LECO FP528 Nitrogen analyzer, LECO cooperation, St. Joseph, MI, USA) by thermal conductivity. The concentrations of the mineral nutrients in the leaf and fruit were expressed as $mg \cdot g^{-1}$ leaf dry weight or $mg \cdot g^{-1}$ fruit fresh weight.

2.4. Statistical analysis

STATISTICA data analysis software version 13 (Dell Inc. 2015, Round Rock, TX, USA) was used to analyse the data. Analysis of variance (ANOVA) or repeated-measures ANOVA was performed when responses were repeated on the same respondent. Mean

separations were carried out using Fisher's least significant difference test where applicable, at $P \leq 0.05$. Relationships between two continuous variables were analysed by regression analysis and the strength of the relationship indicated by Spearman's correlation coefficient. The percentage variation explained is $100*R^2$ % which is indicated as $(-)R^2$ if the correlation was negative.

3. Results and discussion

The crop removal factor, i.e. the g mineral nutrients removed per kg fruit per tree, was higher for each mineral element in "off" trees – one kg fruit removed 2.3 g N, 0.3 g P, 3.1 g K, 1 g Ca and 0.4 g Mg , compared to 1.3 g N, 0.2 g P, 1.7 g K, 0.6 g Ca and 0.2 g Mg per one kg fruit in "on" trees (data not shown). Total fruit load, i.e. the kg fruit per tree, however, was 6-fold higher in "on" trees in season 1, and 1.5-fold higher in "on" trees in season 2 (Table 1). Fruit loads of 84, 110 and 52 kg fruit per tree in "on" trees therefore removed on average 217 g N, 28 g P, 296 g K, 100 g Ca and 35 g Mg per tree, which were 1.5 to 7 times more than that removed by fruit loads of 14, 71 and 16 kg fruit per tree in "off" trees (Table 2). "Off" trees on the other hand, sprouted two- to three-times more new vegetative shoots than "on" trees (Table 1), but the demand for macro-nutrients by these vegetative shoot flushes was substantially lower than that required by the demand of a heavy fruit load in "on" trees. Nitrogen, P and K accumulated in leaves in "off" trees to concentrations of between 20% and 30% higher than those of "on" trees (Fig. 1). Therefore, fruit load affected the concentration of the macro-nutrients in leaves, but not to the detriment of vegetative shoot flush or flowering.

This concurs with studies in heavy-fruiting 'Michal', 'Murcott' and 'Wilking' mandarin trees, where up to 32%, 44% and 58% of the total N, P and K tree dry-matter were removed by the harvest of a heavy fruit load (Golomb and Goldschmidt, 1987; Monselise et al., 1983;

Smith, 1976). In low-fruiting trees, mineral nutrients accumulated in roots, shoots and leaves (Golomb and Goldschmidt, 1987; Monselise et al., 1983; Smith, 1976). Judging from the drop in the respective N, P, and K concentrations in old leaves from June in winter of season 1, to September in spring of season 2 (Fig. 1), the accumulated elements appeared to have been rapidly used by growth of developing shoots, flowers and fruit, to support an approximate ≈230-fold higher flower number in "on" trees, and double the amount of new vegetative shoots in "off" trees (Table 1). This concurs with Roccuzzo et al. (2017), who recently confirmed earlier findings of Sanz et al. (1987) and Legaz et al. (1995) that during spring, citrus trees mobilise more than 60% of the total required N from reserves that are stored in one-year-old leaves.

In this study, however, the concentration of macro-nutrients in leaves of 'Nadorcott' mandarin trees showed no consistent relationship with return bloom flowering, and/or with fruit load of the subsequent season (Tables 3 and 4). Nitrogen concentration in leaves correlated positively, but weakly with summer vegetative shoot development in both seasons (season 1: R^2=0.49, P=0.050; season 2: R^2=0.51, P=0.049) (Tables 3 and 4). Concentrations of N and P in leaves correlated positively with flowering in season 1 (N: R^2=0.66, P=0.010; P: R^2=0.52, P=0.040) (Table 3), but leaf N concentration had a strong negative correlation with flowering [R^2=(-)0.79, P=0.001], and leaf P concentration showed no correlation with flowering (R^2=0.10, P=0.750) in season 2 (Table 4). Leaf K concentration during flower induction was positively correlated with subsequent return bloom in both seasons (season 1: R^2=0.67, P=0.001; 2016: R^2=0.65, P=0.001) (Tables 3 and 4), but foliar spray treatments during flower induction at the end of the first season, to validate these positive correlations, failed to improve flowering (Fig. 4). It should however be noted that treatments were applied late in the alternate bearing cycle. An earlier timing of foliar nutrient sprays should be considered in future research targeting the induction of root and/or shoot flushes.

Results from the de-fruiting experiment furthermore substantiated the lack of a relationship between leaf mineral nutrient concentration and vegetative shoot development or flowering. There were no differences in the concentrations of any of the leaf mineral nutrients that were induced by the different fruit removal treatments in "on" trees, except for some anomalies (Table 5). Those that did differ from the others were not related to any vegetative or flowering responses that resulted from the foliar nutritional spray treatments (Table 6). For example, a defruiting treatment of "on" trees in summer increased vegetative shoot development by almost 9-fold compared to the control "on" trees, but had no effect on leaf mineral nutrient concentrations (Tables 5 and 6).

Although these results contradict some of those obtained in deciduous fruit and nut trees, e.g. apple [*Malus* × *sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] (Neilsen et al., 1990) and pistachio (Rosecrance et al., 1998), they concur with research in other evergreens, e.g. olive (Jiménez-Moreno and Fernández-Escobar, 2017). There may be a minimum level for each respective mineral element required by a citrus tree to maintain metabolism and general physiological functioning, however, all the commercial orchards used as experimental sites in this study were well-fertilised and never subjected to any deficiencies that may have affected primary metabolism and contributed to alternate bearing in such a manner. In this study, mineral nutrients appeared not to have played a regulative role in the perpetuating habit of alternate bearing in 'Nadorcott' mandarin trees. Alternative factors such as a hormonal regulation of vegetative shoot development, root growth and flowering might be of more relevance (Ulger et al., 2004; Shalom et al., 2012, 2014; Goldberg-Moeller et al., 2013).

These results therefore also question the relevance of leaf mineral nutrient norms that are currently used in citrus production, especially in orchards or trees under conditions of an alternate bearing cycle. To support a maximum fruit yield of between 60 to 70 ton fruit per

hectare, Raveh (2013) proposed that the optimal concentrations of the macro-nutrients in mandarin leaves should be 20 to 24 $mg \cdot g^{-1}$ leaf DW for N, 0.9 to 1.2 $mg \cdot g^{-1}$ leaf DW for P, 5.5 to 6.9 $mg \cdot g^{-1}$ leaf DW for K, and 1.9 to 2.6 $mg \cdot g^{-1}$ leaf DW for Mg. Alva et al. (2006) reported that optimum N, P and K concentrations in 4 to 6-month-old mandarin spring flush leaves are 26 to 30 $mg \cdot g^{-1}$ leaf DW for N, 0.8 to 2.4 $mg \cdot g^{-1}$ leaf DW for P, and 15 to 18 $mg \cdot g^{-1}$ leaf DW for K. Throughout all the experiments in this study, however, trees with leaf nutrient concentrations within these ranges did not consistently produce flowers or sprouted new vegetative shoots. For example, from season 2 to season 3, fruit load in "on" trees decreased from 110 kg fruit per tree to 16 kg fruit per tree, while the average leaf N, P, K, Ca and Mg concentrations were all well within the ranges of their suggested mineral nutrient norms over this period. The same inconsistent relationships between fruit load and the current leaf mineral nutrient norms occurred in "off" trees, but the complete opposite response manifested in terms of crop size. This lack of a direct relationship, positive or negative, between the concentration of mineral nutrients and the subsequent flowering and fruit load response implies that mineral nutrients are not the determining factor of flower prevalence.

Root starvation and malfunction due to carbohydrate consumption by fruit was proposed as the main consequential cause of mineral nutrient depletion of vegetative tissues in "on" 'Murcott' trees (Smith, 1976). In the current study, a major portion of the annual mineral nutrient applications to trees was supplied via the soil, but no new root growth occurred in "on" trees (see Chapter 2, Figs. 2 and 3). Considering that a major portion of mineral nutrients are taken up by citrus trees through the soil (Roccuzzo et al., 2017), and that active root growth is important for scavenging and uptake of certain soil-applied mineral nutrients such as Ca (Castle, 1978), the lack of root growth in "on" trees could have impacted on leaf mineral nutrient concentration. However, in this study, Ca, which was only applied

via the soil, accumulated in leaves of "on" trees to greater concentrations than that in "off" trees (Fig. 1). This is in concurrence with results obtained in heavy-fruiting 'Wilking' mandarin trees (Golomb and Goldschmidt, 1987). Transport of Ca from the soil is a passive process and dependent on the strength of the transpiration stream through the xylem (Hanger, 1979). Once taken up by the leaf, Ca is weakly translocated to other plant organs such as newly developing leaves, meristems and fruit (Hanger, 1979). Similar reduced root growth compared to that in the current study was reported in heavy-fruiting sweet orange trees by Lenz (2000), but in that study root growth did not negatively affect mineral nutrient uptake from the soil. In fact, Lenz (2000) reported that although root growth was almost completely lacking, the uptake of Ca and other mineral nutrients were much higher in heavy-fruiting trees due to an apparent increased transpiration rate induced by a heavy fruit load. This strongly concurs with results from the current study in which leaf photosynthesis, leaf stomatal conductance and leaf transpiration rates were all higher in "on" trees (see Chapter 2). In fact, the higher concentration of total mineral nutrients (Fig. 3), i.e. the sum of the individual elements in leaves, and an approximately 2-fold higher crop removal factor by fruit in "on" trees compared to "off" trees suggest that uptake of mineral nutrients by "on" 'Nadorcott' trees must have been substantially higher, and that new root growth was not related to root function.

4. Conclusion

Mineral nutrients apparently do not play a regulative role in the perpetuating habit of alternate bearing. Fruit load influenced the concentration of the macro-nutrients in leaves, but not to the detriment of vegetative shoot flush or flowering of the subsequent season. The crop removal factor was higher for each mineral in "off" trees, but an ≈8-fold higher fruit load in "on" trees removed 1.5 to 7 times more mineral nutrients than fruit load in "off" trees.

In “off” trees N, P and K accumulated in old leaves to concentrations of between 20% and 30% higher compared to “on” trees, and in some cases correlated positively with the intensity of vegetative shoot flushes and flower number. However, the lack of significance of the apparent relationships between mineral nutrients, vegetative shoots and flowering in subsequent experiments failed to confirm these correlations. Apart from some anomalies, concentrations of mineral nutrients in leaves were unaffected by defruiting and foliar spray applications of mineral nutrients to “on” trees, and showed no consistent relationship with treatment effects on parameters of vegetative shoot flush and/or flowering. This study does not support a direct regulative role for mineral nutrients in alternate bearing in ‘Nadorcott’ mandarin, and changes in leaf mineral nutrient status can be considered a consequence, rather than a cause of this phenomenon.

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Table 1. Fruit yield, return bloom and vegetative response of ten-year-old alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees over three seasons.

Tree fruiting status	Fruit yield in current year (kg per tree)	Fruit per tree in current year (no.)	Return bloom and vegetative response in the following year (no. per tree)			
			Total flowers	Total new spring vegetative shoots	Total new summer vegetative shoots	Total new vegetative shoots
Season 1						
B[z]: "Off"	14 b[y]	126 b	51 097 a	163 b	144 b	306 b
W: "On"	84 a	918 a	30 034 b	493 a	369 a	863 a
P value	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Season 2						
B: "On"	110 a	1225 a	165 b	1018 a	420 a	1439 a
W: "Off"	71 b	657 b	32 712 a	598 b	167 b	766 b
P value	0.0005	<0.0001	0.0004	0.0007	<0.0001	<0.0001
Season 3						
B: "Off"	16 b	144 b				
W: "On"	52 a	621 a				
P value	<0.0001	<0.0001				

[z] For easier interpretation of results over three seasons, treatments were assigned colours blue (B) and white (W).

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=8).

Table 2. The total amount (g) of nitrogen (N), phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg) removed by crop load from "on" and "off" 'Nadorcott' mandarin (*C. reticulata*) trees over three seasons.

Tree fruiting status	Mineral elements removed by fruit load (g per tree)				
	N	P	K	Ca	Mg
Season 1					
B[z]: "Off"	32b	4 b	43 b	14 b	5 b
W: "On"	106a	14 a	145 a	49 a	17 a
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Season 2					
B: "On"	217a	28 a	296 a	100 a	35 a
W: "Off"	152b	19 b	204 b	68 b	23 b
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Season 3					
B: "Off"	38b	5 b	52 b	17 b	6 b
W: "On"	93a	12 a	127 a	43 a	15 a
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[z] For easier interpretation of results over two seasons, treatments were assigned colours blue (B) and white (W).

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=8).

Table 3. The relationship between measurements of tree phenological events and the concentrations of leaf nitrogen (N), phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg) in alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during season 1.

