Physiological and molecular mechanisms underlying shoot manipulation strategies with the plant growth regulator ethephon for the improvement of flowering in litchi (*Litchi chinensis* Sonn.) cv. 'Mauritius'

by

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated) that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2023

DEDICATION

To my Mom and Dad

Thank you for teaching me diligence and perseverance

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SUMMARY

Erratic flowering is a major challenge in most litchi (*Litchi chinensis* Sonn.) producing countries, including South Africa. In recent decades, above-average temperatures in autumn and early winter have increased the incidence of vegetative shoot growth prior to floral induction, further exacerbating irregular flowering and rendering the conventional ethephon shoot control measures less effective. Therefore, this study aimed to investigate the effect of ethephon applied to mature terminal shoots for its potential to prevent shoot growth prior to floral induction, to delay panicle emergence to a period with consistently low temperature and to promote carbohydrate accumulation for improved flowering and yield compared with conventional spot spray applications to emerging, immature vegetative shoots.

In a four-year orchard-based study, ethephon was applied to 'Mauritius' trees, either as single or double whole-canopy spray at 500, 750 and 1000 $mg \cdot L^{-1}$, each at three different dates between autumn and early winter, or as a combination of whole-canopy spray at first signs of shoot growth and spot sprays on developing shoots, in two climatically different production areas of South Africa. All whole-canopy treatments were compared with the conventional spot spray applications at 1000 $mg \cdot L^{-1}$ and untreated trees. Results revealed that whole-canopy ethephon applications to mature terminal shoots successfully inhibited pre-induction vegetative shoot growth in a dose-dependent manner, subsequently delaying panicle emergence to a cooler period, which promoted carbohydrate accumulation, improved inflorescence quality, flowering rate and yield without delaying fruit maturity, compared with spot-sprayed and untreated trees. The date of application played an important role in the efficacy of ethephon applications, with higher ethephon concentrations being more beneficial under warmer conditions, such as in early autumn or generally in warm areas, while lower concentrations were sufficient for applications later in autumn and early winter.

In a second study, ethylene evolution as well as phenological, physiological and molecular changes underlying ethephon application and its associations with bud dormancy and flowering were investigated, by comparing a single whole-canopy ethephon application at 1000 $mg \cdot L^{-1}$ to mature terminal shoots with untreated trees. Ethylene evolution peaked in leaves on the day of application with a rapid decline thereafter, but persisted in buds for seven days before gradually declining. Ethephon application significantly increased relative expression of *LcEIN3* and *LcFLC* in terminal buds one day after application, while *LcFT2* expression in leaves and *LcAP1* expression in terminal buds were significantly increased at the bud break stage. The ethephon treatment also significantly increased soluble sugar concentrations in leaves and shoots at the bud break or floral initiation stage.

The current study provided evidence that ethephon application plays an important role in the physiological and molecular regulation of bud dormancy and subsequent floral regulation of litchi. By preventing pre-flowering shoot growth and influencing the time of bud break, ethephon application to mature terminal shoots proved to be a more effective than conventional spot spray applications and can be a powerful tool to manage panicle emergence under less inductive conditions. Moreover, the modified use of ethephon contributes to the sustainability and expansion of the South African litchi industry.

OPSOMMING

Wisselvallige blom is 'n groot uitdaging in die meeste lietsjie (*Litchi chinensis* Sonn.) produserende lande, insluitend Suid-Afrika. In die afgelope dekades het bogemiddelde temperature in die herfs en vroeë winter die voorkoms van vegetatiewe lootgroei voor blominduksie verhoog, wat onreëlmatige blom vererger het en die konvensionele lootbeheermaatreëls met etefon minder doeltreffend gemaak het. Die doel van hierdie studie was dus om die effek van etefon wat op volwasse terminale lote toegedien word, te ondersoek vir die potensiaal om lootgroei voor blominduksie te voorkom, om die opkoms van bloeiwyses tot 'n tydperk met konstante lae temperatuur te vertraag, en om koolhidraatakkumulasie te bevorder vir verbeterde blom en opbrengs in vergelyking met die konvensionele kolbespuitings op ontwikkelende, onvolwasse vegetatiewe lote.

In hierdie vier-jaar boord-gebaseerde studie, is etefon op 'Mauritius' bome toegedien in twee klimaatverskillende produksiegebiede van Suid-Afrika, hetsy as enkel- of dubbel heelboom bespuiting teen 500, 750 en 1000 mg·L^{-1}, elk op drie verskillende datums tussen herfs en vroeë winter, of as 'n kombinasie van heelboom bespuiting by eerste tekens van lootgroei en kolbespuitings op ontwikkelende lote,. Alle heelboom behandelings is vergelyk met konvensionele kolbespuitings teen 1000 mg·L^{-1} asook onbehandelde bome. Resultate het getoon dat heelboom toedienings op volwasse terminale lote voor blominduksie die vegetatiewe lootgroei suksesvol geïnhibeer het, wat die opkoms van blompluime na 'n koeler tydperk vertraag het, koolhidraatakkumulasie bevorder het, kwaliteit van bloeiwyses, blompersentasie en opbrengs verbeter het sonder om vrugrypheid te vertraag, in vergelyking met kolbespuiting en onbehandelde bome. Die datum van toediening het 'n belangrike rol gespeel in die doeltreffendheid van etefontoedienings. Hoër etefonkonsentrasies was meer voordelig onder warmer toestande, soos in die vroeë herfs of algemeen in warm gebiede, terwyl laer konsentrasies voldoende was vir toedienings later in die herfs en vroeë winter.

In 'n tweede studie is etileen evolusie sowel as fenologiese, fisiologiese en molekulêre veranderinge onderliggend aan etefontoediening en in assosiasies met dormansie van ogies en blomvorming ondersoek, deur 'n enkele heelboom etefontoediening teen 1000 mg·L^{-1} met volwasse terminale lote te vergelyk met onbehandelde bome. Etileen evolusie het 'n hoogtepunt bereik in blare op die dag van toediening met 'n vinnige afname daarna, maar het vir sewe dae in ogies voortgeduur voordat dit geleidelik afgeneem het. Etefon toediening het die relatiewe uitdrukking van *LcEIN3* en *LcFLC* in terminale ogies een dag na toediening aansienlik verhoog, terwyl *LcFT2* uitdrukking in blare en *LcAP1* uitdrukking in terminale ogies aansienlik toegeneem het tydens die

knopbreekstadium. Die etefon behandeling het ook die oplosbare suikerkonsentrasies in blare en lote aansienlik verhoog tydens die knopbreek- of blominisiasiestadium.

Die huidige studie het bewys dat etefontoediening 'n belangrike rol speel in die fisiologiese en molekulêre regulering van knopdormansie en daaropvolgende blomregulering van lietsjie. Deur lootgroei te voorkom en die tyd van knopbreek te beïnvloed, het etefontoediening op volwasse eindlote bewys dat dit meer effektief is as konvensionele kolbespuitings en dat dit 'n kragtige hulpmiddel kan wees om die tyd van blomontwikkeling onder minder induktiewe toestande te bestuur. Verder dra die gewysigde gebruik van etefon by tot die volhoubaarheid en uitbreiding van die Suid-Afrikaanse lietsjiebedryf.

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Peer-reviewed Publications

Cronje, R.B. and Ratlapane, I.M. 2018. Effect of full-cover ethephon applications on flowering and yield of 'Mauritius' litchi in South Africa. Acta Horticulturae 1211:71-78. https://doi.org/10.17660/ActaHortic.2018.1211.10. (Appendix I)

Cronje, R.B., Ratlapane, I.M., Rohwer, E.A., Hoffman, E.W. and Huang, X.M. 2020. Carbohydrate reserve dynamics as influenced by shoot control strategies and climatic conditions prior to flowering in 'Mauritius' litchi. Acta Horticulturae 1293:155-165. https://doi.org/ 10.17660/ActaHortic.2020.1293.22. (Appendix II)

Cronje, R.B., Hajari, E., Jonker, A., Ratlapane, I.M. Huang, X., Theron, K.I. and Hoffman, E.W. 2022. Foliar application of ethephon induces bud dormancy and affects gene expression of dormancy- and flowering-related genes in 'Mauritius' litchi (*Litchi chinensis* Sonn.). Journal of Plant Physiology, 276: 153768. https://doi.org/10.1016/j.jplph.2022.153768. (Appendix III)

Cronje, R.B. and Jonker, A.J. 2023. Quantification of ethylene production in leaf and bud tissue of the subtropical tree crop litchi (*Litchi chinensis* Sonn.) using gas chromatography and flame ionization detection. Bio-protocol 13(xx): exxxx. (in print). (Appendix IV)

Semi-scientific Publications

Cronje, R.B. and Ratlapane, I.M. 2017. Optimizing the use of ethephon for cooler mid-season production areas. South African Litchi Growers' Association Yearbook, 23:24-32.

Cronje, R.B. and Ratlapane, I.M. 2017. Optimizing the use of ethephon for warm early-season production areas. South African Litchi Growers' Association Yearbook, 23:33-40.

Cronje, R.B. and Ratlapane, I.M. 2021. Full canopy ethephon applications for litchi autumn shoot control as effective alternative to spot spray applications. South African Litchi Growers' Association Yearbook, Vol 24: 24-32.

Cronje, R.B., Hajari, E., Ratlapane, I.M. and Jonker, A.2021. Effect of ethephon application on ethylene evolution and flower gene expression in 'Mauritius' litchi. South African Litchi Growers' Association Yearbook, Vol 24: 34-40.

Conference Presentations

Cronje, R.B., Ratlapane, I.M. and Froneman, I.J. Full-cover Ethapon sprays applied to dormant trees affect time of flower panicle emergence and flowering of litchi, cv. 'Mauritius'. Poster presentation at the 2015 Combined Congress, George, South Africa. 19-22 January 2015.

Cronje, R.B. and Ratlapane, I.M. Effect of full-cover ethephon applications on flowering and yield of 'Mauritius' litchi in South Africa. Oral presentation at the 5th International Symposium on Lychee, Longan and Other Sapindaceae Fruit, Sabour, Bihar, India. 31 May to 3 June 2016.

Cronje, R.B. and Ratlapane, I.M. Effect of various shoot control strategies with Ethapon on 'Mauritius' litchi tree starch reserves in South Africa. Poster presentation at the 5th International Symposium on Lychee, Longan and Other Sapindaceae Fruit, Sabour, Bihar, India. 31 May to 3 June 2016.

Cronje, R.B., Ratlapane, I.M., Hajari, E., Hannweg K.F. and Booyse, M. Manipulation of litchi phenology using ethephon to mitigate changes in seasonal weather patterns in South Africa. Poster presentation at the 1st International ISHS Symposium on Flowering, Fruit Set and Alternate Bearing, Palermo, Italy. 19-22 June 2017.

Cronje, R.B., Ratlapane, I.M., Jonker, A.J., Huang, X.M. and Hoffman, E.W. Effect of full-cover ethephon applications on shoot inhibition and flower induction of litchi, cv. 'Mauritius' ('Tai So') in South Africa. Oral presentation at the 2018 China International Litchi Conference, Conghua, China. 28-30 June 2018.

Cronje, R.B., Ratlapane, I.M., Rohwer, E.A., Hoffman, E.W. and Huang, X.M. Carbohydrate reserve dynamics as influenced by shoot control strategies and climatic conditions prior to flowering in litchi, cv. 'Mauritius'. Oral presentation at the 6th International ISHS Symposium on Lychee, Longan and Other Sapindaceae Fruits, Hanoi, Vietnam. 7-12 June 2019.

Cronje, R.B., Hajari, E., Ratlapane, I.M., Jonker, A.J. and Hoffman, E.W. Ethephon application affects gene expression of ethylene-, dormancy- and flowering-related genes and alters tree phenology and physiology in litchi, cv. 'Mauritius'. Oral presentation at the 2022 Combined Congress (virtual), South Africa. 24-26 January 2022.

Industry Symposium Presentations

Cronje, R.B. and Ratlapane, I.M. Effect of full-cover Ethapon sprays on litchi flowering and yield. Oral presentation at the 2015 SALGA Research Symposium, Mbombela, South Africa. 11 November 2015.

Cronje, R.B. and Ratlapane, I.M. Shoot control strategies with Ethapon for improved flowering and yield. Oral presentation at the 2016 SALGA Research Symposium, Hoedspruit, South Africa. 26 October 2016.

Cronje, R.B. and Ratlapane, I.M. Optimizing the use of ethephon. Oral presentation at the 2017 SALGA Research Symposium, Mbombela, South Africa. 25 October 2017.

Cronje, R.B., Ratlapane, I.M., Jonker, A.J. and Hajari, E. Optimizing ethephon applications. Oral presentation at the 2018 SALGA Research Symposium, Mbombela, South Africa. 24 October 2018.

Cronje, R.B., Ratlapane, I.M. and Rohwer, E.A. Influence of climatic conditions on carbohydrate reserve dynamics prior to flowering in litchi. Oral presentation at the 2019 SALGA Research Symposium, Mbombela, South Africa. 30 October 2019.

Cronje, R.B., Hajari, E. and Ratlapane, I.M. Effect of ethephon on flower gene expression. Oral presentation at the 2020 SALGA Research Symposium, Mbombela, South Africa. 22 October 2020.

Cronje, R.B., Ratlapane, I.M., Hajari, E. and Jonker, A.J. Ethephon application promotes bud dormancy and gene expression of flowering related genes in ‘Mauritius’ litchi. Oral presentation at the 2021 SALGA Research Symposium, Nelspruit, South Africa. 21 October 2021.

PREFACE

This dissertation is a compilation of chapters starting with a general introduction, followed by a literature review (Paper 1) and three research papers (Papers 2 to 4), and lastly a general discussion and conclusion. Each paper is introduced separately and is written according to the style specifications of the Journal of the American Society for Horticultural Science. Repetition or duplication between papers was therefore inevitable.

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(F). Untreated trees or trees treated with whole-canopy ethephon applications were applied to fully mature shoots (A).

Fig. 3. Percentage shoots (out of total number of tagged terminal shoots) developing from dormant (A) and active (B) apical buds following treatment with ethephon (Eth750, Eth1000) or no treatment (Untreated) of terminal shoots in 'Mauritius' litchi. Ethephon was applied to mature terminal shoots at concentrations of 750 $mg{\cdot}L^{-1}$ (Eth750) or 1000 $mg{\cdot}L^{-1}$ (Eth1000) during April 2013 at the experimental site near Mbombela. Data was collected on the day of application and over a 120-day period after ethephon application. Values represent means (n = 20). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$), for dormant and actively growing bud data, respectively. Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

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years. Agglomerative hierarchical cluster analysis was performed on PCA factor scores. Eth: ethephon. T1: early application date (end-March/early April), T2: mid application date (mid-April), T3: late application date (end-April/early May).

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Paper 4: Foliar application of ethephon induces bud dormancy and affects gene expression of dormancy- and flowering-related genes in 'Mauritius' litchi (*Litchi chinensis* Sonn.)

Fig. 1. Morphology of terminal buds at different developmental stages between dormancy and floral initiation in 'Mauritius' litchi. Sub-figures specify the developmental stages used for sample collection for ethylene and gene expression analysis: (A) dormant bud stage, (B) bud break stage, and (C) floral initiation stage.

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Fig. 3. Leaf chlorophyll content index of 'Mauritius' litchi leaves at different developmental stages. Leaf chlorophyll content index from a leaflet of the compound leaf closest to the terminal bud was determined at the indicated developmental stages following ethephon treatment at 1000 $mg \cdot L^{-1}$ (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds.

Columns represent mean values ± *SE* (*n* = 5). Statistical analysis of data was assessed with repeated measures ANOVA (*P* < 0.05).

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General Introduction

Litchi (*Litchi chinensis* Sonn.), also known as lychee, is a subtropical evergreen fruit tree that belongs to the Sapindaceae family. Most species in this family, which comprises about 2000 species from 150 genera, are trees or shrubs that are widely distributed in the tropics and warm subtropical regions (Subhadrabandhu and Stern, 2005). *Litchi chinensis* is the only known member in the genus *Litchi* and is divided into three subspecies based on the morphological characteristics of the shoot, flower and fruit: *Litchi chinensis* ssp. *chinensis*, *L. chinensis* ssp. *philippinensis* and *L. chinenesis* ssp. *javensis*. Only *Litchi chinensis* ssp. *chinensis* is commercially cultivated (Menzel and Simpson, 1994; Subhadrabandhu and Stern, 2005). *Litchi chinensis* is native to southern China and northern Vietnam, where its cultivation dates back more than 3000 years (Menzel, 2002; Menzel and Simpson, 1994; Subhadrabandhu and Stern, 2005). Today, litchi is mainly cultivated between 17° and 32° latitude of the Northern and Southern Hemispheres. The leading litchi-producing countries in the Northern Hemisphere are China, India, Vietnam, Thailand, Bangladesh, and Mexico, while countries in the Southern Hemisphere include Madagascar, South Africa, and Australia (Huang et al., 2005). With its attractive exotic appearance and taste, as well as rich nutritional value, fresh litchi fruit have become increasingly popular on international markets (Huang et al., 2005). However, despite being an economically important fruit crop in many tropical and subtropical regions, litchi is still considered a minor crop worldwide, mainly due to its low climatic adaptability (Menzel and Simpson, 1994; Stern and Gazit, 2003).

Litchi is best adapted to warm subtropical areas with hot, humid summers and dry, cool winters (Carr and Menzel, 2014; Huang et al., 2005; Menzel and Simpson, 1994). One of the most climate-dependent growth stages in litchi is the reproductive stage, in particular floral induction and initiation (Batten and Lahav, 1994; Chen and Huang, 2005; Menzel and Simpson, 1988). Each floral developmental stage has different temperature requirements. Floral induction and initiation generally require temperatures below 15°C, while floral differentiation necessitates gradual rising temperature conditions (Huang and Chen, 2005; Menzel et al., 1989). As the litchi floral bud is a mixed bud with leaf and panicle primordia, as well as rudimentary leaves, its final morphological characteristics strongly depend on the prevailing temperature during floral initiation. At low temperatures, floral buds develop into a richly branched inflorescence without leaves, while at slightly elevated temperatures, mixed shoots, also called leafy inflorescences, develop with both leaves and inflorescences present at each node. At temperatures higher than those required for inflorescence development, the panicle primordia atrophy and form vegetative shoots (Davenport and Stern, 2005; Zhou et al., 2008, 2010).

Successful floral development in litchi requires mature terminal shoots to coincide with low inductive temperatures. Litchi inflorescences are generally produced terminally (Robbertse et al., 1995). Therefore, any new vegetative shoots emerging immediately prior to the floral induction period will not mature in time to produce flowers, thus, causing poor flowering (Batten and McConchie, 1995; Davenport and Stern, 2005; Huang and Chen, 2005, 2014; O'Hare, 2002; Singh et al., 2017; Stern and Gazit, 2003). As flowering and fruiting are carbon-costly developmental processes, sufficient carbohydrate accumulation prior to flowering is an important strategy of fruit trees to sustain reproductive growth (Huang and Chen, 2014). Carbohydrate accumulation in litchi is also closely linked with shoot maturity (Fu et al., 2022). Continuous vegetative shoot growth into early winter often occurs in litchi under climatic conditions that are less favorable for flowering and is a major concern for growers. Various horticultural practices have been used in the past to address unwanted vegetative shoot growth, albeit with various degrees of success. These include manipulating the timing of postharvest flush cycles, e.g., by timely pruning and nitrogen fertilizer applications after harvest, as well as suppressing or eliminating untimely vegetative shoot growth prior to the floral induction period, e.g., by applying water stress, girdling or plant growth regulators (Chaitrakulsub et al., 1992; Mandal et al., 2014; Menzel et al., 1988; Olesen et al., 2013; Ramburn, 2001; Roets et al., 2010; Stern et al., 1998; Subhadrabandhu and Koo-Duang, 1987; Zhang et al., 1997).