Month	Leaf mineral nutrient concentration ($mg \cdot g^{-1}$ leaf DW[z])	Flowers		Spring vegetative shoots		Summer vegetative shoots		Total shoots		Total tree fruit yield		Return bloom		Spring vegetative shoots	
		R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
September	N	(-)0.21	0.430	0.35	0.190	0.49	0.050	0.35	0.180	(-)0.45	0.080	0.62	0.010	(-)0.40	0.130
	P	(-)0.55	0.030	0.26	0.330	0.17	0.530	0.15	0.570	(-)0.16	0.560	0.28	0.300	(-)0.41	0.120
	K	(-)0.47	0.060	(-)0.20	0.460	(-)0.29	0.280	(-)0.25	0.360	0.19	0.480	(-)0.25	0.360	(-)0.14	0.600
	Ca	0.06	0.820	(-)0.13	0.630	(-)0.19	0.490	(-)0.17	0.520	0.26	0.340	(-)0.15	0.590	0.11	0.680
	Mg	0.05	0.860	0.15	0.590	0.17	0.530	0.18	0.510	(-)0.22	0.400	0.28	0.290	0.07	0.810
December	N	(-)0.11	0.670	0.01	0.980	0.21	0.440	0.16	0.560	(-)0.18	0.500	0.24	0.360	(-)0.07	0.800
	P	(-)0.46	0.070	(-)0.01	0.960	0.04	0.890	(-)0.08	0.760	0.10	0.700	(-)0.08	0.770	(-)0.29	0.280
	K	(-)0.20	0.450	(-)0.06	0.820	(-)0.22	0.420	(-)0.22	0.400	0.22	0.410	(-)0.18	0.500	0.00	1.000
	Ca	0.00	1.000	(-)0.19	0.490	(-)0.23	0.400	(-)0.21	0.440	0.14	0.610	(-)0.28	0.290	(-)0.01	0.970
	Mg	0.15	0.580	0.02	0.940	0.07	0.810	0.03	0.920	(-)0.16	0.560	0.15	0.570	0.06	0.810
March	N	-	-	-	-	0.23	0.400	0.14	0.590	(-).031	0.250	0.20	0.450	(-).45	0.080
	P	-	-	-	-	0.51	0.040	0.41	0.110	(-)0.59	0.020	0.48	0.060	(-)0.70	0.001
	K	-	-	-	-	0.45	0.080	0.40	0.130	(-)0.58	0.020	0.45	0.080	(-)0.76	0.001
	Ca	-	-	-	-	(-)0.57	0.020	(-)0.48	0.060	0.62	0.010	(-)0.61	0.010	0.61	0.010
	Mg	-	-	-	-	0.14	0.600	0.08	0.760	(-)0.19	0.470	0.27	0.320	0.00	0.990
June	N	-	-	-	-	-	-	-	-	(-)0.62	0.010	0.66	0.010	(-)0.51	0.050
	P	-	-	-	-	-	-	-	-	(-)0.63	0.010	0.52	0.040	(-)0.62	0.010
	K	-	-	-	-	-	-	-	-	(-)0.60	0.010	0.67	0.001	(-)0.50	0.050
	Ca	-	-	-	-	-	-	-	-	0.63	0.010	(-)0.68	0.001	0.45	0.080
	Mg	-	-	-	-	-	-	-	-	(-)0.28	0.290	0.34	0.190	(-)0.05	0.850

[z] Dry weight.

The data were analysed using regression analysis. The strength of the relationship is indicated by Spearman's correlation coefficient. The percentage variation explained is $100*R^2$, which is indicated as $(-)R^2$ if the correlation was negative. Significance at the 95% level.

Table 4. The relationship between measurements of tree phenological events and the concentrations of leaf nitrogen (N), phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg) in alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees during season 2.

Month	Leaf mineral nutrient concentration (mg·g^{-1} leaf DW[z])	Flowers		Spring vegetative shoots		Summer vegetative shoots		Total shoots		Total tree fruit yield		Return bloom		Spring vegetative shoots	
		R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
September	N	(-)0.51	0.040	0.32	0.220	0.51	0.040	0.37	0.160	(-)0.44	0.090	0.51	0.040	(-)0.35	0.180
	P	0.23	0.390	(-)0.50	0.050	(-)0.46	0.070	(-)0.49	0.050	0.30	0.250	0.10	0.750	(-)0.20	0.620
	K	0.01	0.970	(-)0.17	0.530	(-)0.22	0.420	(-)0.21	0.440	0.12	0.660	0.01	0.950	0.10	0.800
	Ca	(-)0.51	0.040	0.25	0.350	0.34	0.200	0.33	0.210	(-)0.51	0.040	(-)0.25	0.310	0.25	0.320
	Mg	0.24	0.380	(-)0.06	0.820	(-)0.19	0.470	(-)0.17	0.540	0.37	0.160	0.15	0.690	0.27	0.520
December	N	(-)0.25	0.360	(-)0.10	0.700	0.15	0.590	0.02	0.950	(-)0.09	0.730	0.04	0.950	0.02	0.980
	P	(-)0.49	0.050	(-)0.12	0.670	0.01	0.980	(-)0.04	0.890	(-)0.32	0.230	0.02	0.970	(-)0.24	0.150
	K	(-)0.57	0.020	0.05	0.850	0.15	0.570	0.11	0.690	(-)0.32	0.230	(-)0.08	0.900	0.12	0.850
	Ca	0.00	0.999	0.06	0.820	0.23	0.400	0.22	0.400	(-)0.02	0.940	0.08	0.920	(-)0.11	0.870
	Mg	0.21	0.450	(-)0.01	0.980	0.00	1.000	(-)0.05	0.860	0.35	0.180	0.05	0.970	0.06	0.810
March	N	-	-	-	-	(-)0.80	0.001	(-)0.68	0.001	0.78	0.001	(-)0.69	0.001	0.65	0.010
	P	-	-	-	-	(-)0.35	0.190	(-)0.40	0.130	0.05	0.850	0.20	0.500	(-)0.15	0.700
	K	-	-	-	-	(-)0.09	0.750	(-)0.31	0.240	0.08	0.760	0.18	0.670	(-)0.20	0.600
	Ca	-	-	-	-	0.16	0.540	0.17	0.530	0.24	0.370	(-)0.12	0.780	0.20	0.670
	Mg	-	-	-	-	(-)0.25	0.350	(-)0.26	0.330	0.37	0.150	(-)0.21	0.620	0.11	0.850
June	N	-	-	-	-	-	-	-	-	0.79	0.001	(-)0.79	0.001	0.69	0.010
	P	-	-	-	-	-	-	-	-	(-)0.07	0.790	0.10	0.750	(-)0.05	0.850
	K	-	-	-	-	-	-	-	-	(-)0.50	0.050	0.65	0.010	(-)0.51	0.040
	Ca	-	-	-	-	-	-	-	-	(-)0.03	0.910	(-)0.12	0.900	0.14	0.960
	Mg	-	-	-	-	-	-	-	-	0.12	0.650	(-)0.15	0.840	0.21	0.800

[z] Dry weight.

The data were analysed using regression analysis. The strength of the relationship is indicated by Spearman's correlation coefficient. The percentage variation explained is 100*R^2, which is indicated as (-)R^2 if the correlation was negative. Significance at the 95% level.

Table 5. The concentrations of leaf nitrogen (N), phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg) determined at monthly intervals in "on" and "off" 'Nadorcott' mandarin (*C. reticulata*) trees, as well as in response to de-fruiting treatments applied to "on" trees applied in Jan. and Apr. 2016.

Treatments	January	February	March	April	May	June
N			mg·g^{-1} leaf DW[z]			
"On" tree	22.2 ns[y]	22.9 a[x]	23.7 ns	24.8 ns	26.4 ns	23.9 ns
"Off" tree	22.9	22.8 a	23.8	25.1	26.0	22.5
"On" tree de-fruited January	22.2	21.3 b	21.4	23.0	23.6	22.3
"On" tree de-fruited April	22.6	23.0 a	23.8	24.3	25.5	22.4
P value	0.5280	0.0190	0.1320	0.2320	0.3270	0.5770
P			mg·g^{-1} leaf DW			
"On" tree	1.08ns	1.16ns	0.98ns	1.18ns	1.26ns	1.20ns
"Off" tree	1.24	1.16	1.14	1.30	1.34	1.14
"On" tree de-fruited January	1.10	1.02	0.94	1.20	1.18	1.16
"On" tree de-fruited April	1.14	1.14	1.02	1.22	1.34	1.20
P value	0.1100	0.0660	0.3270	0.4800	0.2660	0.7610
K			mg·g^{-1} leaf DW			
"On" tree	12.4b	11.4ns	10.5ns	10.9b	11.0ns	7.6ns
"Off" tree	15.00a	13.1	12.5	13.7a	13.3	9.8
"On" tree de-fruited January	12.00b	9.5	9.5	11.1b	10.7	8.9
"On" tree de-fruited April	12.2b	11.6	10.3	10.3b	11.9	8.7
P value	0.0029	0.1000	0.2040	0.0178	0.3920	0.2630
Ca			mg·g^{-1} leaf DW			
"On" tree	31.8ns	29.5ns	28.8ab	31.9a	33.4ab	33.2ns
"Off" tree	27.0	27.5	24.5b	22.5b	27.0b	30.3
"On" tree de-fruited January	35.9	34.5	36.1a	30.6a	38.8a	36.2
"On" tree de-fruited April	35.5	35.7	33.4a	32.6a	34.1ab	30.7
P value	0.0600	0.1950	0.0235	0.0178	0.0327	0.3200
Mg			mg·g^{-1} leaf DW			
"On" tree	4.1ns	3.6ns	3.2ns	3.6ns	4.0ns	3.6ab
"Off" tree	3.6	3.4	3.4	3.1	3.6	3.2bc
"On" tree de-fruited January	4.0	4.2	4.0	3.7	4.0	4.1a
"On" tree de-fruited April	3.8	3.6	3.5	3.8	3.5	2.8c
P value	0.2190	0.1480	0.2560	0.0820	0.5280	0.0099

[z] Dry weight.

[y] No significant differences.

[x] Different letters in the same column denote significant differences between values ($P>0.05$; Fisher's LSD test; n=6).

Table 6. The vegetative and reproductive responses to de-fruiting treatments in "on" 'Nadorcott' mandarin (*C. reticultata*) trees in Jan. and Apr. 2016, compared to "on" and "off" trees.

Treatments	New summer vegetative shoots	Spring flowering response
	(no. flush per tree)	(no. flowers per tree)
"On" tree	9 c[z]	18 942 c
"Off" tree	34 b	43 110 a
"On" tree de-fruited January	79 a	32 324 b
"On" tree de-fruited April	11 c	22 991 c
P value	<0.0001	0.0020

[z] Different letters in the same column denote significant differences between values (*P*<0.05; Fisher's LSD test; n=6).

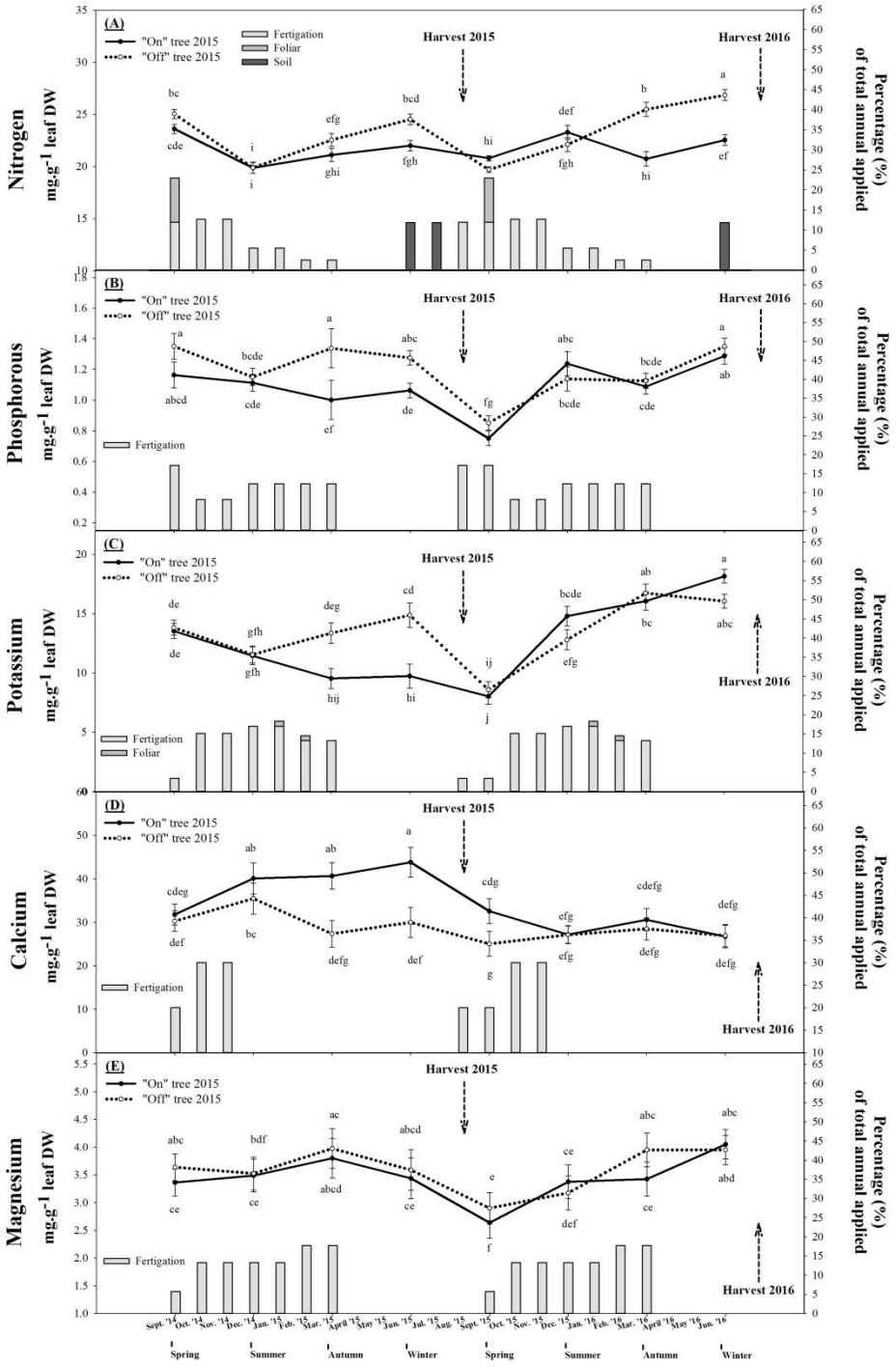

Fig 1. The concentrations of leaf nitrogen (A), phosphorous (B), potassium (C), calcium (D) and magnesium (E), determined at three-monthly intervals over two seasons in alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees. The line graph corresponds to the left Y-axis and represents the concentration of the mineral elements in the leaf expressed as $mg \cdot g^{-1}$ leaf dry weight (DW), whereas the bar graph corresponds to the right Y-axis and represents the rate and distribution of the annual nutrient application as a percentage of the total annual application. The arrows indicate the time of harvest. Bars denote standard errors of the means and different letters significant differences between values ($P<0.05$; Fisher's LSD test; n=8). DW = dry weight.