Litchi was introduced to South Africa from Mauritius in 1875 (De Villiers, 2010; Huang et al., 2005; Maity and Mitra, 1990; Stern and Gazit, 2003). Compared with other subtropical fruit crops, such as avocado and mango, litchi is only a minor crop in South Africa with approximately 1500 hectares and an average production of 6000 tons (Subtrop, 2020, 2022). However, as a highly profitable crop with a total annual industry value of around 185 million South African Rand (Derek Donkin, Subtrop, personal communication), the opportunities for the South African litchi industry to increase the market share on the local market as well as to gain access to new international markets are enormous. The main commercial cultivar in South Africa is 'Mauritius', comprising about 75% of the South African litchi production. The litchi production regions in South Africa are located in the frost-free areas of Mpumalanga and Limpopo Province in the northeastern part of the country, with minor plantings in the KwaZulu-Natal Province (Subtrop, 2020, 2022). These production regions are classified as subtropical regions with warm, wet summers and cool, dry winters. However, in the past two decades, the weather patterns have changed to drier summers, later-than-usual rainfall, which last well into autumn (March and April, and sometimes May), as well as winters with milder temperatures and delayed onset of chilling temperatures across most subtropical regions of South Africa (Weather Online, 2021).

To achieve effective floral induction and initiation in the cultivar 'Mauritius', the trees require a firm drop in temperatures below a daily mean of 20°C for several days, provided the terminal buds are at the correct stage of development, although a longer cold period is preferable (Huang and Chen, 2005; Kift and Roets, 2001). However, these temperature requirements are no longer consistently met during the traditional floral induction and initiation periods, which are between mid-April and mid-May for the mid-season production areas (Mbombela, Hazyview, and Tzaneen areas) and between mid-May to mid-June for early production areas (Malalane) (Cronje et al., 2017). Recurring new vegetative shoot growth during autumn (April) and early winter (May/June), linked to above-average temperatures and late rainfall, has thus become a common phenomenon in 'Mauritius' litchi trees cultivated under South African conditions. South African growers have been using the plant growth regulator ethephon since the early 2000s to control continuous shoot growth during autumn and early winter (Roets et al., 2010). For this purpose, ethephon is traditionally applied as a spot spray application, only targeting young emerging vegetative shoots when they appear during autumn and early winter. This chemical treatment causes abscission of young leaves and eventually dieback of the entire shoot, including the terminal bud. Four to six weeks later, when consistently low temperatures prevail, inflorescences develop from axillary buds (Kift and Roets, 2001). However, shoot control by spot spraying generally requires several applications due to uneven flush emergence within a tree and between trees within an orchard. With more frequent, warmer autumns and winters, the prolonged vegetative shoot growth period prior to floral development requires even more spot spray applications for effective control, thus, rendering this control mechanism less economical.

As developing vegetative shoots in litchi have a high water and nutritional demand (Huang and Chen, 2014) as well as consume carbohydrate reserves (Cronje and Mostert, 2009; Menzel et al., 1995), a reduction of reserve carbohydrates shortly before floral development may influence the flowering potential of the trees. In addition, inconsistent low temperatures during winter increase the incidence of leafy inflorescences, which were found to produce fewer female flowers and fruit compared with leafless inflorescences (Huang and Chen, 2014; Lee and Chang, 2019; Menzel and Simpson, 1992), and are therefore likely to reduce yields. As growers prefer not to spray ethephon beyond the first signs of floral development out of concern to damage the emerging flowers, irrespective of the presence of young vegetative shoots, the traditional shoot control measures are not considered effective anymore, because of progressively warm winter conditions. Hence, this challenge necessitated a different approach to shoot control prior to floral development, to maintain sustainability and promote expansion of the litchi industry in South Africa.

Therefore, the aim of this study was to find a practical solution for South African litchi growers that could effectively prevent pre-flowering shoot growth when exposed to warm autumns and

winters with sub-optimal inductive temperatures and consequently improve flowering in the cultivar 'Mauritius'. For this purpose, the traditional ethephon spot spray application to already emerged young vegetative shoots was modified to applying ethephon as whole-canopy spray to trees with mature terminal shoots and dormant apical buds, i.e., prior to any prospective new shoot growth. It was hypothesized that ethephon will induce bud dormancy and therefore, inhibit new shoot growth prior to the floral induction period. By delaying bud break and panicle emergence, it was anticipated that the trees would be less responsive to high temperatures during the pre-floral induction period, and only allow growth when temperatures have become conducive for floral induction and initiation, thus, leading to improved flowering and yield. In addition, it was speculated that inhibiting new vegetative shoot growth and extending the dormancy period can provide additional opportunity for trees to accumulate carbohydrate reserves as necessary energy resource to support the flowering process.

To provide sound advice to growers, the first objective of the study was to evaluate the effectiveness of a range of ethephon concentrations on shoot inhibition when applied to trees with fully mature terminal shoots and dormant buds, in comparison with the commercial practice of applying ethephon as spot sprays to already growing young vegetative shoots. Due to annual changes in postharvest flushing cycles, three different application dates were included. The influence of climate on the shoot control strategies was addressed by conducting the experiments in two production regions that differed distinctly in their winter climates, i.e., cool and moderate, respectively. Treatment assessments at both trial sites focused on the rate of vegetative shoot growth prior to floral induction, the time interval between application and bud break as indicator of dormancy duration, the rate of leafy panicles as indicator of intermittent temperature fluctuations during floral initiation, flowering rate, certain fruit characteristics and yield.

The second objective of the study was to investigate the influence of prevailing vegetative shoot or flower development, depending on the type of ethephon application strategy, on the changes in reserve carbohydrate concentrations of small branches, and whether the inhibition of shoot growth can promote carbohydrate accumulation. Furthermore, it was explored whether there is a positive correlation between effective treatments, flowering rate and possibly chill responsiveness of the trees. The data for this investigation were only collected from the experimental site with the cool winter climate as it provided more variation in terms of environmental conditions and consequently phenological differences. The focus was placed on the three contrasting ethephon application strategies, i.e., spot spray applications to remove young developing shoots, whole-canopy applications to inhibit all new shoot growth, and no treatment to allow unrestricted shoot growth.

Following the outcomes of the first two objectives, the third objective was to study the underlying physiological and molecular mechanism involved in bud dormancy and floral initiation

in response to the ethephon treatment. This included investigating the rate and duration of ethylene evolution after ethephon application, and studying various downstream processes following ethephon application, which included changes in leaf chlorophyll content index (as shoot maturity indicator), carbohydrate concentrations and gene expression levels of dormancy-, ethylene- and flowering-related genes in the leaves and apical buds of terminal shoots at various phenological stages between bud dormancy and floral initiation.

It is envisioned that this study will significantly increase the knowledge of flowering mechanisms of litchi when subjected to plant growth regulator manipulation and contribute to improved crop manipulation techniques to mitigate adverse climatic conditions that influence litchi flowering. Increasing the productivity and profitability of litchi production will be key to expanding litchi production in South Africa and increasing its footprint on international markets.

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Paper 1: Environmental and endogenous regulation of floral development in litchi (*Litchi chinensis* Sonn.)

Keywords. Carbohydrate accumulation, crop manipulation techniques, flower bud formation, inductive temperatures, plant hormones, regulatory networks, shoot maturity

Abstract. **Litchi (*Litchi chinensis* Sonn.) is an exotic fruit that has become very popular on international markets. However, erratic flowering is a major challenge and one of the main factors leading to poor productivity. Intricate and temperature-specific flowering processes contribute to the low adaptability of litchi to a wider range of climates and create a challenge for effective orchard management and expansion of production despite high consumer demand. Moreover, warmer winters due to climatic changes in the past few decades have increased the incidence of vegetative shoot growth during late autumn and early winter, and this has interfered with floral initiation and development, further exacerbating flowering irregularity in litchi. Hence, most studies focused on investigating environmental and endogenous factors affecting floral formation in litchi and the underlying physiological, biochemical and molecular processes. Molecular studies, particularly RNA-sequencing based on transcriptome analysis and gene expression profiling, have brought a broader understanding of a number of mechanisms and pathways involved in litchi floral induction and initiation. While great progress has been made in many areas, certain questions remain unanswered, particularly the role of carbohydrates, plant hormones and certain biochemical constituents in floral bud development, as well as how to mitigate insufficient chilling during warm winters. Therefore, the aim of this review is to provide an overview on floral transition and development in litchi, including the current knowledge on hormonal and molecular regulation. Furthermore, the review elaborates on current horticultural practices and discusses their contribution, limitations and relevance to solving challenges in floral formation of litchi. Special reference is given to the role of ethephon and ethylene in floral induction. Potential new approaches to address erratic flowering, particularly under less inductive conditions, are suggested.**

A. Introduction

Litchi (*Litchi chinensis* Sonn.) is an evergreen subtropical tree crop native to southern China and northern Vietnam. It has a long history of cultivation and is of high economic importance in several Southeast Asian countries, such as China, India, Vietnam and Thailand (Huang et al., 2005;

Maity and Mitra, 1990; Menzel, 2002). Litchi is best adapted to warm subtropical regions with hot, humid summers and cool, dry winters (Table 1) (Carr and Menzel, 2014; Huang et al., 2005; Menzel and Simpson, 1994). In the last century, litchi has also become an important tree crop in other countries with subtropical climates, with significant plantings in Madagascar, Mexico, South Africa and Australia, due to the increased popularity of litchi on new international markets (Huang et al., 2005). Nevertheless, litchi is still considered a minor crop worldwide due to low, irregular flowering and fruiting, which can be mainly attributed to its low climatic adaptability (Menzel, 1983; Menzel and Simpson, 1994). Furthermore, the frequent occurrence of warm winters in the past decades poses an additional challenge for regular flowering and production of litchi (Nghi et al., 2020; Wang et al., 2017).