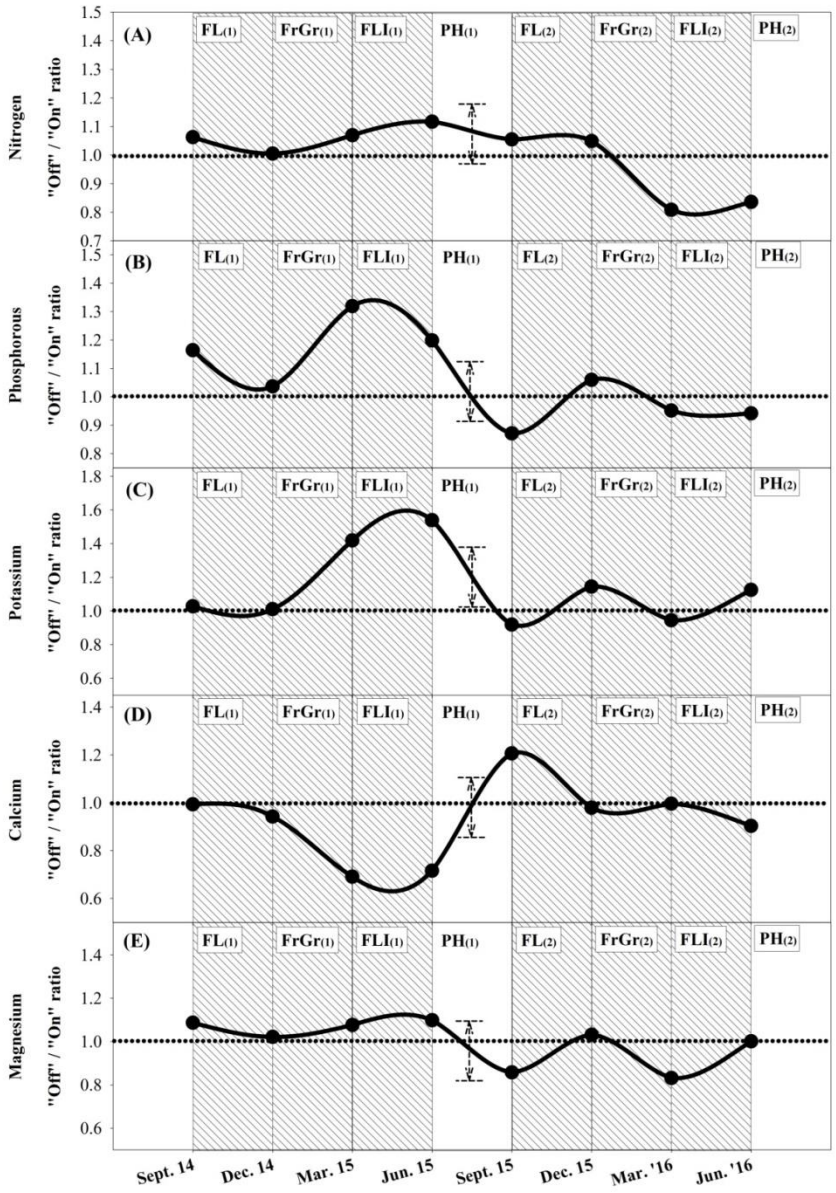

Fig. 2. The "off"/"on" ratios of concentrations of nitrogen, phosphorous, potassium, calcium and magnesium, in leaves of alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees over a period of two seasons (1 and 2). A ratio higher than one means the concentration of the mineral element of interest was higher in leaves from "off" trees on the particular sampling date, whereas ratios lower than one mean the concentration of the element was higher in "on" trees. FL = Flowering; FrGr = Fruit growth; FLI = Flower induction; PH = Post harvest.

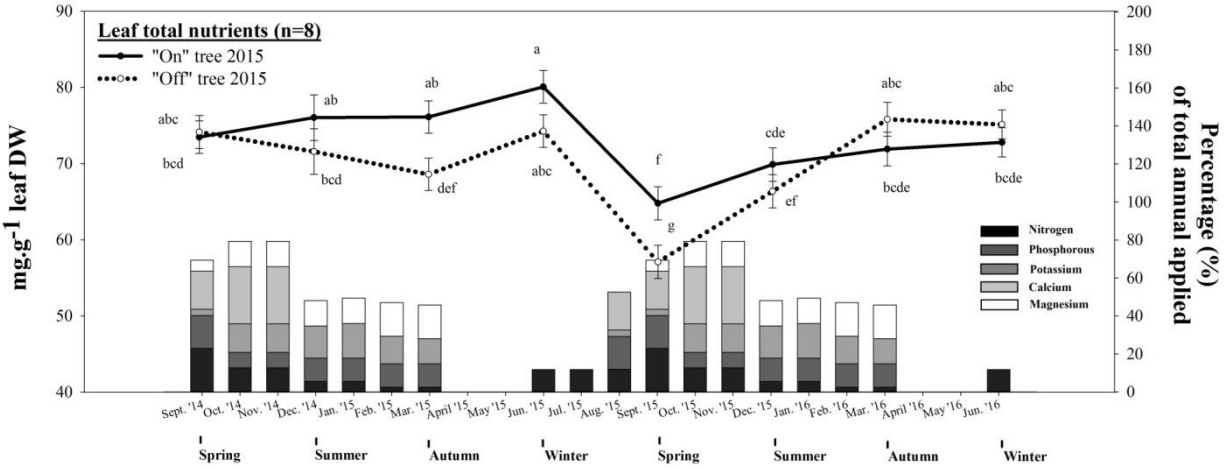

Fig 3. The sum of the concentrations (mg·g^{-1} leaf dry weight DW) of macronutrients in leaves of alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees over a period of two seasons. The line graph corresponds to the left Y-axis and represents the total nutrient concentration in the leaf, whereas the bar graph corresponds to the right Y-axis and represents the distribution of annual application of the various mineral nutrients. Bars denote standard errors of the means and different letters, significant differences between values (P<0.05; Fisher's LSD test; n=8).

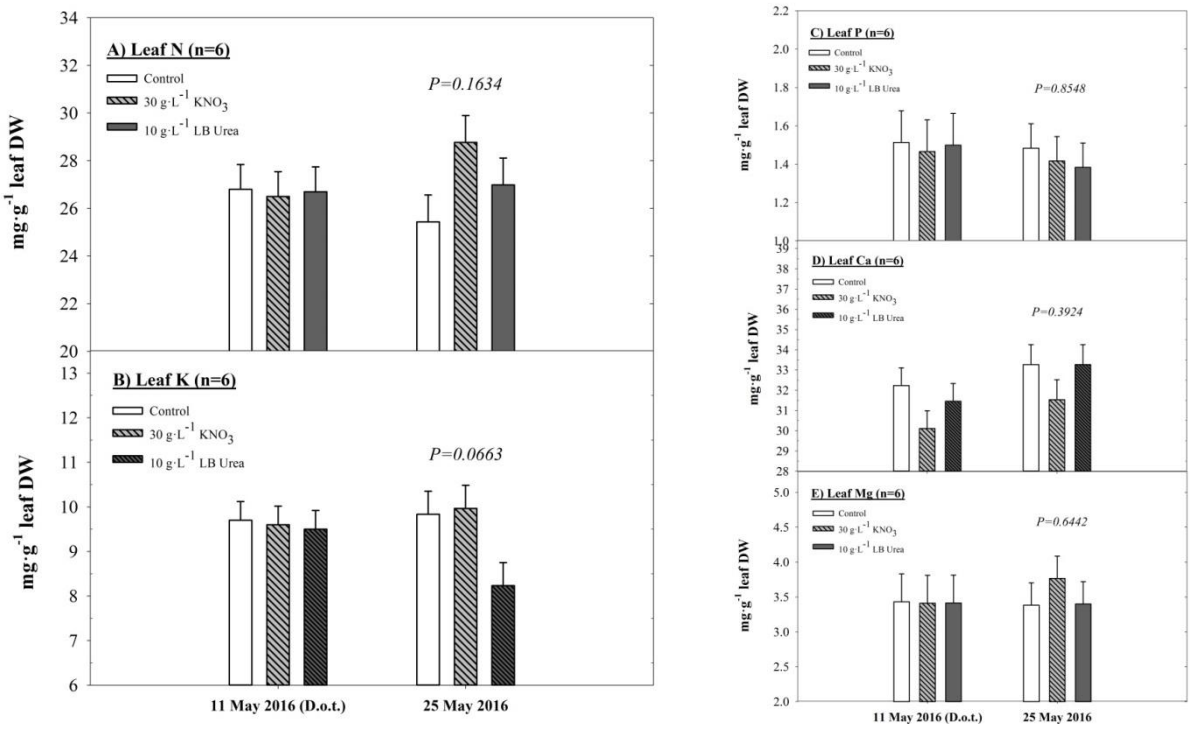

Fig 4. The response of concentration (mg·g^{-1} leaf dry weight DW) of nitrogen (A), potassium (B), phosphorous (C), calcium (D) and magnesium (E) in leaves of alternate bearing 'Nadorcott' mandarin (*C. reticulata*) trees after foliar sprays of 10 g·L^{-1} low (0.25%) biuret urea (N source) and 30 g·L^{-1} potassium nitrate (KNO_3) (K source). Bars denote standard errors of the means and different letters significant differences between values (P<0.05; Fisher's LSD test; n=6).

Chapter 5: The phyto-hormone profile of heavy- and low-fruiting 'Nadorcott' mandarin trees in relation to alternate bearing

Abstract. The objective of this study was to investigate the role of phyto-hormones in alternate bearing in citrus (*Citrus* spp.). Profiling of phyto-hormone content in leaves and roots of representative heavy- and low-fruiting ("on" and "off") 'Nadorcott' mandarin (*C. reticulata* Blanco) trees was conducted during two distinct phenological stages in which flowering in citrus is known to be affected by endogenous phyto-hormones, viz. vegetative shoot development in summer, and floral bud development in winter. Given that the two most important determinants of return bloom flowering in alternate bearing 'Nadorcott' mandarin trees is firstly the number of new potential floral positions that developed from parent shoots during summer, and secondly, the inhibition of floral bud development by fruit during early winter, this study demonstrated the role of phyto-hormones in the imposition of both these mechanisms of floral inhibition. The auxin 1 *H*-indole-3-acetic acid (IAA) was the primary substance causing the inhibition of new summer vegetative shoot development in the presence of fruit, but probably did not act alone. High concentrations of the products of abscisic acid (ABA) metabolism, viz. dihydrophaseic acid and the ABA glucose ester in leaves provided evidence that IAA acted synergistically with high ABA concentration to create an inhibitory signal in shoots of "on" trees, thereby not allowing cytokinins to participate in bud sprouting. During early winter, high gibberellin (GA) concentration in leaves possibly inhibited floral bud development of "on" trees. Treatment of "off" trees and shoots with 40 $mg \cdot L^{-1}$ gibberellic acid inhibited floral bud development, whereas soil and foliar treatments of "on" trees with 1000 $mg \cdot L^{-1}$ paclobutrazol and uniconazole increased flowering and fruit development of "on" shoots. This study confirmed the roles of IAA and

GA in the hormonal theory of alternate bearing, but also adds support to a 'hormonal balance' concept involving ABA and cytokinin.