Table 1. Ideal climatic conditions for various phenological phases in litchi.

Growth stage	Optimal climatic conditions
Vegetative growth	25-30°C, high water supply and humidity
Dormancy	< 20°C, low water supply
Flowering	Flower induction: < 15°C, low water supply Floral development: > 15°C, increasing water supply
Pollination and fruit set	23-28°C, high water supply, humidity > 50%
Fruit development	> 25°C, high water supply and humidity

Data Source: Batten and Lahav, 1994; Batten and McConchie, 1995; Chen and Huang, 2005.

In litchi, one of the growth stages most influenced by unfavorable weather conditions is the reproductive stage, in particular floral bud formation (Batten and Lahav, 1994; Chen and Huang, 2005; Menzel and Simpson, 1988). In the past, there have been some controversies on the exact mechanisms involved in litchi floral formation due to fragmentary knowledge relating to floral induction and initiation, as well as the factors that promote or impair floral bud differentiation (Huang and Chen, 2005). Hence, many research studies focused on elucidating the factors involved in the failure to flower, e.g. temperature, soil moisture and shoot maturity. Numerous studies also investigated the morphological and physiological mechanisms influencing floral induction and flower bud development, as well as the effect of horticultural manipulation techniques, e.g. water stress, girdling and the use of plant growth regulators (PGRs) as mitigation strategies (Davenport and Stern, 2005; Stern and Gazit, 2003). In recent years, more emphasis has been placed on studying the molecular mechanisms and pathways involved in litchi flowering using RNA sequencing, transcriptome profiling and gene expression analysis under different experimental conditions (Lu

et al., 2017, 2020; Shen et al., 2016; Xiao et al, 2018; Zhang et al., 2014, 2017). In light of the increasing influence of adverse weather conditions on reproductive growth of litchi, gaining deeper insight into the physiological, hormonal, molecular and genetic mechanisms underlying normal and abnormal flowering processes is essential for the development of new strategies, or modification of existing orchard practices, that will allow for mitigation of adverse climatic conditions.

Therefore, this review presents the current knowledge of the reproductive growth stages in litchi with emphasis on floral bud development. It discusses the morphological, physiological, hormonal and molecular regulatory mechanisms involved in litchi flower induction and initiation as well as endogenous and exogenous factors influencing these processes as far as current knowledge permits. Furthermore, studies on manipulation techniques to improve flowering in litchi are critically reviewed for their relevance and practicability in current farm management. New approaches to mitigate the effects of climate change will be elaborated upon and recommendations for further research are suggested.

B. Floral development in litchi

1. Development of bearing shoots in litchi

As a terminal flowering species, litchi develops inflorescences, also commonly described as panicles (Robbertse et al., 1995), on the apex of the most recent, mature terminal shoot formed after harvest (Huang and Chen, 2014). Therefore, the postharvest vegetative shoot growth period is the most important time to foster strong bearing branches for the next season's floral development and fruiting, since high quality bearing shoots are the basis for a productive tree (Huang and Chen, 2014). Strong, productive bearing branches are characterized by thick stems with abundant healthy leaves, shoot maturation at the most favorable time prior to floral induction and high carbohydrate accumulation prior to flowering (Chang and Lin, 2008; Hieke et al., 2002a, 2002b; Huang and Chen, 2014).

Litchi trees produce a number of growth flushes per year that can be either vegetative (summer) or floral (winter) (Fig. 1). Vegetative shoot growth generally occurs after harvest or during the growing season when flowering or fruit set have failed due to unfavorable weather conditions (Wei et al., 2013). These flush cycles follow one another more rapidly at higher temperatures (25-30°C) than at lower temperatures (15-20°C) (Subhadrabandhu and Stern, 2005). The intervals between the start of successive flushes can be as short as five weeks. Under warm climatic conditions, flush growth frequency can be encouraged by regular irrigation and fertilization (Huang and Chen, 2014; Subhadrabandhu and Stern, 2005; Wei et al., 2013). The vegetative growth phases are interrupted by quiescent periods during which shoot elongation stops, the meristem of terminal buds enters

dormancy and the expanded leaves darken and thicken. New bud break occurs spontaneously when the leaves have fully matured or have been removed (Fu et al., 2014; Zhang et al., 2016).

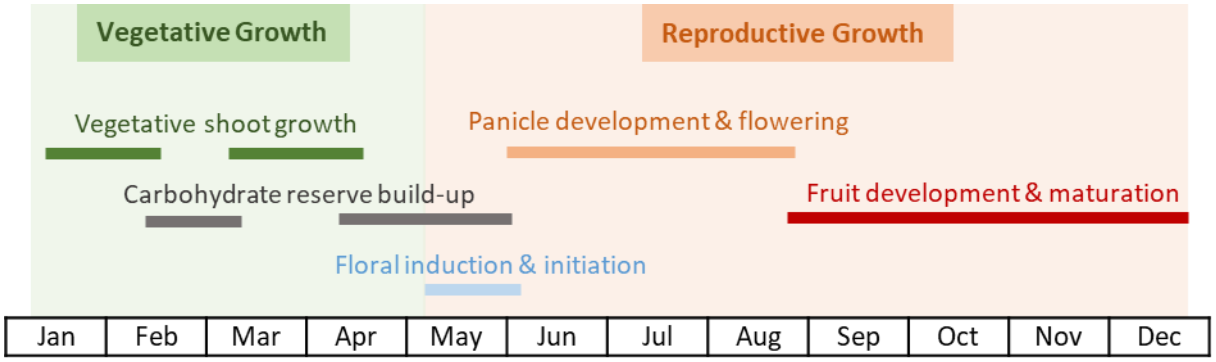

Fig. 1. Vegetative and reproductive growth cycles of litchi trees grown in the Southern Hemisphere, including periods of carbohydrate reserve build-up and floral induction/initiation based on Cronje and Mostert (2009).

The vigor of postharvest flushes in litchi is linked to the previous season's crop load, severity of pruning, as well as water and nutrient supply (Huang and Chen, 2014; Nath et al., 2014). High crop load causes a higher depletion of tree reserves, which leads to weaker and delayed postharvest shoot growth (Cronje and Mostert, 2009; Stern and Gazit, 2003; Yuan et al., 2009). Similar responses were found in citrus (Martínez-Alcántara et al., 2015; Martínez-Fuentes et al., 2010; Stander et al., 2018; Verreynne and Lovatt, 2009), mango (Normand et al., 2018) and loquat (Reig et al., 2014), although the vegetative shoot growth cycles in relation to floral formation in these crops differ distinctly from litchi. In addition, the presence of fruit exerts an apical dominance-type paradormancy, which suppresses bud break and delays vegetative flush growth after harvest (Huang and Chen, 2014; Lang et al., 1987; Reig et al., 2014). Pruning may advance postharvest shoot growth in litchi by removing the inhibiting substances produced by the fruit, thus releasing bud growth. In general, a heavier pruning will lead to a stronger vigor of the new growth according to the response of compensatory growth (Bevington, 1980; Cronje et al., 2010). However, heavy pruning also removes a considerable amount of tree resources and functional leaves, which may affect flowering and fruiting, if not replaced by an adequate amount of nutrient input (Hieke et al., 2002a; Huang and Chen, 2014). Nevertheless, pruning improves light exposure in the canopy and thus enhances photosynthetic capacity and net CO_2 assimilation in the leaves (Hieke et al., 2002d; Menzel and Simpson, 1994). In particular, the leaves of the latest flush exhibit significantly higher photosynthetic capacity and net CO_2 assimilation compared with previous flushes, and therefore, are the main carbohydrate contributors for flowering and fruiting (Hieke et al., 2002a, 2002b; Roe et al., 1997).

The development of strong shoots with ample functional leaves demands high water and nutrient supply to support both the growth processes as well as allow for efficient stomatal conductance and photosynthesis. Optimum water supply is necessary to maintain high stomatal conductance under elevated temperatures, whereas drought or water stress restricts vegetative growth and reduces stomatal conductance and therefore also net CO_2 assimilation (Menzel and Simpson, 1994; Menzel, 2005a). Nutrients, such as nitrogen (N), magnesium (Mg), potassium (K), phosphorus (P), sulphur (S), molybdenum (Mo), zinc (Zn) and boron (B) are essential for optimum growth and metabolic functionality of postharvest shoots in litchi (Menzel, 2005b), but are also required in sufficient amounts for future translocation to flower panicles, as nutrient absorption between maturation of the last vegetative flush and flowering is generally limited (Yao et al., 2020). In the annual growth cycle, leaf N, P and K nutrient levels of terminal shoots are generally lowest around harvest, but reach their highest levels prior to floral initiation, whereafter these levels decrease again during inflorescence and fruit development (Cronje et al., 2021b; Fan et al., 2005; Mandal et al., 2014; Yao et al., 2020). The development of inflorescences, in particular, requires high amounts of P and K (Fan et al., 2005).

Leaf nitrogen levels during the winter dormancy period were also positively correlated with shoot starch reserves at floral induction and fruit set where nitrogen was applied prior to each postharvest leaf flush (Cronje et al., 2017a, 2021). In China, fostering strong bearing branches by applying fertilizer to support the growth of each postharvest flush has long been considered good agricultural practice (Huang and Chen, 2014). Studies showed that the bearing potential of a terminal shoot was positively correlated with shoot thickness and high carbohydrate reserve levels (Chang and Lin, 2008; Huang and Chen, 2014; Pan et al., 2014; Zhang et al., 1997). Hence, to support the growth of bearing branches as well as future floral development, fertilizers can be applied during or after harvest, and prior to the growth of each postharvest flush, while organic fertilizer can be applied before harvest (Cronje et al., 2021; Huang and Chen, 2014).

Since flowering and fruiting are carbon-costly developmental processes for the plant, sufficient carbohydrate accumulation prior to flowering is an important strategy of fruit trees to sustain reproductive growth (Huang and Chen, 2014). High carbohydrate reserves in litchi trees, and particularly terminal shoots, prior to flowering were associated with higher flowering rates (Cronje and Mostert, 2009; Menzel et al., 1995; Yang et al., 2014). Carbohydrate accumulation to support flowering processes primarily takes place during the pre-flowering dormancy period following the maturation of the last postharvest vegetative flush, a time when carbohydrate demand is generally low (Cronje and Mostert, 2009; Yuan et al., 2009) (Fig. 1). Inhibiting new shoot growth during this period by use of various horticultural techniques can promote carbohydrate accumulation and is discussed further under the section “Horticultural practices to promote floral formation in litchi”.

2. Floral induction, floral initiation, and inflorescence development in litchi

Litchi trees pass through several stages of reproductive development, which last about six to eight months. The reproductive phase starts with floral bud development, which includes floral induction, floral initiation and panicle differentiation. This is followed by panicle elongation and the flowering stage, comprising anthesis, anther dehiscence and pollination, leading to fruit set and development, and finally ending in fruit maturity (Menzel, 1984). The inflorescence development and the flowering stage encompasses the period between floral induction and pollination, which take place during winter and spring, respectively. For countries in the Northern Hemisphere, this period occurs between December and March and in the Southern Hemisphere between May and August. The duration of each phenological stage can last between two to eight weeks, depending on cultivar and environmental conditions (Menzel, 1984; Singh et al., 2012a; Stern and Gazit, 2003). Wei et al. (2013) developed an extended *Biologische Bundesanstalt, Bundessortenanstalt und Chemische Industrie* (BBCH)-scale for all major growth stages in litchi, and therein provided a detailed and graphic description of inflorescence development, which can be used to define floral development and flowering for different cultivars.

Similar to mango, litchi inflorescences are generally produced terminally, but can also develop from axillary buds under certain conditions, e.g., after removal of the apical bud (Davenport and Núñez-Eliséa, 1997; Stern and Gazit, 2003). Both terminal and axillary buds in litchi can be chill-induced to form flower panicles (Huang and Chen, 2005). However, terminal flower panicles are generally stronger and more productive compared with panicles developing from axillary buds (Singh et al., 2012a). Floral bud development occurs in mature shoots under low inductive temperatures and can be divided into floral induction and floral initiation (Fig. 2). Floral induction involves physiological and biochemical changes that take place in the quiescent apical meristem, whereas floral initiation is characterized by morphological differentiation of the actively growing shoot apex, making it highly responsive to chilling temperatures (Huang and Chen, 2005). The floral initiation stage is defined as the stage when the inflorescence primordium becomes visible in the form of little white ‘granules’, also commonly known as the “white millet” stage (Huang and Chen, 2005; Wei et al., 2013).

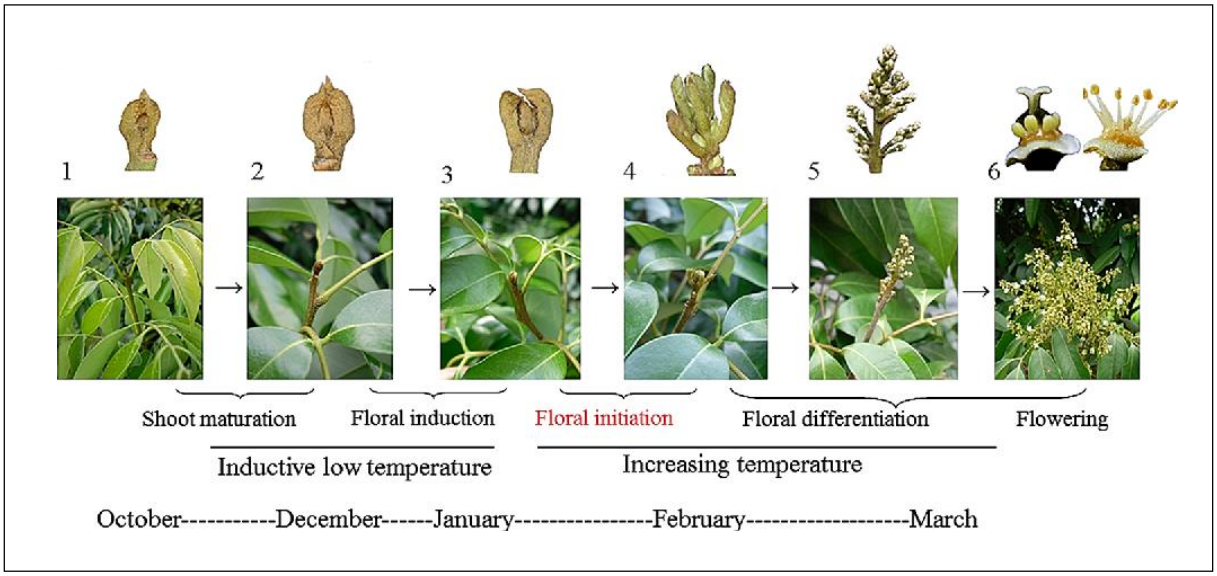

Fig. 2. Developmental stages in inflorescence development of litchi, adapted for 'Feizixiao' litchi (Northern Hemisphere) Phenological stages are numbered based on the BBCH-scale for litchi (Wei et al. 2013). Reprinted from Zhang et al. (2014).

At the floral initiation stage, litchi floral buds are mixed, consisting of panicle and leaf primordia as well as rudimentary leaves (Zhou et al., 2008; Zhou et al., 2010). The fate of the mixed bud is largely dependent on the temperature prevailing during the floral initiation stage and can give rise to three different types of floral shoots, viz., purely floral (generative; Fig. 3A), mixed (leafy; Fig. 3B) or transitional (vegetative/floral; Fig. 3C) shoots (Davenport and Stern, 2005). When floral buds are exposed to floral-inducing chilling temperatures for a lengthy period, generally below a day maximum of 20°C and night minimum of 15°C, the rudimentary leaves will abscise and axillary panicle primordia will develop, leading to a pure floral, richly branched inflorescence without leaves (Batten and McConchie, 1995; Menzel and Simpson, 1995) (Table 1). In contrast, mixed shoots form when exposed for a few hours per day to elevated temperatures, which are, however, lower than those required for purely vegetative shoots (Davenport and Stern, 2005; Menzel and Simpson, 1995). Mixed shoots arise from both leaf primordia and lateral meristems, resulting in the so-called leafy inflorescences or panicles. Leafy inflorescences have both leaves and lateral inflorescences, all developing at the same node along the main inflorescence axis (Batten and McConchie, 1995; Davenport and Stern, 2005; McConchie and Batten, 1991; Olesen et al., 2002; Zhou et al., 2008, 2010). Generally, leafy inflorescences are weaker and less fruitful than pure panicles (Huang and Chen, 2014; Lee and Chang, 2019). Transitional shoots may develop when rapidly changing temperatures prevail during shoot emergence, and generally start as vegetative shoots during warm temperatures, but then transition to inflorescences at the distal end, when temperatures drop (Davenport and Stern, 2005). However, when temperatures during the floral

initiation stage rise far above inductive temperatures, the leaf primordia and rudimentary leaves will fully develop and the floral organs atrophy, producing a purely vegetative shoot (Huang and Chen, 2005; Zhou et al., 2008).

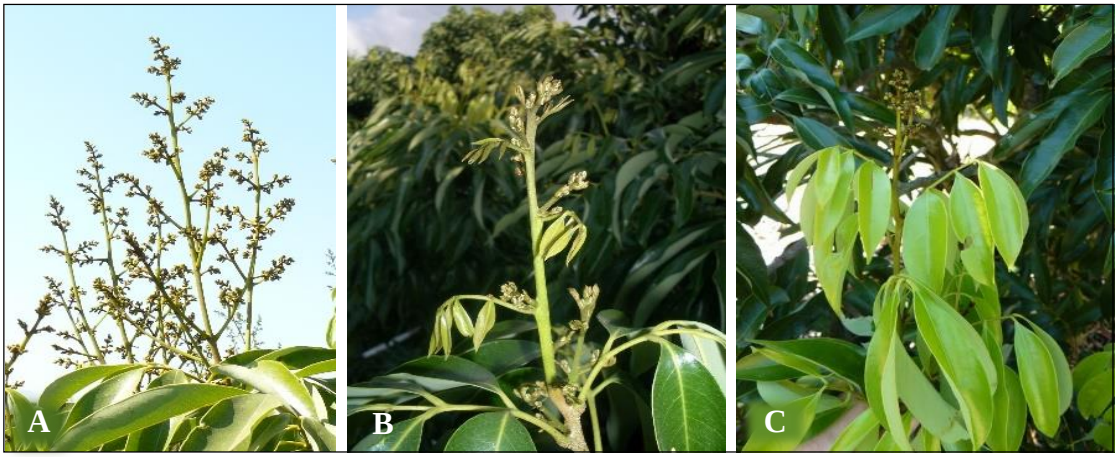

Fig. 3. The three types of litchi floral shoots: A) purely floral, B) mixed or 'leafy', and C) transitional (vegetative to floral).