1. Introduction

Alternate or biennial bearing in perennial fruit trees is the synchronised tendency to flower profusely and produce an abundance of fruit in one season, followed by a scant number of flowers and fruit in the following season (Monselise and Goldschmidt, 1982). Alternate bearing is a natural phenomenon in deciduous fruit and nut trees such as apple [*Malus* × *sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] (Guitton et al., 2012), pear (*Pyris communis* L.) (Jonkers, 1979), pecan [*Carya illinoinensis* (Wangenh.) C. Koch] (Wood et al., 2004), pistachio (*Pistachia vera* L.) (Rosecrance et al., 1998) and prune (*Prunus domestica* L.) (Davis, 1931), but is more frequent in evergreen fruit trees, e.g. avocado (*Persea americana* Mill.) (Garner and Lovatt, 2008), citrus (*Citrus* spp.) (Monselise and Goldschmidt, 1982), coffee (*Caffea arabica* L.) (Vaast et al., 2005), litchi (*Litchi chinensis* Sonn.) (Menzel, 1983), mango (*Mangifera indica* L.) (Souza et al., 2004) and olive (*Olea europaea* L.) (Bustan et al., 2011). Alternate bearing undermines the consistency of orchard management practices and leads to costly challenges in the production, harvesting, transport, packing and marketing of fruit. The "on" crop is generally distinguished by a large number of small fruit, whereas the "off" crop constitutes large unappealing fruit (Galliani et al., 1975; Hield and Hilgeman, 1969; Monselise and Goldschmidt, 1982; Moss et al., 1974).

Factors responsible for the initiation and continuation of alternate bearing appear to be complex and of a combinative nature. In citrus the phenomenon first seemed to have conspicuous causal factors, viz. the level of seediness and time of harvest (Monselise and Goldschmidt, 1982), but strong discrepancies have since been reported for these factors to be accepted as a rule (Iwasaki and Owada, 1960; Okuda, 2000; Sanderson and Treeby, 2014;

Schaffer et al., 1985). The fundamental cause(s) of alternate bearing in citrus therefore remains an enigma (Bangerth, 2009).

In contrast to the cause, the mechanism perpetuating alternate bearing appears to be conclusive – the level of fruiting is a factor of the degree of flowering in the following season and vice versa. Heavy fruiting in citrus limits the sprouting of new and potential flowering sites during summer (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009) and inhibits the expression of citrus flowering genes during flower induction (Muñoz-Fambuena et al., 2011).

Studies on how fruit modulate these effects have resulted in the development of the nutritional and the hormonal theories of alternate bearing (Bangerth, 2009; Barnett and Mielke, 1981; Davenport, 2000; Goldschmidt, 1999). In the nutritional theory the flowering response is dependent on mineral nutrients and available plant metabolic energy, viz. carbohydrates as determined by fruit load. Effects of girdling and fruit removal treatments corroborate the merits of the nutritional theory since an increase in flower number usually correlates with high carbohydrate concentration (Cohen, 1981; García-Luís et al., 1995a; Goldschmidt et al., 1985; Schaffer et al., 1986). The correlative evidence that normally results from studies on this theory, however, is not particularly convincing since the use of treatments such as girdling or de-fruiting would also have hormonal effects on flowering or vegetative responses that are unrelated to carbohydrates or mineral nutrients (Erner, 1988; García-Luís et al. 1995a; Goldschmidt et al., 1985; Koshita et al., 1999). The direct control of flowering and other roles for carbohydrates in the nutritional theory of alternate bearing have therefore not been unequivocally established.

The hormonal theory of alternate bearing, on the other hand, suggests that high levels of endogenous phyto-hormones such as abscisic acid (ABA), auxins [1 *H*-indole-3-acetic acid (IAA)] and gibberellins (GAs) relates to the inhibition in the formation of new vegetative

shoots during summer and newly available flowering positions during spring (Martínez-Alcántara et al., 2015; Verreynne and Lovatt, 2009), as well as the suppression in citrus floral genes and inhibition of flower induction (Goldberg-Moeller et al., 2013; Koshita et al., 1999; Muñoz-Fambuena et al., 2011; Tang, 2017).

Shedding light on the role of phyto-hormones in alternate bearing is challenging since the physiological processes related to the alternate bearing phenomenon are closely intertwined. In the hormonal theory, inhibition of flowering and fruit development by GAs (Goldberg-Moeller et al., 2013; Monselise and Halevy, 1964; Muñoz-Fambuena et al., 2012) and vegetative growth by IAA (Verreynne, 2005; Verreynne and Lovatt, 2009) have been established and accredited to one specific plant hormone, but few studies have investigated an enigmatic 'hormonal balance' concept (Goldschmidt, 1999, 2015). Studies with cytokinins have mostly been conducted in tissue-culture or in potted and non-fruiting citrus trees (Hendry et al., 1982a, 1982b; Van Staden and Davey, 1979) and the role of ABA are yet to be demonstrated in alternate bearing (Goldschmidt; 1984; Jones et al., 1976; Shalom et al., 2014).

The objective of this study was to investigate the significance of the phyto-hormones ABA, cytokinins, GAs and IAA and their derivatives in the hormonal theory of alternate bearing in citrus. A phyto-hormone profile of representative alternate bearing trees was compiled during two distinct phenological stages in which flowering is known to be affected by endogenous phyto-hormone content, viz. in summer and winter. Leaves and roots of natural, heavy-fruiting, i.e. "on", and low-fruiting, i.e. "off", 'Nadorcott' mandarin (*C. reticulata* Blanco) trees grown under field-conditions in commercial orchards were used. Results from exogenous phyto-hormone treatments and/or fruit removal during summer and winter were compared to significant results obtained from endogenous phyto-hormone measurements.

2. Materials and methods

2.1. Plant material, treatments and experimental design

Profiling of phyto-hormones was done using leaf and root tissues that were sampled from 'Nadorcott' mandarin trees budded onto 'Carrizo' citrange [*C. sinensis* L. (Osb.) × *Poncirus trifoliata* (L.) Raf.] rootstock and grown under commercial production conditions in De Doorns (lat. 33°51'S, long. 19°52'E) in the Western Cape Province of South Africa. The trees were selected from a 10-year-old commercial orchard that was cultivated, pruned and sprayed according to good agricultural practices. Most of the trees in the orchard bore similar fruit yields, but for the purpose of this experiment, individual trees that showed an opposite and natural alternate bearing trend were selected during full bloom in the middle of Oct. during spring of 2016. The trees were representative of heavy- or low-fruiting trees and subsequently included as single-tree replicates of "on" and "off" treatments in a completely randomised design (n=5).

Separate trees from the same orchard were used in an experiment to test the effects of summer foliar applications of synthetic auxins and cytokinins on vegetative shoot development in "on" and "off" trees, as well as that of a de-fruiting treatment of "on" trees. The foliar sprays were applied to whole-tree replicates ≈90 d after full bloom on 05 Jan. 2017 between 08:00 and 10:00 AM and all fruit were removed from five separate "on" trees in the de-fruiting treatment. The following treatments formed part of a randomised complete block design (n=5): 1) "Off" (low-fruiting) trees; 2) "On" (heavy-fruiting) trees; 3) Complete de-fruiting of "on" trees; 4) "Off" trees + 40 $mg \cdot L^{-1}$ of the amine formulation of 2,4-dichlorophenoxy acetic acid [2,4-D amine; Avima (Pty) Ltd, Krugersdorp, South Africa; containing 720 $g \cdot L^{-1}$ a.i.]; and 5) "On" trees + 7 $mg \cdot L^{-1}$ forchlorfenuron (CPPU) [Sitofex 10 EC; Philagro SA (Pty) Ltd, Somerset West, South Africa; containing 10 $g \cdot L^{-1}$ a.i. in an

emulsifiable concentrate (EC) formulation]. Foliar sprays were applied at a rate of ≈4 L spray solution per tree using a backpack mist-blow sprayer [Stihl SR430; Andreas Stihl (Pty) Ltd, Pietermaritzburg, South Africa] set at droplet size 1 (1 – fine droplet size, 5 – coarse droplet size). Buffer trees were left untreated between treated and control trees in the same row, as well as buffer rows between treated rows to avoid drift between different treatments. The replicate trees were selected for uniformity in tree condition, based on a dark, green leaf colour. All trees were uniform in canopy volume and had a height of approximately 3.5 to 4.0 m and an across-row width of 2.5 to 3.0 m. The trees had a trunk circumference of ≈320 mm as measured above the bud union.

To evaluate the effects on flowering of different timings of foliar-applied gibberellic acid (GA_3) [ProGibb®; Philagro SA (Pty) Ltd; containing 400 $g \cdot kg^{-1}$ a.i.] treatments during winter, three-year-old, previously non-bearing 'Nadorcott' mandarin trees budded on 'Carrizo' citrange rootstock were used in an experiment in a commercial orchard in De Doorns. Uniform, non-bearing trees were selected to eliminate any possible interference of inhibition of fruit on flowering (pers. comm. Goldschmidt). The whole-tree experiment consisted of six treatments that included an untreated control and 10 single-tree replicates, and was set up in a randomised complete block design (n=10). Foliar GA_3 was applied at a rate of 40 $mg \cdot L^{-1}$ with a non-ionic polyether-polymethylsiloxanecopolymer wetting agent [Break-Thru®; Villa Crop Protection (Pty) Ltd, Aston Manor, South Africa; containing 1000 $g \cdot L^{-1}$ a.i.] added to the spray solution at a rate of 5 $ml \cdot 100\ L^{-1}$ water. Treatments were applied to individual whole-tree replicates in 2-week intervals at a rate of ≈1 L spray solution per tree using a backpack mist-blow sprayer set at droplet size 1. Buffer trees were left untreated between treated and control trees in the same row, as well as buffer rows between treated rows. One treatment consisted of two applications of 40 $mg \cdot L^{-1}$ GA_3 to one whole-tree

replicate. The treatments were applied 2 weeks apart on 01 and 15 May, 15 May and 01 Jun., 01 Jun. and 15 Jun., 15 Jun. and 01 Jul., and 01 Jul. and 15 Jul. 2014, respectively.

Shoot experiments consisted of treatments applied to five individual shoots in eight trees (n=8) at the same experimental site. The treatments were applied to shoots that were approximately 12 months of age and had triangular internodes, a length of ≈12 cm and were located on the outside of the tree canopy at a height of ≈1.5 m above the orchard floor. In 2014 the treatments consisted of the following: 1) Untreated control ("off" shoot); 2) "Off" shoot + 40 $mg \cdot L^{-1}$ GA_3 applied at ≈2-week intervals to same shoots starting on 1 May 2014 and continued until 15 Jul. 2014; and 3) "On" shoot with one fruit located at the terminal position of the shoot. In 2015 the following treatments were applied to five individual shoots in eight moderately bearing trees (n=8): 1) Untreated control ("off" shoot); 2) "Off" shoot + 40 $mg \cdot L^{-1}$ GA_3 applied to same shoots on 29 Apr. 2015 and 15 May 2015; 3) "On" shoot with one fruit located at the terminal position of the shoot; and 4) "On" shoot with terminal fruit removed on 29 Apr. 2015. The GA_3 treatments were applied to individual shoots using a 500 mL handgun-sprayer.

To evaluate the effects of different treatments of GA biosynthesis inhibitors during winter on return fruit yield in "on" trees, treatments were applied to "on" 'Nadorcott' mandarin trees during the period in which GA_3 treatments previously resulted in the strongest floral inhibition response, viz. in May and June. The treatments were applied to 7-year-old 'Nadorcott' mandarin trees topworked onto a 'Navel' sweet orange interstock that was budded onto 'rough lemon' [*C. jambhiri* (Lush.)] rootstock between Citrusdal and Clanwilliam (lat. 32°33'S, long. 18°83'E) in the Western Cape Province of South Africa. The experiment was set up in a randomised complete block design and the following treatments were applied to whole-tree treatment replicates (n=8) on 25 May and 16 Jun. 2016: 1) Untreated control ("on" tree); 2) 1000 $mg \cdot L^{-1}$ uniconazole [Sunny® 50 SC; Philagro

SA (Pty) Ltd; containing 50 g·L^{-1} a.i.] soil drench in 3 L water per tree; 3) 1000 mg·L^{-1} uniconazole foliar spray at 4 L water spray-mixture per tree; 4) 1000 mg·L^{-1} paclobutrazol [Cultar® 250 SC; Syngenta SA (Pty) Ltd, Halfway house, South Africa; containing 250 g·L^{-1} a.i.] soil drench at 3 L per tree; and 5) 1000 mg·L^{-1} paclobutrazol foliar spray at 4 L spray-mixture per tree.

Foliar spray treatments were applied to individual whole-tree replicates using a backpack mist-blow sprayer set at droplet size 1. No wetting agent was added to spray mixtures of foliar treatments. The soil drench treatments were applied with a watering can around the tree trunk after scraping away all leaf debris and subsequent to at least 30 min of commercial irrigation. In each tree, ten fruiting shoots with one terminal fruit were tagged on the day of application of the first treatment. Buffer trees were left untreated between treated and control trees in the same row, as well as buffer rows between treated rows. One treatment consisted of two applications of which the first was applied on 26 May and again on 16 Jun. 2016.

2.2. Data collection

2.2.1. Vegetative and flowering phenology

The phenological pattern of different shoot types in “on” and “off” trees was followed by randomly selecting five vegetative (“off”) and five reproductive (“on”) shoots in each tree. All shoots were approximately 12 months of age and had triangular internodes, a length of ≈15 cm and were located on the outside of the tree canopy at a height of ≈1.5 m above the orchard floor. On each shoot the number of nodes, vegetative shoots and flowers were counted in addition to the classification of inflorescence type. Inflorescences were classified as leafy, i.e. buds sprouting both flowers and leaves, or leafless, i.e. buds sprouting flowers only. In February and March the numbers of persistent fruit and new vegetative shoots that

developed during the subsequent vegetative shoot flushes were recorded for each shoot and return bloom and vegetative response were determined on the same shoots during the subsequent spring.