After successful floral initiation, panicle and flower development continues, and inflorescences elongate. Panicles develop faster at higher temperatures, and can grow up to 35 cm in length and 30 cm in width (Chen et al., 2013; Davenport and Stern, 2005; Wei et al., 2013). The litchi inflorescence is composed of a cluster of panicles (dichasia) and is defined as a determinate, compound thyse, because the side branches are cymose (Robbertse et al., 1995). Each dichasium produces tens to hundreds of functional male (M1 and M2, according to the order of their opening) and functional female (F) flowers. The male 1 (M1) flowers have 6 to 8 stamens, a rudimentary pistil and an ovary with partially formed ovules, but with no embryo sac (Fig. 4A). The female (F) flowers have a fully functional two-lobed ovary with a single style and bifurcated stigma, which is receptive for several days, and short stamens that do not dehisce (Fig. 4B). The male 2 (M2) flowers have six to eight functional stamens, a prominent, but non-functional pistil and a rudimentary ovary (Fig. 4C). The differentiation of individual flowers takes place in an acropetal manner with first the calyx, then the stamens and finally the carpels (Davenport and Stern, 2005). The sex ratio varies greatly with cultivar and environmental conditions. Lower temperatures during inflorescence development promote the formation of female flowers (Davenport and Stern, 2005). Likewise, long or leafy inflorescences were found to have a higher proportion of male flowers, while short or leafless inflorescences showed a higher female-to-male flower ratio (Huang and Chen, 2014; Lee and Chang, 2019).

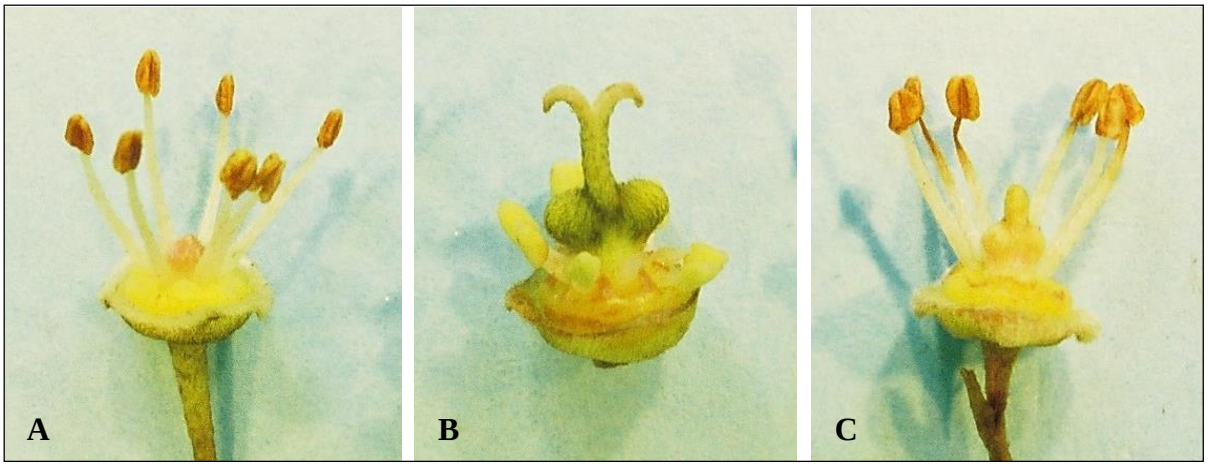

Fig. 4. The three litchi flower types in sequential order of opening: A) male 1 (M1), B) female, C) male 2 (M2). Adapted from Cronje (2010).

Although individual flowers are self-sterile, because pistil and stamens are not functional in the same flower, litchi is self-compatible, due to an overlap between flower opening of the same and different inflorescences within a tree or of trees within an orchard (Davenport and Stern, 2005; Stern and Gazit, 2003). Flower opening of each flower type lasts for about seven to twelve days, with an overlap of one to three days between flower types, promoting cross-pollination (Davenport and Stern, 2005; Menzel, 1984; Singh et al., 2012a). Fertilization takes place two to three days after pollination, followed by the division of the nucleus of the primary endosperm (Stern et al., 1997). After fertilization, usually only one of the two ovules in the two-lobed ovary develops to form a single seeded fruit. The second lobe remains attached, but reduces to a tiny appendix. In rare cases, both ovules develop into fully sized fruit, which are borne on one pedicel (Davenport and Stern, 2005; Stern and Gazit, 2003).

3. Environmental and endogenous factors governing floral induction and initiation in litchi

3.1 Pathways involved in floral induction of horticultural crops

Floral induction and initiation are regulated by a range of factors, such as environmental stimuli and endogenous signals, but also interactions between them. In herbaceous plants, flowering is controlled by four major pathways, which include the photoperiod and vernalization pathways, both of which respond to environmental cues, as well as the gibberellin and autonomous pathways, which are endogenous (Cho et al., 2017; Wilke et al., 2008). In most horticultural tree crops, however, flowering is initiated mainly in response to environmental stimuli, such as chilling temperatures and drought, or autonomously (Albrigo and Galán Saúco, 2004; Cho et al., 2017; Wilke et al., 2008). Recently, a newly described endogenous pathway, involving microRNA156 and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factors, has been added to the list of

pathways that regulate and control flowering-related processes, such as the induction of floral competence, flowering timing and the development of floral organs (Galvão and Schmid, 2014; Waheed and Zeng, 2020).

Among horticultural tree crops, temperate and subtropical tree crops differ in two major areas concerning floral initiation. Firstly, floral initiation in temperate crops is mostly autonomous, while in subtropical crops floral initiation is in response to environmental stimuli, such as exposure to low temperature. Secondly, in temperate tree crops, the period between floral initiation and anthesis is separated by a winter dormancy period, while in subtropical crops, floral initiation leads uninterrupted to anthesis, and in most cases is preceded by a dormancy period (Wilke et al., 2008). In both cases, the dormancy period plays an important role for successful floral initiation.

3.2 Prerequisites for floral induction and initiation in litchi

Floral bud formation is the most critical time in litchi floral development. Due to the complex differentiation processes associated with litchi floral bud formation, specific environmental and endogenous factors are required for successful floral induction and initiation. Similar to some other subtropical crops, for example mango, avocado and macadamia (Chaikiattiyos et al., 1994; Olesen et al., 2013), low temperatures and shoot maturity are undoubtedly prerequisites for effective flower induction and initiation in litchi (Menzel, 1983; Huang and Chen, 2005; O'Hare, 2002). Furthermore, high carbohydrate reserves in leaves and terminal shoots prior to floral induction are considered important for successful flowering, but whether they play an inductive role or merely assist the growth processes after floral induction, such as bud break and panicle development, still needs further investigation (Huang and Chen, 2014; Yang et al., 2014). Low soil moisture status was reported to assist with shoot maturation and growth check, but has not been regarded as essential for floral induction and initiation (Davenport and Stern, 2005; Huang and Chen, 2005; Huang and Chen, 2014; Stern and Gazit, 2003) and therefore, can only be considered as having a "modulating" effect (Bangerth, 2006). Nakata and Watanabe (1966) classified litchi as a day-neutral plant, because daylength did not influence floral induction and initiation. However, gene expression profiling during bud development, under non-inductive and inductive conditions, revealed upregulation of genes in the circadian rhythm pathway and of the floral promoter *CONSTANS* (*LcCO*), respectively, suggesting that certain processes in dormant and breaking buds might be subject to circadian control (Yang et al., 2014; Zhang et al., 2016).

3.2.1 Temperature as an environmental stimulus

Litchi floral induction and initiation depends mainly on low temperatures (Nataka and Watanabe, 1966). Groff (1921, 1943) and Nakata and Watanabe (1966) were some of the first

researchers that emphasized the importance of cool winters for adequate litchi flowering. Later studies confirmed the low temperature requirement for successful flowering in litchi and further investigated the specific maximum and minimum temperature requirements for different cultivars under controlled temperature, with temperature regimes ranging from maxima/minima of 15°/5°C to 30°/25°C (Joubert, 1986; Menzel and Simpson, 1988, 1991, 1994, 1995; O'Hare, 2002; Subhadrabandhu, 1990; Subhadrabandhu and Yapwattanaphun, 2001). In general, successful floral induction and initiation in litchi requires exposure to minimum air temperatures below 15°C and average air temperatures below 20°C for several weeks prior to and during panicle emergence (Table 1). However, high temperatures (> 25°C) have always been associated with vegetative shoot growth (Batten and McConchie, 1995; Huang and Chen, 2005; Menzel and Simpson, 1988; O'Hare, 2002). Subtropical litchi cultivars generally have a higher cold requirement compared with litchi cultivars growing under low-elevation tropical conditions (Sukhvibul et al., 2014). Even within subtropical cultivars there are significant differences in their cold requirements (Huang and Chen, 2005; Mitra and Irenus, 2018; Stern and Gazit, 2003). The vigor of a cultivar also appears to affect flowering, with low-vigor cultivars generally flowering more profusely than high-vigor cultivars (Menzel and Simpson, 1988).

The temperature regime during floral initiation determines the type of inflorescence that will develop, as already alluded to under the section "Floral induction, floral initiation, and inflorescence development". Strong inductive temperatures during floral bud differentiation promote the development of leafless panicles with high numbers of female flowers, as well as a high percentage of axillary panicles (Menzel and Simpson, 1988, 1991, 1995; O'Hare, 2002). However, exposure to less inductive temperatures during floral initiation will cause the rudimentary leaves and leaf primordia to develop and flowers to atrophy, giving rise to leafy panicles, or even leaf flushes under non-inductive temperatures (Huang and Chen, 2005; Zhou et al., 2008; Zhou et al., 2010). Menzel and Simpson (1988) further found that panicles emerged earlier at a maximum/minimum temperature regime of 20°/15°C than at 15°/10°C, but required a longer time to reach anthesis. Moreover, root temperature was shown to influence floral induction. High root temperatures (> 20°C) reduced and prevented floral initiation in 'Tai So' litchi even when the shoots were exposed to inductive air temperatures, whereas low root temperatures (< 15°C) were able to induce flowers at partial inductive air temperatures (~ 20°C), but not at non-inductive (> 20°C) temperatures (Menzel et al., 1989; O'Hare, 2004).