2.2.2. Yield

To determine the fruit yield of each treatment all fruit were harvested separately from individual trees on the day prior to the start of commercial harvest in the first week of August. Total fruit weight of each tree was recorded using an electronic scale (W22 Series; UWE Co., Hsin Tien, Taiwan) and a sample of 100 fruit was randomly collected from each tree replicate to measure the transverse diameter of each fruit using an electronic fruit size measuring calliper (CD-6" C; Mitutoyo Corp, Tokyo, Japan). Each fruit was assigned to a fruit size category of which the average fruit weight was determined and for which the fruit size distribution from each treatment replicate was extrapolated for the total number of fruit per tree.

2.2.3. Analysis of phyto-hormones

Leaf and root samples were collected between 05:00 and 06:00 AM on each sampling date, viz. on 04 Jan. 2017 in summer, and on 27 Apr. 2017 in autumn. For each sample, eight fully-intact shoots were removed from each treatment replicate, placed in individual paper bags and transported on ice to the laboratory in Stellenbosch. In the laboratory, one leaf was removed from each of the eight shoots to compile a leaf sample that consisted of eight leaves per sample for fruiting "on" shoots of "on" trees and vegetative "off" shoots of "off" trees. Only fully-hardened leaves were sampled from the first to third position in the apical region of each shoot since flowering is most likely to occur at these positions (Abbott, 1935; Sauer, 1951). A sample of fine fibrous roots (<0.5 mm in diameter) was collected from a pooled

root sample that was sampled from four different areas around the trunk of each tree. The leaves and roots were separated, immediately frozen in liquid nitrogen and lyophilised using a pestle and mortar. The ground leaf and root samples were immediately transferred to 15 mL centrifuge tubes sealed with screw caps and stored in a –80 °C freezer and freeze-dried (Christ Beta 1–8 LD Freeze Dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The samples were shipped to the Plant Biotechnology Institute of the National Research Council of Canada in Saskatoon, SK, Canada for determination of the hormone concentrations using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) with multiple reaction monitoring (MRM) (Waters Corp., Medford, MA, USA) (Ross et al., 2004).

The procedure for quantification of multiple hormones and metabolites, including 1 *H*-indole-3-acetic acid (IAA) and IAA-conjugates (IAA-aspartate, IAA-glutamate, IAA-alanine, and IAA-leucine), and indole-3-butyric acid (IBA), abscisic acid (ABA) and its metabolites [phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-ABA (7'-OH-ABA), neo-phaseic acid (neoPA), and ABA-glucose ester (ABA-GE)], cytokinins [isopentenyladenine (2iP), iso-pentenyladenosine (iPA), trans- and cis-zeatin (t- and c-Z), trans- and cis-zeatin riboside (t- and c-ZR), dihydrozeatin (dhZ), dihydrozeatin riboside (dhZR), and trans-and cis-zeatin-O-glucoside (t-and c-ZOG)], and gibberellins was described in detail by Chiwocha et al. (2003; 2005).

Briefly, a 100 mL aliquot containing all the hormone internal standards (ISs), each at a concentration of 0.2 pg·mL^{-1}, was added to 50 mg of ground leaf or root sample, followed by 3 mL of the extraction solvent that consisted of isopropanol:water:glacial acetic acid (80:19:1, v/v/v). The samples were agitated in the dark for 24 h at 4 °C. After centrifugation, the supernatant was isolated and dried on a distillation evaporator (Syncore Polyvap, Büchi Labortechnik, Flawil, Switzerland) and reconstituted in 100 mL of acidified methanol,

adjusted to 1 mL with acidified water, and partitioned against 2 mL hexane. After 30 min, the aqueous layer (bottom phase) was isolated and dried as above. The dried sample was reconstituted in 800 mL of acidified methanol and adjusted to 1 mL with acidified-water, passed through equilibrated Sep-Pak C18 cartridge (Waters Corp.) and the eluates were dried in a centrifuge vacuum concentrator (Labconco Corp., Kansas City, MO, USA). An IS was prepared with 100 mL of the deuterated ISs mixture. A quality control standard (QC) was prepared by adding 100 mL of a mixture containing all the analytes of interest, each at a concentration of 0.2 $pg \cdot mL^{-1}$, to 100 mL of the IS mix. Finally, the sample, IS and QC were reconstituted in an aqueous solution of 40% methanol (v/v), containing 0.5% acetic acid and 0.1 $pg \cdot mL^{-1}$ of each of the recovery standards. The samples were analysed by injection onto an ACQUITY UPLC® HSS C18 SB column (2.1 x 100 mm, 1.8 mm, Waters Corp.) with an in-line filter and separated by a gradient elution of water containing 0.02% formic acid against an increasing percentage of a solution of acetonitrile and methanol (50:50, v/v).

The analysis uses the MRM function of the MassLynx v.4.1 control software (Waters Corp.) with the resulting chromatographic traces quantified off-line by the QuanLynx software (v.4.1, Waters Corp.). By this method, each trace is integrated and the resulting ratio of signals (non-deuterated/IS) is compared with a previously constructed calibration curve to yield the amount of analyte present [nanograms (ng) per sample]. Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding IS.

2.3. Statistical analysis

Analysis of variance (ANOVA) was performed using STATISTICA data analysis software version 13 (Dell Inc. 2015, Round Rock, TX, USA). Mean separations were carried out using Fisher's least significant difference test where applicable, at $P \leq 0.05$.

3. Results and discussion

During the summer vegetative shoot flush, "off" parent shoots sprouted more new vegetative shoots (see Chapter 2, Table 1) and had a ≈47% lower IAA concentration in leaves compared with "on" parent shoots (Table 1), from which very few new vegetative shoots sprouted (see Chapter 2, Table 1). Of all the gibberellins, only GA_3 was detected in leaves during summer, with no difference between the concentration of GA_3 in leaves of "on" and "off" trees (Table 1). There were no differences between the concentrations of the active cytokinins, t-Z and iPA in leaves of "on" and "off" trees during summer (Table 1), and the concentration of active ABA in leaves was similar for "on" and "off" trees (Tables 1) and not related to the difference between their vegetative responses, viz. reduced summer vegetative shoot development in "on" trees (see Chapter 2, Table 1). In leaves, c-ZOG, an inactive and storage form of cytokinin (Letham and Palni, 1983; Palmer et al., 1981; Van Staden and Davey, 1981) made up 95% of the total cytokinin concentration and was more than 80% higher in "on" trees compared with "off" trees during summer (Table 1). Similarly, for ABA, the concentration of ABA-GE, an ABA glucose ester and an ABA storage form (Goodger and Schactman, 2010; Priest et al., 2006), was higher in leaves of "on" trees, as well as that of the end-product of ABA catabolism, viz. DPA (Seiler et al., 2011) (Table 1). These results suggest that the concentration of gibberellins and active cytokinin and ABA in leaves was not affected by fruit load during summer, and a compensation mechanism possibly adjusted bioactive forms of cytokinin and ABA in leaves to inactive storage froms. When buds are under inhibition, cytokinins accumulate mostly in the form of the O-glucoside metabolites (Letham and Palni, 1983; Palmer et al., 1981; Van Staden and Davey, 1981). These storage cytokinins can accumulate to a high concentration in mature leaves, but once bud dormancy is released, the level of free bases or non-polar cytokinins such as t-Z increases and those of

O-glucosides such as c-ZOG decreases rapidly (Hendry et al., 1982b; Palmer et al., 1981). The high level of c-ZOG in leaves is probably a result of the sampling of leaves between two distinct vegetative shoot flush periods, when shoot growth was inactive. For ABA, a coordination of the two metabolic pathways in lowering active ABA levels possibly resulted in the higher concentration of DPA and ABA-GE in leaves of "on" trees (Jiang and Hartung, 2007). The glucose ester ABA, however, was only detected in leaves of "on" trees (Table 1) and has been reported to be an efficient long-distance signal molecule from roots to shoots, since it is translocated in the xylem without loss to surrounding tissues (Jiang and Hartung, 2007). During xylem transport, ABA-GE remains stable because of its hydrophilic properties and the extremely low permeability of bio-membranes for this conjugate (Baier et al., 1990; Sauter and Hartung, 2002). In the current study, roots of "on" trees might have been responsible for ABA-GE signalling to leaves, but de novo biosynthesis in leaves or transport of excess ABA from fruit, although unlikely, cannot be ruled out as the possible source of the increased ABA-GE synthesis.

There were no differences in the concentration of IAA and its conjugates in roots of "on" and "off" trees during summer (Table 2). The concentration of GA_3 and the active cytokinin, t-Z was higher in roots of "off" trees during summer (Table 2), which concurs with the substantialy higher root growth activity observed in "off" trees compared to "on" trees during the corresponding period (see Chapter 2, Figs. 2 and 3) (Malladi and Burns, 2007). The concentration of cis-zeatin riboside, a transport form of the active cis-zeatin base (Bassil et al, 1993; Mok et al. 2000), however, was more than double in roots of "on" trees (Table 2), which was surprising, since roots of "on" trees were inactive at this stage (see Chapter 2, Figs. 2 and 3). The result suggests that although roots of "on" trees were not actively growing, they might still have been a source of phyto-hormone synthesis.

The lack of significant differences between active cytokinin and ABA in leaves of "on" and "off" trees, and higher concentration of IAA in leaves of "on" trees during summer, support the concept that in the presence of fruit, the inhibiting effects of fruit on summer vegetative shoot development is imposed directly by the basipetal flux of IAA from fruit in the phloem, which manifests in a high IAA concentration in leaves (Koshita et al., 1999). The result is consistent with other studies reporting on the inhibition of bud sprouting and vegetative shoot growth by a high concentration of IAA in buds (Verreynne, 2005). In two other mandarin cultivars, viz. 'Pixie' (Verreynne and Lovatt, 2009) and 'Satsuma' (*C. unshiu* Marc.) (Ehara et al., 1981; García-Luís et al., 1995b), as well as in 'Washington Navel' (Lenz, 1967) and 'Valencia' (Plummer et al., 1989) sweet oranges [*C. sinensis* L. (Osb.)], fruit were shown to inhibit budbreak and the subsequent sprouting of new vegetative shoots during vegetative shoot flush in summer. While IAA has been shown to inhibit and delay in vivo sprouting of 'Shamouti' sweet orange buds (Altman and Goren, 1974), Yuan et al. (2003) provided convincing evidence for the movement of IAA from fruit to lateral buds and leaves of a citrus tree under field conditions. Verreynne (2005) showed that this basipetal phloem-transport of IAA from young fruitlets to buds during summer was responsible for the perpetuation of the lack of flowering in "on" 'Pixie' mandarin trees, since high endogenous IAA concentration inhibited vegetative shoot development from lateral buds. This is a similar mechanism to the correlative inhibition of a terminal shoot tip on lateral or axillary bud development, a phenomenon known as apical dominance (Bangerth, 1989; Cline, 1991; Dun et al., 2006).

In the classical apical dominance theory (Dun et al., 2006), IAA is loaded into the shoot by the terminal bud or fruit at the shoot apical meristem and establishes an IAA transport stream that is necessary to manifest the bud's competence as a carbohydrate sink (Cline, 1991; Dun et al., 2006). Once loaded in the phloem, the IAA transport stream from the

terminal bud or fruit limits the outflow of IAA from axillary buds and inhibits the formation of the lateral bud's own IAA transport stream into the main stem (Bangerth, 1989; Li and Bangerth, 1999; Morris, 1977). Since in this study a higher IAA concentration accumulated in leaves that were sampled from lateral positions in "on" shoots (Table 1), the shoot growth inhibition response might have been similar to other results reporting on the response caused by high IAA in buds. Treatment of fruitless, vegetative shoots at the onset of summer with the synthetic auxin 2,4-D resulted in little to no development of new summer vegetative shoots (Table 3, Fig. 1) and fruit removal resulted in increased sprouting of new summer vegetative shoots (Table 3, Fig. 1). Treatment of "on" trees with CPPU, a highly active N-pyridyl-N'-phenylurea with cytokinin activity that acts at the same binding sites as the purine-based cytokinins (Kurosaki et al., 1981), on the other hand, was unable to stimulate the development of new vegetative shoots (Table 3). In fact, CPPU treatments caused phytotoxicity in leaves of fruiting shoots only (Fig. 2), probably because the shoots were under impediment by growth inhibitors and the endogenous storage cytokinin concentration was therefore already high.

Despite the importance of the contribution of summer vegetative shoots to flowering and the role of IAA, the lack of flowers that sprouted from vegetative and/or fruiting-shoots during spring suggested that an alternative endogenous substance(s) was also responsible for the imposition of floral bud inhibition. In this study, GA_3 concentration in leaves of "on" shoots, from which no flowers developed, was higher during winter compared to "off" shoots (Table 4), from which flowers sprouted in abundance (see Chapter 2, Table 1). In fact, no GA_3 was detected in "off" leaves. No differences in the concentrations of other GAs were detected between leaves of "on" and "off" trees during winter (Table 4), which indicates that the flowering inhibition response to GA might be specific to GA_3 in citrus. In studies in regular and alternate bearing apple cultivars, Stephan et al. (1999, 2001) found six different

GAs and mostly GA_4 in exudates of fruit of the regular bearing cultivar. In fruit exudates of the alternate bearing cultivar 'Elstar', however, GA_3 was the predominant signal. Treatment of "off" trees and shoots with 40 $mg \cdot L^{-1}$ GA_3 in this study inhibited flowering (Figs. 3 and 4, and Table 6), and the period when citrus buds were most sensitive to GA_3, i.e. when maximum inhibition on flowering by exogenous GA_3 treatments was obtained was when GA_3 was applied during May and June (Fig. 3 and Table 6). In spring, buds from "off" trees and shoots that were treated with 40 $mg \cdot L^{-1}$ GA_3 sprouted mostly vegetative shoots and became "off" trees, whereas those of "off" untreated trees sprouted mostly flowers and became "on" trees. The strength of the flowering inhibition response to winter GA_3 treatments became progressively weaker with time (Fig. 3). Lord and Eckard (1985) showed that in 'Washington Navel' sweet orange, sepal formation was the microscopic developmental marker for an irreversible commitment of a citrus bud to produce a flower. Once the floral bud produced its sepals, the bud lost its vegetative competence and exogenous GA_3 no longer had an inhibitory effect on flowering (Lord and Eckard, 1987). This could, therefore, explain why, in this study, later treatments of buds with GA_3, e.g. 15 Jun. or 15 Jul. were ineffective, probably because more buds became progressively determined and unresponsive to GA_3 during the winter period.

An explanation for the response of buds to GA treatment is the suppression of expression of key citrus floral genes (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012; Nishikawa et al., 2007). In the presence of fruit, or during warm and wet growth conditions, floral gene expression is suppressed and flowering is completely absent (Chica and Albrigo 2013; Nishikawa et al., 2007). In citrus, GAs are the only compounds that have conclusively been proven to directly suppress the expression of the floral genes *FLOWERING LOCUS T* (*FT*) and *LEAFY* (Goldberg-Moeller et al., 2013; Muñoz-Fambuena

et al., 2012) and reduce floral bud development (Guardiola et al., 1982; Koshita et al., 1999; Monselise and Halevy, 1964).

An hypothesis generated from these results is that endogenous GA is exported from fruit to leaves and buds and/or the synthesis of GA is upregulated in leaves and buds of heavy-fruiting trees. A high GA_3 concentration is responsible for inhibition of floral bud development in "on" trees during a period in which trees are most sensitive to these effects, viz. from May to June or during early winter. Following on from this hypothesis, a treatment that would reduce GA biosynthesis and lower the endogenous GA concentration in plant tissues of heavy-fruiting trees would likely play an indirect role in the stimulation of floral gene expression and the intensity of return bloom flowering.

In "on" trees treated during May and June with GA biosynthesis inhibitors, i.e. the triazoles paclobutrazol and uniconazole, fruit again developed from "on" parent shoots (Fig. 5). All four triazole treatments increased fruit yield by 30% to 50% compared with the untreated control (Table 7). Although an evaluation of the effects of the treatments on flowering was not conducted in this experiment, the sprouting habit of "on" parent shoots during the previous season revealed that fruit developed from lateral buds located proximally to where the terminal fruit was located in the previous season. This would mean that one or more flowers sprouted from positions behind fruit during spring, approximately 2 months after the fruit were harvested. This suggests that GA was likely the primary phyto-hormone responsible for the inhibition of floral bud development from "on" fruiting shoots and/or trees (Table 7). Shoots from "on" trees that did not receive the treatment sprouted only vegetative and "off" shoots (Fig. 5).

Paclobutrazol has been reported to inhibit GA biosynthesis in evergreens such as mango (Blaikie et al., 2004) and in deciduous fruit trees such as pear (*Pyrus communis* L.) (Asín et al., 2007). In citrus, a foliar spray with paclobutrazol during floral bud development

in ‘Satsuma’ mandarin reduced the activities of GA_{20} and GA_{19}, both intermediates in the synthesis of active GAs such as GA_1, GA_3 and GA_7 (Yamaguchi, 2008) in leaves, and increased the number of flowers (Ogata et al., 1996). Muñoz-Fambuena et al. (2012) reported that a treatment of citrus trees with paclobutrazol increased the expression of flowering genes and conversion of apical meristems to flowers. Because the effect of triazoles on flowering is reversible by endogenous GA (Martínez-Fuentes et al., 2013) a variation in effectivity in flowering promotion have been reported in sweet orange (Delgado et al., 1986a; Martínez-Fuentes et al., 2004; Moss, 1970), mandarin (Delgado et al., 1986b; Martínez-Fuentes et al., 2004), lime (*C. aurantifolia* Christm.) (Davenport, 1983) and in kumquat (*Fortunella crassifolia* Swingle × *F. margarita* Swingle), a *Citrus* relative (Iwahori and Tominaga, 1986). Martínez-Fuentes et al. (2013) suggested that endogenous flowering inhibitors, i.e. GA synthesised in fruit or leaves prevail over exogenous promoters, and at a too high fruit load, application of growth retardants are ineffective. Monselise and Goldschmidt (1982), however, mentioned that for these chemicals to be effective, the antagonists of GA synthesis should reach the site of synthesis before GA is produced. The timing of application is therefore critical to achieving a significant response.

4. Conclusion

Given that the two most important determinants of return bloom flowering in alternate bearing ‘Nadorcott’ mandarin trees is firstly the number of new potential floral positions that developed from parent shoots during summer and secondly, the inhibition of floral bud development by fruit during early winter, this study demonstrated the role of phyto-hormones in the imposition of both these mechanisms of floral inhibition. Regarding the first limitation to regular bearing, viz. the number of new flowering sites, results showed that auxin was the primary phyto-hormone causing the inhibition of new summer vegetative shoots. The

concentration of IAA was significantly higher in leaves of "on" trees compared with "off" trees during summer. A foliar treatment of "off" trees with a synthetic auxin during summer, viz. 2,4-D, inhibited new summer vegetative shoot development from vegetative parent shoots. The lack of new summer vegetative shoots in "on" trees was not related to low cytokinin concentration. On the contrary, the storage and inactive cytokinin concentration in leaves in "on" trees was higher compared to "off" trees. Exogenous cytokinin application was unable to stimulate bud sprouting and new summer vegetative shoot growth from "on" parent shoots, and when fruit were removed from "on" parent shoots new vegetative shoots sprouted freely. Differences in the concentrations of various end-products of ABA metabolism indicate that ABA and IAA may have acted synergistically and were responsible for the maintenance of buds from "on" trees in an inactive state. "On" trees not only had fewer positions available from which a flower could sprout during spring, but no inflorescences developed from available flower bud positions. Therefore regarding the second limitation to regular bearing, viz. the inhibition of floral bud development, high GA concentration in leaves during early winter likely contributed to the inhibition of floral bud development, particularly in "on" parent shoots during spring. Treatment of "off" trees and shoots with 40 $mg \cdot L^{-1}$ synthetic GA_3 inhibited floral bud development and resulted in the sprouting of mostly vegetative shoots. Untreated trees sprouted mostly flowers and became "on" trees. The period during which buds were most sensitive to GA was from May to June. In heavy-fruiting 'Nadorcott' mandarin trees in this study, fruit occupied the terminal positions of the majority of shoots during this period and were most likely responsible for the higher GA concentration in "on" parent shoots which inhibited floral bud development throughout the winter. Soil and foliar treatments of "on" trees with 1000 $mg \cdot L^{-1}$ of the GA biosynthesis-inhibitors during May and June, viz. paclobutrazol and uniconazole resulted in increased flowering and fruit development from buds of "on" parent shoots. This study

confirmed the roles of IAA on inhibition of summer vegetative shoot development and GA on inhibition of floral bud development, but also provides insights to the 'hormonal balance' concept involving ABA and cytokinin in the hormonal theory of alternate bearing as proposed by Goldschmidt (2015).

Table 1. The phyto-hormone profile of endogenous auxin (IAA), cytokinin, abscisic acid (ABA) and gibberellin (GA), during summer in leaves of heavy-fruiting, i.e. "on" and low-fruiting, i.e. "off" 'Nadorcott' mandarin (*C. reticulata*) trees representative of alternate bearing. Values are expressed in $ng \cdot g^{-1}$ leaf dry weight.

Auxins	IAA			
"On" leaves	12.5 a[z]			
"Off" leaves	6.6 b			
P value	0.0239			
Cytokinins	t-ZR	iPA	t-ZOG	c-ZOG
"On" leaves	0.74 ns	5.4 ns	120.9 ns[y]	1862.7a
"Off" leaves	0.47	6.1	134.5	1092.5b
P value	0.6270	0.5640	0.7339	
Abscisic acid	ABA	ABA-GE	PA	DPA
"On" leaves	24.3 ns	113.1 a	17.4 ns	761.6a
"Off" leaves	23.9	0.0 b	20.7	530.3b
P value	0.9115	0.0001	0.5848	0.0261
Gibberellins	GA_3			
"On" leaves	3.1 ns			
"Off" leaves	2.4			
P value	0.2645			

[z] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=5).

[y] No significant differences.

Table 2. The phyto-hormone profile of endogenous auxin (IAA), cytokinin, abscisic acid (ABA) and gibberellin (GA), during summer in roots of heavy-fruiting, i.e. "on" and low-fruiting, i.e. "off" 'Nadorcott' mandarin (*C. reticulata*) trees representative of alternate bearing. Values are expressed in $ng \cdot g^{-1}$ leaf dry weight.

Auxins	IAA	IAA-Asp	IAA-Glu			
"On" roots	58.0 ns[z]	40.2 ns	0.9 ns			
"Off" roots	58.7	59.4	3.0			
P value	0.9632	0.1609	0.3517			
Cytokinins	t-Z	2iP	iPA	t-ZR	c-ZR	c-ZOG
"On" roots	2.3 b[y]	0.0 ns	11.0 ns	25.3 ns	14.0 a	52.7ns
"Off" roots	3.7 a	1.6	13.1	30.6	6.2 b	26.3
P value	0.0308	0.2637	0.2304	0.6342	0.0086	0.1066
Abscisic acid	ABA	7'-OH-ABA	PA	DPA		
"On" roots	29.8ns	4.0 ns	8.4b	1661.9 ns		
"Off" roots	34.5	7.6	16.3a	1832.8		
P value	0.4861	0.1481	0.0142	0.7119		
Gibberellins	GA_{29}	GA_3	GA_8			
"On" roots	5.5 ns	2.3 b	16.6 ns			
"Off" roots	4.9	5.5 a	36.2			
P value	0.8442	0.0343	0.0453			

[z] No significant differences.

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=5).

Table 3. The effects of fruit load and foliar applications of a synthetic auxin and cytokinin on vegetative shoot development in "on" and "off" 'Nadorcott' mandarin (*C. reticulata*) trees during summer in 2017.

Treatments	New vegetative shoots (no. per parent shoot)
"Off" tree	0.93b[z]
"Off" tree + 2,4-D[y]	0.63bc
"On" tree	0.00c
"On" tree + CPPU[x]	0.00c
"On" tree defruited 05 Jan. 2017	1.70a
P value	<0.0001

[z] Different letters in the same column denote significant differences between values [$P<0.05$; Fisher's LSD test; n=8].

[y] 40 mg·L^{-1} 2,4-D amine [Avima (Pty) Ltd; containing 720 g·L^{-1} a.i.].

[x] 7 mg·L^{-1} CPPU [Sitofex®; Phillagro SA (Pty) Ltd; containing 10 g·L^{-1}a.i.].

Table 4. The phyto-hormone profile of endogenous auxin (IAA), cytokinin, abscisic acid (ABA) and gibberellin (GA), during winter in leaves of heavy-fruiting, i.e. "on" and low-fruiting, i.e. "off" 'Nadorcott' mandarin (*C. reticulata*) trees representative of alternate bearing during winter. Values are expressed in $ng \cdot g^{-1}$ leaf dry weight.

Auxins	IAA			
"On" leaves	6.9 ns[z]			
"Off" leaves	5.6			
P value	0.6755			
Cytokinins	iPA	t-ZR	t-ZOG	c-ZOG
"On" leaves	10.2 b[y]	0.51 ns	138.7 ns	2633.1 a[y]
"Off" leaves	20.3 a	0.39	103.3	1353.6 b
P value	0.0231	0.4110	0.3127	0.0018
Abscisic acid	ABA	ABA-GE	PA	DPA
"On" leaves	41.5 ns	195.7 ns	15.3b	671.7 a
"Off" leaves	47.6	130.8	50.5a	445.3 b
P value	0.2227	0.4495	0.0107	0.0252
Gibberellins	GA_3			
"On" leaves	5.3 a			
"Off" leaves	0.0 b			
P value	0.0270			

[z] No significant differences.

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=5).

Table 5. The phyto-hormone profile of endogenous auxin (IAA), cytokinin, abscisic acid (ABA) and gibberellin (GA), during winter in roots of heavy-fruiting, i.e. "on" and low-fruiting, i.e. "off" 'Nadorcott' mandarin (*C. reticulata*) trees representative of alternate bearing during winter. Values are expressed in $ng \cdot g^{-1}$ leaf dry weight.