To account for the differences in temperature requirements for the various floral development stages in litchi, Chen et al. (2016) developed specific temperature models for floral induction, inflorescence length and anthesis, for the cultivar 'Yu Her Pau' by calculating the optimum chilling or thermal degree hours for each stage. These models established that floral induction and

inflorescence length were positively correlated with chilling degree hour accumulation, while the time to anthesis was associated with accumulation of thermal degree hours.

3.2.2 Shoot maturity and bud dormancy as endogenous signals

Various studies on temperate and subtropical tree crops suggest that there is an interaction as well as a competition between vegetative and reproductive growth, where the presence, time of appearance and certain morphological characteristics of either affect the development of the other (Gaaliche et al., 2011; Huang and Chen, 2014; Martínez-Fuentes et al., 2010; Normand et al., 2016, 2018; Reig et al., 2014; Stander et al., 2018; Stern and Gazit, 2003). While most temperate crops have a distinct growth cessation period in autumn and a dormancy period in winter, many subtropical crops display a recurrent growth pattern throughout the entire year at a much higher frequency, with only short rest phases in-between (Fu et al., 2014).

In litchi, the cambium in the terminal shoots remains active during these rest phases, and dormancy is only limited to the terminal bud meristem (Fu et al., 2014). According to the classifications given by Lang et al. (1987), litchi bud dormancy can be characterized as endodormancy, as it is regulated by physiological factors inside the apical bud (Zhang et al., 2016). However, unlike the release of bud dormancy in temperate tree crops, which is primarily induced or released by environmental cues, such as the accumulation of sufficient chilling temperatures, the release of bud dormancy in litchi occurs spontaneously when the leaves of the terminal shoot have fully matured. Therefore, endodormancy in litchi is a purely endogenous process and limited to the apical bud (Fu et al., 2014; Zhang et al., 2016). Furthermore, the release of bud dormancy in litchi is not associated with flower development as is the case with most temperate crops. Only when the breaking buds are exposed to floral-inducing chilling temperatures, can floral development take place (Batten and McConchie, 1995; Chen and Huang, 2005).

Where minimum temperatures are not achieved to allow for floral induction by the time apical buds are ready to grow, adequate horticultural measures may be necessary to extend bud dormancy. Conversely, low inductive temperatures do not always lead to successful flowering in subtropical crops like litchi and mango if the terminal shoots have not fully matured (Davenport and Stern, 2005; Wilke et al., 2008). Since the breaking buds in litchi are most responsive to low inductive temperatures, there is only a narrow time window for floral induction and initiation to occur (Batten and McConchie, 1995; Huang and Chen, 2005; Zhang et al., 2016). The high reliance of floral induction and initiation on the correct timing for shoot maturity, bud break and low inductive temperatures, make litchi production extremely vulnerable to flower and crop failure in the presence of unfavorable weather conditions, such as warm winters. In this regard, timely cessation of shoot

growth with its associated shoot maturation processes are critical for chilling responsiveness and successful floral induction in litchi (Huang and Chen, 2005; Wilke et al., 2008; Zhang et al., 2016).

Some of the earliest studies on the litchi phenological cycle recognized the importance of shoot age and a dormancy period prior to panicle emergence for good panicle development (Nakata, 1950; Shigeura, 1948; Zheng et al., 2001). In contrast, developing vegetative shoots just prior to floral induction had a negative effect on floral bud development (Lynch, 1958; Nakata, 1955). Negative correlations between vegetative shoot growth, four to six weeks prior to panicle emergence, and litchi flowering were also reported in an Australian study (Menzel and Simpson, 1992). Similarly, poor flowering and yields due to late-season flushing in various litchi cultivars in India (Pereira et al., 2005), China (Pan et al., 2014) and South Africa (Oosthuyse, 2014) were attributed to the immaturity of these shoots, as they prevented floral bud development.

Leaf greenness is a good indicator of shoot maturity in litchi and can easily be determined using hand-held chlorophyll meters, such as a SPAD (Special Products Analysis Division) or CCI (Chlorophyll Content Index) meters. By determining the full maturity point, using the growing degree days with 10°C as the basal growth temperature in litchi, shoot growth can be calculated for different climatic regions or cultivars, and the number of flush cycles can be predicted (Fu et al., 2014; Huang and Chen, 2014). Based on this knowledge, the timing of shoot growth and the duration of bud dormancy can be manipulated by the application of appropriate horticultural practices during late autumn and early winter, if necessary.

The development of late-appearing shoots during autumn and early winter can be caused by a heavy crop in the preceding fruiting season (Stern and Gazit, 2003), as well as ill-timed pruning (Oosthuyse, 2014), both of which tend to delay the postharvest flushing cycle. Furthermore, the occurrence of late autumn vegetative shoot growth is associated with warm and humid weather conditions, while cool and dry autumns and winters naturally reduce the vigor of trees (Crane, 2004; Huang and Chen, 2014). Therefore, terminal shoots at different developmental stages are commonly found in litchi prior to panicle emergence, causing variations in the flowering ability of shoots, in a leaf maturity-dependent manner (Xiao et al., 2018). Detailed studies conducted on various shoot maturity stages, at different temperature regimes, clearly showed that the combination of high shoot maturity and low inductive temperatures produced the strongest response for floral induction and initiation (Batten and McConchie, 1995; Menzel, 1983; Menzel and Simpson, 1988; O'Hare, 2002). However, exposure of young immature shoots to low inductive temperatures resulted in little or no flowers. Such shoots only matured and started to grow again past the inductive period, giving rise to leaf flush instead of flowers (Batten and McConchie, 1995; Huang and Chen, 2005; Huang and Chen, 2014; O'Hare, 2002; Singh et al., 2017; Stern and Gazit, 2003).

The maturity status of terminal shoots also played a role in the type of shoots that emerged under less inductive temperatures, with mature shoots still leading to adequate panicle development, but shoots of intermediate shoot maturity only producing small, stunted panicles (O'Hare, 2002). In contrast, high temperatures (> 25°C) always resulted in vegetative shoot growth, irrespective of the shoot maturity status (O'Hare, 2002). The differences in response of shoots of different maturity stages to low temperatures are related to the expression levels of the floral promoter gene *FLOWERING LOCUS T*, as well as the carbohydrate levels in the leaves of terminal shoots (Yang et al., 2014), as will be further discussed in the section "Regulatory mechanisms and networks associated with floral development in litchi".

3.2.3 Carbohydrate status as an endogenous signal

The role of carbohydrate reserves for crop productivity has been studied in many subtropical crops, such as citrus (Garcia-Louis et al., 1995; Goldschmidt and Golomb, 1982; Goldschmidt, 1999), avocado (Scholefield et al., 1985; Whiley and Wolstenholme, 1990; Wolstenholme and Whiley, 1997), mango (Davie et al., 2000; Prasad et al., 2014) and papaya (Allan, 2001). In these crops, carbohydrate concentrations generally accumulated during the winter dormancy and pre-flowering period, with the highest concentration of carbohydrates being recorded prior to or during flower formation and flowering, followed by a decline with fruit set and development, indicating that the reproductive growth period has a higher carbohydrate demand than the vegetative growth period. While there is consensus that carbohydrates are an important energy resource for flowering and fruiting (Huang and Chen, 2014; Martinez-Fuentes et al., 2010; Ortiz-Marchena et al., 2014), the specific function of carbohydrates in floral induction and bud differentiation is not fully understood.

In litchi, similar phenology-dependent reserve carbohydrate dynamics were found as in the above-mentioned fruit tree crops. Carbohydrate concentrations in litchi typically accumulated during the winter dormancy period, but declined during flower development and fruit growth (Chen et al., 2004; Cronje and Mostert, 2009; Menzel et al., 1995; Yuan et al., 2009). However, while crop load-dependent carbohydrate levels affected floral induction and return bloom, for example, in citrus (Stander et al., 2018; Verreynne and Lovatt, 2009), pre-flowering carbohydrate reserve levels in litchi were not influenced by crop load, but rather depended on strong postharvest shoot growth (Huang and Chen, 2014; Yuan et al, 2009). Carbohydrate accumulation in litchi is closely linked to shoot maturity. Newly emerging shoots are initially consumers of assimilates. They only reach maximum CO_2 assimilation and are able to contribute to carbohydrate reserves upon reaching 50% of their maximum photosynthetic capacity, i.e., at sink-to-source transition, when the leaves have fully expanded and start to turn dark green in color (Fu et al., 2022; Hieke et al., 2002c; Menzel et

al., 1995). Recent studies confirmed the positive correlation between shoot maturity and carbohydrate levels in the leaves of terminal shoots, as well as with expression of flower promoting genes under inductive temperatures (Xiao et al., 2018; Yang et al., 2014; Zhang et al., 2017).

In litchi, already early on Nakata and Watanabe (1966) related floral initiation with the starch concentration of leaves and stems. Later on, several other studies correlated a high pre-flowering carbohydrate status with high flowering rates, suggesting their importance for litchi flowering and fruiting (Cronje and Mostert, 2009; Jiang et al., 2012; Yang et al., 2014; Yuan et al., 2009). The main reserve carbohydrate in litchi is starch (Huang and Chen, 2014; Menzel et al., 1995; Yang et al., 2014; Yuan et al., 2009). The highest concentrations of starch were found in small to medium branches (Chen et al., 2004; Hieke et al., 2002a; Menzel et al., 1995; Yuan et al., 2009), whereas soluble sugar concentrations were highest in the terminal shoots (Chen et al., 2004). While starch is the stored reserve energy for future metabolic activities, soluble sugars, such as sucrose, glucose and fructose are involved in the regulation of general growth, photosynthesis, carbohydrate partitioning, osmotic homeostasis, or act as signaling molecules during abiotic stresses and in the regulation of source-sink activities to name but a few (Sami et al., 2016).