Auxins	IAA	IAA-Asp	IAA-Glu			
"On" roots	116.8 ns[z]	105.5 ns	3.0 ns			
"Off" roots	89.5	143.7	5.4			
P value	0.4634	0.4728	0.4190			
Cytokinins	t-Z	2iP	iPA	t-ZR	c-ZR	c-ZOG
"On" roots	1.5 ns	0.96 ns	10.0ns	30.3 ns	15.5 a[y]	62.0ns
"Off" roots	1.8	1.14	8.0	20.6	6.8 b	61.6
P value	0.3546	0.5708	0.1191	0.5937	0.0426	0.9262
Abscisic acid	ABA	7'OH-ABA	PA	DPA		
"On" roots	33.3ns	5.0 ns	8.4 b	665.8ns		
"Off" roots	52.5	6.8	14.6 a	445.3		
P value	0.2202	0.2506	0.0345	0.2082		
Gibberellins	GA_{29}	GA_3	GA_8	GA_{19}		
"On" roots	3.6 ns	2.9 ns	29.2 ns	0.0 ns		
"Off" roots	0.8	2.1	30.6	1.8		
P value	0.1468	0.7244	0.8672	0.1413		

[z] No significant differences.

[y] Different letters in the same column denote significant differences between values ($P<0.05$; Fisher's LSD test; n=5).

Table 6. The effects of fruiting status ("off" or "on"), foliar application of gibberellic acid (GA_3) and fruit removal (de-fruiting) treatments on bud sprouting and flowering characteristics of individual 'Nadorcott' mandarin (*C. reticulata*) shoots during return bloom in spring 2014 and 2015.

Shoot treatments	Total flowers	Leafless inflorescences	Leafy inflorescences	Vegetative shoots	Dormant buds
	No. per shoot				
			2014		
"Off" (control)	12.84 ns[z]	2.28a[y]	3.80a	1.36b	2.96b
"On"	1.08	0.20b	0.40b	3.80a	5.84ab
"Off" + GA_3[x]	0.56	0.00b	0.40b	3.00a	7.52a
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
			2015		
"Off" (control)	15.38a	3.75a	1.69a	0.38b	4.48ab
"On"	0.50b	0.56b	0.19bc	2.50a	5.69a
"Off" + GA_3[w]	1.13b	0.38b	0.18c	2.63a	5.70a
"On" defruited[v]	1.75b	1.19b	0.69ab	3.00a	4.00b
P value	<0.0001	<0.0001	0.0172	0.0007	0.0410

[z] No significant differences.

[y] Different letters in the same column denote significant differences between values [$P<0.05$; Fisher's LSD test; n=8].

[x] 40 $mg \cdot L^{-1}$ gibberellic acid [Progibb®; Phillagro SA (Pty) Ltd; containing 400 $g \cdot kg^{-1}$ a.i.]. Treatment applied at ≈2-week intervals to same shoots, starting on 1 May 2014 and continued until 15 Jul. 2014.

[w] 40 $mg \cdot L^{-1}$ gibberellic acid. Treatment applied to same shoots on 29 Apr. 2015 and 15 May 2015.

[v] Terminal fruit removed on 29 Apr. 2015.

Table 7. The effects of different treatments of gibberellin biosynthesis inhibitors during winter 2016 on return fruit yield of "on" 'Nadorcott' mandarin (*C. reticulata*) trees in 2017.

Treatments	Fruit yield		Fruit	Vegetative shoots	Shoot length
	Kg per tree	No. fruit per tree	No. per shoot	No. per shoot	mm
"On" tree (control)	109.7 b[z]	1108 b	0.00 b	3.21 a	19.2 a
"On" tree + Uniconazole foliar[y]	157.1 a	1861 a	0.61 a	2.17 ab	14.4 ab
"On" tree + Uniconazole soil[x]	159.2 a	1967 a	0.81 a	1.09 b	13.2 ab
"On" tree + Paclobutrazol foliar[w]	139.2 a	1622 a	0.44 a	3.10 a	10.0 ab
"On" tree + Paclobutrazol soil[v]	152.6 a	1778 a	0.74 a	2.55 ab	5.5 c
P value	0.0063	0.0021	0.0172	0.0341	0.0012

[z] Different letters in the same column denote significant differences between values [$P<0.05$; Fisher's LSD test; n=8].

[y]1000 mg·L^{-1} uniconazole [Sunny® 50 SC; Philagro SA (Pty) Ltd; containing 50 g·L^{-1} a.i.] foliar spray in 4 L water per tree.

[x]1000 mg·L^{-1} uniconazole soil drench in 3 L water per tree.

[w]1000 mg·L^{-1} paclobutrazol [Cultar® 250 SC; Syngenta SA (Pty) Ltd; containing 250 g·L^{-1} a.i.] foliar spray in 4 L water per tree.

[v]1000 mg·L^{-1} paclobutrazol soil drench in 3 L water per tree.

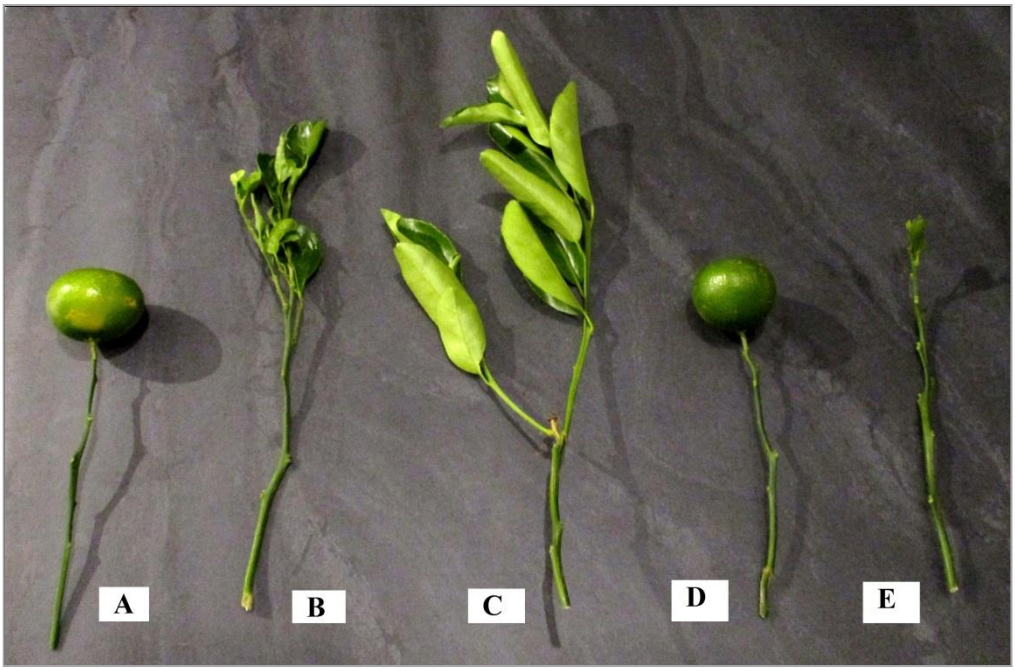

Fig. 1. The vegetative re-growth response to A) fruit load in an "on" 'Nadorcott' mandarin (*C. reticulata*) tree; B) fruit load in an "off" tree; C) de-fruiting in summer on 05 Jan. 2017; D) a foliar treatment of "on" trees with 7 $mg \cdot L^{-1}$ CPPU; and E) a foliar treatment of "off" trees with 40 $mg \cdot L^{-1}$ 2,4-D amine. Old leaves were removed and new leaves were kept intact to indicate new vegetative shoot development subsequent to treatment.

Fig. 2. The effect of a foliar treatment of "on" 'Nadorcott' mandarin (*C. reticulata*) trees with 7 mg·L^{-1} CPPU. The treatment was unable to stimulate development of new vegetative shoots and resulted in phytotoxicity in leaves of fruiting shoots only, possibly because the shoots were under impediment by endogenous growth inhibitors and the endogenous cytokinin concentration in leaves was already high.

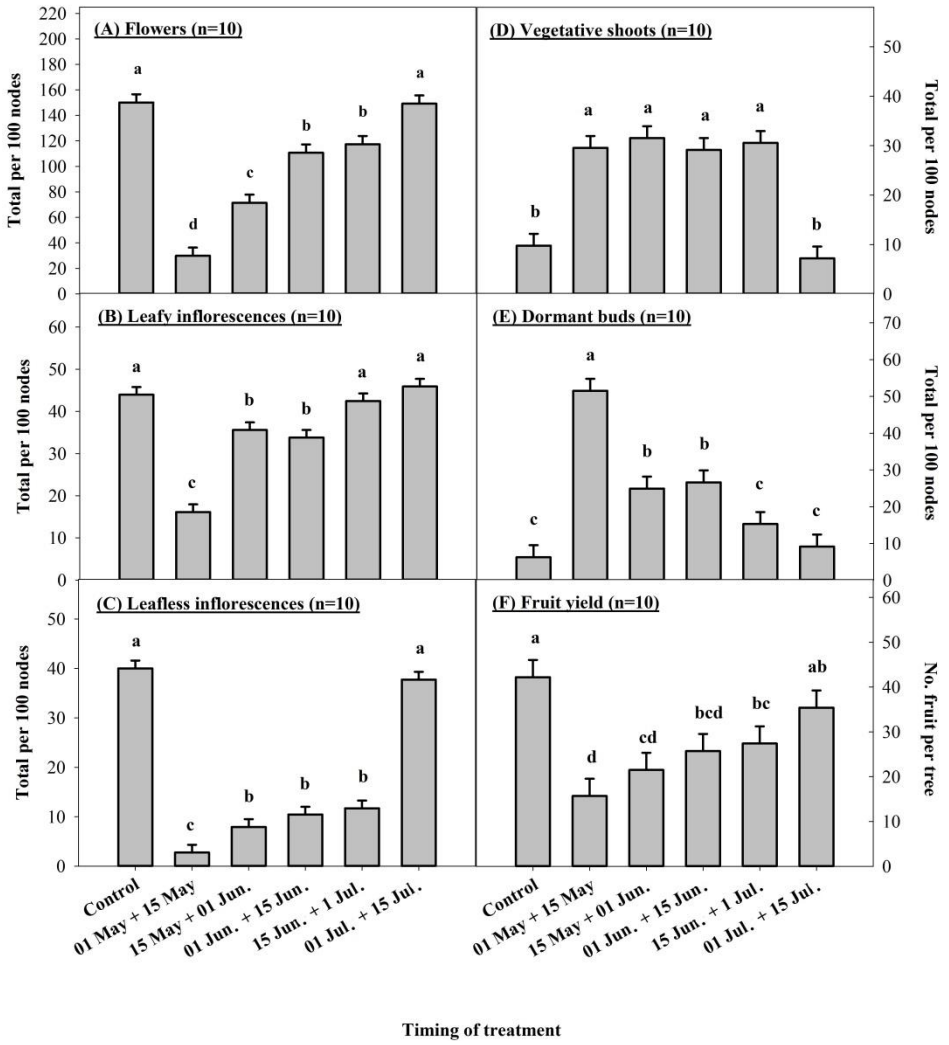

Fig. 3. The effects of different timings of foliar gibberellic acid (GA_3) treatments during winter in 2014, on flowering characteristics and fruit yield during return bloom and time of harvest in three-year-old, non-bearing 'Nadorcott' mandarin (*C. reticulata*) trees.

Fig. 4. Bud sprouting and flowering characteristics in individual 'Nadorcott' mandarin (*C. reticulata*) shoots during return bloom in spring 2015: A) "off" shoot (control); B) "off" shoot + 40 mg·L^{-1} foliar gibberellic acid on 29 Apr. 2015 and 15 May 2015; C) "on" shoot; and D) "on" shoot de-fruited on 29 Apr. 2015.

Fig. 5. The effects of: A) fruit presence in an “on” shoot; B) 1000 mg·L^{-1} uniconazole foliar spray; C) 1000 mg·L^{-1} uniconazole soil drench; D) 1000 mg·L^{-1} paclobutrazol foliar spray; and E) 1000 mg·L^{-1} paclobutrazol soil drench treatments applied to “on” ‘Nadorcott’ mandarin (*C. reticulata*) trees on 26 May and 16 Jun. 2016 on return bloom and fruit yield in 2017.

General discussion and overall conclusions

The aim of this study was to improve the understanding of the mechanism perpetuating alternate bearing in *Citrus* spp. and to establish the underlying cause(s) in the context of the recognised nutritional and hormonal theories of alternate bearing in citrus. 'Nadorcott' mandarin (*C. reticulata* Blanco) was selected as a model cultivar to use in this study.

Fruit load in 'Nadorcott' mandarin trees was the central factor determining return bloom flowering in subsequent seasons [R^2=(-)0.80 and R^2=(-)0.73 in seasons 1 and 2, respectively; (P<0.001)]. The quantity of flowers and fruit also had a strong inverse relationship with the number of new vegetative shoots in spring [R^2=(-)0.80 and R^2=(-)0.79 in seasons 1 and 2, respectively; (P<0.001)], summer [R^2=(-)0.81 R^2=(-)0.78 in seasons 1 and 2, respectively; (P<0.001)] and with total new vegetative shoots [R^2=(-)0.79 and R^2=(-)0.85 in seasons 1 and 2, respectively; (P<0.001)]. The number of new vegetative shoots that developed in "off" trees was 2- to 3-fold higher in spring and summer, and the number of total new vegetative shoots that developed in "off" trees was almost double that in "on" trees ("off" = 863 and 1439 vs. "on" = 306 and 766). Therefore, fewer new vegetative shoots developed when fruit load was high, i.e. in "on" trees, than when fruit load was low, i.e. in "off" trees.

The higher number of new vegetative shoots in "off" trees affected flowering in the subsequent spring; "off" trees had more nodes and more potential sites available from which flowers could develop. Hence, tree flower number was 1.7-fold higher in "off" trees in spring of season 1 ("off" = 51 097 flowers per tree vs. "on" = 30 034 flowers per tree) and ≈230-fold higher in spring of season 2 ("off" = 37 712 flowers per tree vs. "on" = 165 flowers per tree). Besides more flowering positions, flowering intensity was consistently higher in individual vegetative ("off") shoots in "off" trees, than in "off" shoots in "on" trees. From "off" shoots in "off" trees ≈50% more nodes developed than from "off" shoots in "on"

trees and, additionally, flower intensity in "off" shoots in "off" trees, i.e. the number of flowers that sprouted from a single node in an individual shoot, was about 5-fold that of "off" shoots in "on" trees. From these results it was concluded that alternate bearing in 'Nadorcott' mandarin trees perpetuates because of an inhibiting effect of fruit load on subsequent flowering. This mechanism firstly manifests because of reduced budbreak and new vegetative shoot growth, and secondly, by a lower number of flowers that develops from a single flowering position in newly developed vegetative shoots.

Neither of these mechanisms affecting return bloom as a result of heavy fruit load were related to parameters of leaf gas exchange, or to leaf carbohydrate concentration. Apart from some anomalies, photosynthesis, stomatal conductance and transpiration rates during spring and summer were always higher in leaves in fruiting ("on") shoots and "on" trees, from which fewer new vegetative shoots developed, than in "off" shoots in "off" trees, which was unsurprising. The relationship between leaf sugar concentration and the number of new vegetative shoots, however, was non-significant and very weak [season 1: R^2=0.49, P=0.050; season 2: R^2=(-)0.01, P=0.970]. Due to a higher starch concentration in leaves in "off" trees, than in "on" trees [season 1: 98 vs. 72 mg·g^{-1} leaf dry weight (DW); season 2: 53 vs. 42 mg·g^{-1} leaf dry weight DW] leaf starch concentration and number of new vegetative shoots showed a stronger relationship and a positive correlation in summer (season 1: R^2=0.53, P=0.040; season 2: R^2=0.71, P<0.001). However, when testing the significance of the apparent relationship using branch experiments, results failed to provide confirmation of the tree-level results. When fruiting branches were girdled, leaf starch concentration increased ≈3-fold compared to non-fruiting branches (298 vs. 112 mg·g^{-1} leaf DW), but very few new vegetative shoots sprouted per branch compared to non-fruiting branches (1.6 vs 8.6). Furthermore, the study showed that although high leaf starch concentration correlated with the number of new vegetative shoots, leaf starch concentration did not contribute to new

vegetative shoot growth, but accumulated to near-toxic levels in the palisade mesophyll parenchyma cells, the spongy mesophyll parenchyma cells and in the phloem cells of the leaf vein. This resulted in the development of an abiotic physiological phenomenon in "off" trees described for the first time by this study as "fruit-load-induced leaf chlorosis".

Fruit load probably disturbed the balance between vegetative shoot development and root growth. In "off" trees, root growth and vegetative shoot flushes showed alternating growth patterns – two distinct root growth peaks and three vegetative shoot flushes occurred in an eloquently synchronised pattern. The first root flush started during early summer in November, after cessation of the first and spring vegetative shoot flush. By mid-summer in December this root flush peaked, but growth ceased towards the end of January and prior to initiation of the second and summer vegetative shoot flush. A second and final root flush started when shoot growth stopped in March and a third, small vegetative shoot flush followed in April.

In "on" trees, root growth was almost completely absent and the number of new vegetative shoots was half that of "off" trees. The lack of root growth in "on" trees appeared to be related to a source-limitation in carbohydrates caused by profuse flowering in spring, and excessive fruiting in summer. The up to 230-fold more flowers in spring and ≈7.3-fold more fruit in summer in "on" trees used the majority of sugars, which likely limited carbohydrate availability in the roots during these periods. This was apparent in the ≈3-fold higher root sugar concentration in "off" trees during full bloom in October (119 vs. 36 $mg \cdot g^{-1}$ leaf DW) and ≈20% higher root sugar concentration during summer in December (61 vs. 49 $mg \cdot g^{-1}$ leaf DW). These results were convincing in terms of the significance in the difference between carbohydrate concentrations, but results were of a correlative nature only and this may be a shortcoming of this aspect on the research on alternate bearing. The results and interpretation nevertheless strongly concur with the well-documented and important inter-

dependent relationship between root growth and vegetative shoot flushes in citrus and for the first time points to a similar relationship under conditions of alternate bearing. This opens up new avenues for horticultural research and could also provide practical opportunities to explore as a potential cultural practice in citrus production, e.g. exploring means to stimulate root growth and vegetative shoot flush in heavy-fruiting trees. More importantly, this paves the way for possible novel research opportunities and a better understanding of alternate bearing in general, e.g. does the same relationship exist in other alternate bearing citrus species and/or cultivars or fruit crops.

Fruit load affected leaf mineral nutrient concentration, but not to the detriment of vegetative shoot flush or flowering. The crop removal factor, i.e. the g mineral element removed per kg fruit per tree, was higher for each mineral element in "off" trees – one kg fruit removed 2.3 g N, 0.3 g P, 3.1 g K, 1 g Ca and 0.4 g Mg, compared to 1.3 g N, 0.2 g P, 1.7 g K, 0.6 g Ca and 0.2 g Mg per one kg fruit in "on" trees. Fruit loads of 84, 110 and 52 kg fruit per tree in "on" trees, however, removed 217 g N, 28 g P, 296 g K, 100 g Ca and 35 g Mg per tree, which were 1.5 to 7 times more than that removed by fruit loads of 14, 71 and 16 kg fruit per tree in "off" trees. In "off" trees, macro-nutrients accumulated in leaves to concentrations between 20% and 30% higher compared with that in "on" trees. In all the experiments, however, leaf mineral nutrient concentrations showed no consistent relationship with return bloom flowering and/or with fruit load in the subsequent season. With the exception of some anomalies, there were no relationships between the concentrations of any of the leaf mineral nutrients and parameters of flowering and vegetative shoot flush in response to different defruiting treatments in "on" trees. In addition, results from foliar nutrient spray treatments dismissed the significance of any ambiguities regarding the role of nutrients. It should, however, be mentioned that foliar spray treatments in this study were applied relatively late in the alternate bearing cycle and that future research on mineral

nutrients should target the induction of root and/or shoot flushes with foliar nutrient sprays applied at an earlier timing. The results on this aspect of the possible cause of alternate bearing nevertheless suggest that tree mineral nutrient status can be considered a consequence, rather than a cause, of fruit load in alternate bearing 'Nadorcott' mandarin trees.

The two primary triggers in the alternate bearing mechanism in 'Nadorcott' mandarin were related to high concentrations of specific endogenous phyto-hormones. High concentrations of 1 *H*-indole-3-acetic acid (IAA) and metabolites of abscisic acid (ABA) in leaves was related to reduced new vegetative shoot development during the summer vegetative shoot flush. "Off" shoots sprouted more new vegetative shoots and had a ≈47% lower IAA concentration in leaves compared with "on" shoots, from which very few new vegetative shoots sprouted. The concentration of the end-product of ABA catabolism, viz. dihydrophaseic acid (DPA) was higher in leaves in "on" trees than in "off" trees (761.6 vs. 530.3 $ng \cdot g^{-1}$ leaf DW), as well as that of ABA-GE (113.1 vs. 0.0 $ng \cdot g^{-1}$ leaf DW), an ABA glucose ester and ABA storage form. On the other hand, the lower number of new summer vegetative shoots in "on" trees was not related to the low concentration of endogenous cytokinins. On the contrary, the concentration of cis-zeatin O-glucoside (c-ZOG), a storage form of active cytokinin, was higher in leaves in "on" trees than in leaves in "off" trees (1862.7 vs. 1092.5 $ng \cdot g^{-1}$ leaf DW). Results suggest that cytokinin availability in "on" trees was not limited, but merely unable to participate in bud sprouting because of high concentrations of IAA in the presence of fruit. Exogenous cytokinin application was unable to stimulate bud sprouting and new summer vegetative shoot growth from "on" parent shoots, and when fruit were removed from "on" parent shoots, new vegetative shoots sprouted freely. A second major outcome was that high gibberellin (GA) concentration in leaves during winter was related to less flower development from shoots in "on" trees. The concentration

of gibberellic acid (GA_3) in “on” shoot leaves was high and no GA_3 was detected in “off” shoot leaves. Treatments of “off” trees and shoots with 40 $mg \cdot L^{-1}$ synthetic GA_3, inhibited flowering. May and June was the period when citrus buds were most sensitive to GA, i.e. when maximum inhibition on flowering was obtained by exogenous GA_3 application, but earlier applications during summer should be tested to determine their effects on flowering response. Nevertheless, soil and foliar treatments of “on” trees during the corresponding period with 1000 $mg \cdot L^{-1}$ of the GA biosynthesis-inhibitors paclobutrazol and uniconazole increased flowering and fruit development in “on” shoots. Considering that alternate bearing in citrus perpetuates due to an inhibition on flowering by fruit, these results on the effects of treatments with GA biosynthesis-inhibitors in May and June could provide a practical mean for citrus producers to overcome the inhibition of fruit on flowering under conditions of alternate bearing.

An overall model is presented that integrates the nutritional and hormonal theories in alternate bearing in ‘Nadorcott’ mandarin (Fig. 1).

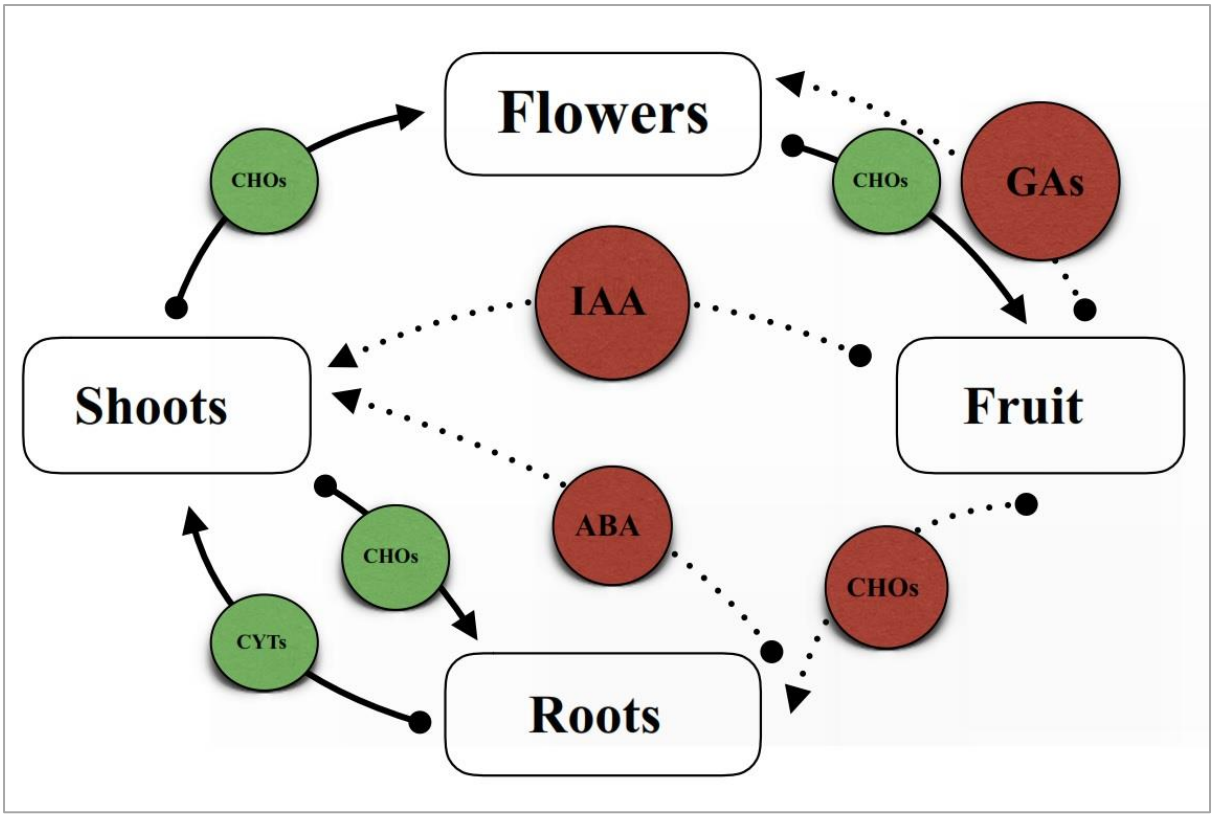

Fig 1. A schematic model proposed for the various factors affecting the alternate bearing habit of 'Nadorcott' mandarin, including carbohydrates (CHOs) in the nutritional theory, and the phyto-hormones abscisic acid (ABA), cytokinins (CYTs), gibberellins (GAs) and the auxin, 1 *H*-indole-3-acetic acid (IAA), in the hormonal theory of alternate bearing. Solid arrows indicate a positive relationship between the organs, viz. roots, vegetative shoots, flowers and fruit, and dotted arrows indicate a negative relationship. A factor in green is responsible for the endogenous stimulation (promotive action) of the organ to which its arrow is pointed, and a factor in red is responsible for the endogenous inhibition (inhibitive action) of the development of the organ to which its arrow is pointed.

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