It has been established for many herbaceous and tree crops that carbohydrate supply, particularly sucrose, plays an important role in dormancy release, bud break and sprouting (Barbier et al., 2015; Lebon et al., 2008; Marquat et al., 1999; Mason et al., 2014; Qin et al., 2012). For example, bud break in pear was associated with starch hydrolysis and a temporary increase in soluble sugars, followed by a rapid decline as bud growth proceeded (Hussain et al., 2016). In the same study, the application of the bud rest breaking agent, hydrogen cyanamide, accelerated bud break and increased the related changes in carbohydrates during this period compared with untreated shoots (Hussain et al., 2016). Some studies suggested that a minimum threshold level of carbohydrates is necessary for sufficient flower formation in subtropical crops like citrus (Garcia-Louis et al., 1995; Goldschmidt et al., 1985; Goldschmidt, 1999; Martínez-Fuentes et al., 2010; Stander et al., 2018), mango (Davenport and Núñez-Eliséa, 1997) and loquat (Reig et al., 2014). However, neither leaf carbohydrate content nor the accumulation of reserve carbohydrates appeared to have an inductive function or limit flower formation in these crops.

In litchi, Huang and Chen (2014) proposed that sufficient carbohydrate accumulation prior to flower induction might increase the responsiveness of the tree to low chill temperatures and thus improve flowering, which could be beneficial particularly under less inductive conditions. This is in contrast to temperate crops, where carbohydrate accumulation, in particular sucrose, prior to and during winter dormancy primarily serves the purpose of providing cold acclimation and frost protection, and is not necessarily associated with floral induction (Kwon et al., 2022; Ma et al., 2009; Van der Schoot and Rinne, 2011). Growing in warmer climates, litchi buds do not require

scales like those of temperate tree crops and are therefore naked with only rudimentary leaves enclosing the apical meristem (Zhang et al., 2016). As such, breaking litchi buds have very high respiration rates, even higher than during active growth, creating a high energy demand (Yang et al., 2014; Zhang et al., 2016). Since floral initiation in litchi is taking place in winter after a resting period during which photosynthetic activity is lower than during summer due to lower temperatures and cold- or drought-induced photoinhibition (Cronje and Mostert, 2009; Herppich, 2000; Menzel, 2005; Rosa et al., 2009), an accumulation of carbohydrates prior to floral development is crucial to support bud break, floral development and initial fruit growth until fruit growth can be supported from current photoassimilates once temperatures have increased again (Hieke et al., 2002c; Roe et al., 1997). Although it is clear from the above-mentioned studies that increased carbohydrate levels in terminal shoots and leaves of litchi are an important energy source for floral formation, still more scientific evidence is needed to determine the role of carbohydrate reserves in cold responsiveness and floral induction processes.

4. Regulatory mechanisms and networks associated with floral development in litchi

4.1 Molecular regulation associated with floral induction and initiation in litchi

In the past few decades, there has been a strong focus on utilizing molecular biology to elucidate the genetic networks that mediate the endogenous and environmental cues that are involved in litchi floral development. The transition from the vegetative phase to the reproductive phase has been extensively studied in the model plant *Arabidopsis thaliana* (Kim et al., 2009). This involves a complex regulatory network of flowering-promoting genes in the leaves and shoot apical meristems, such as *FLOWERING LOCUS T* (*FT*), *CONSTANS* (*CO*), S*UPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), *AGAMOUS-LIKE 24* (*AGL24*) *APETALA1* (*AP1*) and *LEAFY* (*LFY*), as well as flowering-repressor genes, such as *FLOWERING LOCUS C* (*FLC*), *TERMINAL FLOWER1* (*TFL1*), and *SHORT VEGETATIVE PHASE* (*SVP*) to prevent floral transition (Galvão and Schmid, 2014; Kim et al., 2009) (Fig. 5).

In *Arabidopsis*, prolonged exposure to low temperature (vernalization) activates the expression of *FLOWERING LOCUS T* (*FT*), which encodes the FT protein (florigen) that acts as mobile flowering signal (Corbesier et al., 2007). At the same time, floral repressors, such as the transcription factor *FLOWERING LOCUS C* (*FLC*), which also represses the expression of the floral promoter *SOC1* under non-inductive conditions, are suppressed. The expression of the *FT* gene is facilitated by *CO*, an important regulator in the photoperiodic flowering pathway (Tiwari et al., 2010). *CONSTANS* and other day length-dependent genes play an important complementary role in temperature signaling for floral initiation (Fernandez et al., 2016). The low temperature signal, which is generally perceived in the leaves, is transmitted via the FT protein, in the vascular

tissue to the shoot apical meristems through a series of signal transduction mechanisms (Galvão and Schmid, 2014). In the apical meristem, the FT protein then reacts with other floral integrators, such as *FLOWERING LOCUS D* (*FD*), to activate *SOC1* and the floral meristem-identity genes *SEPALATA* (*SEP*), *FRUITFUL* (*FUL*), *LEAFY* (*LFY*) and *AP1* to initiate floral transition and floral bud development (Galvão and Schmid, 2014; Kim et al., 2009; Lu et al., 2017; Nakagawa et al., 2012).

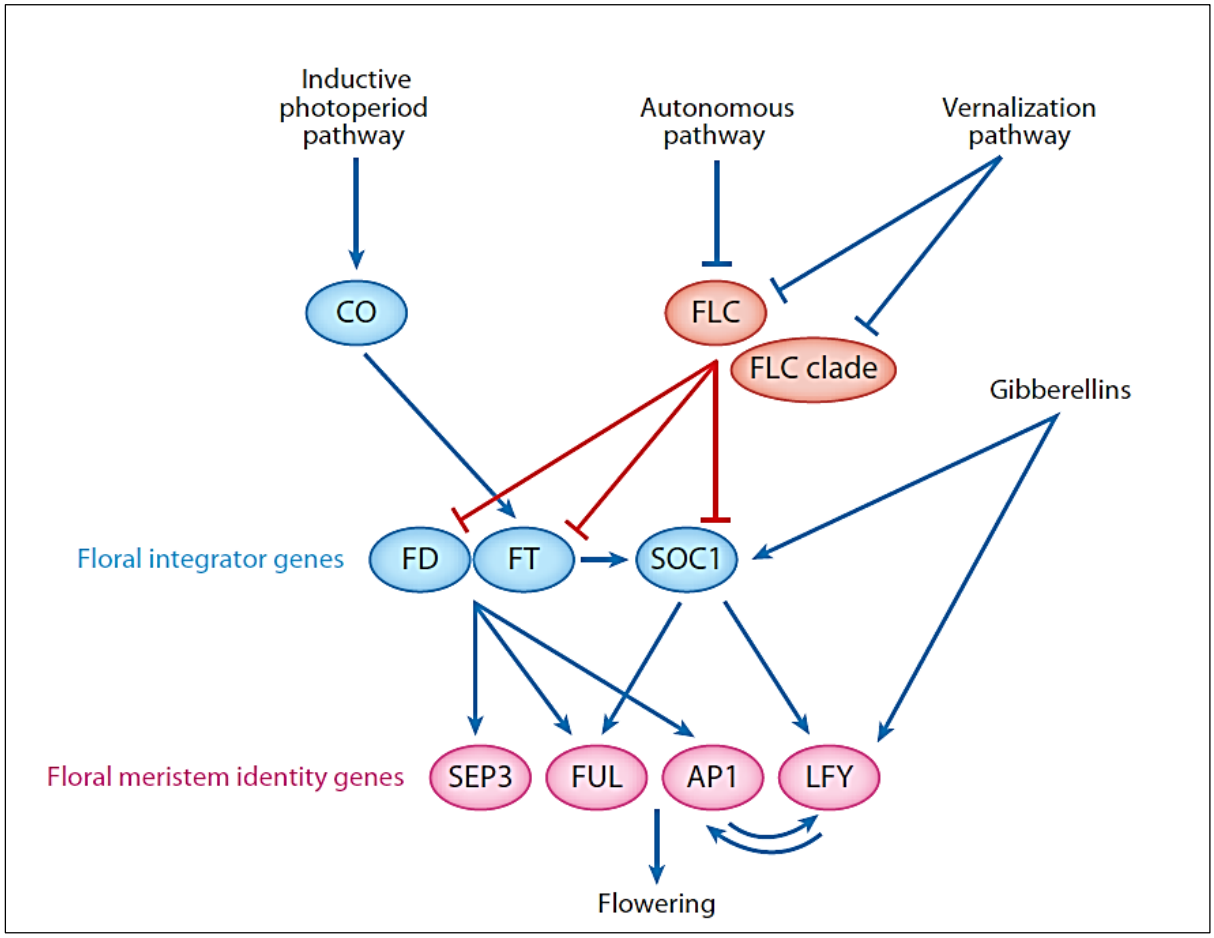

Fig. 5. Simplified diagram of flowering pathways in *Arabidopsis*. Pathways and genetic interactions that promote flowering are indicated in blue; those that repress flowering are indicated in red. *CO*: *CONSTANS*, *FLC*: *FLOWERING LOCUS C*, *FD*: *FLOWERING LOCUS D*, *FT*: *FLOWERING LOCUS T*, *SOC1*: *SUPPRESSOR OF CONSTANS 1*, *SEP*: *SEPALATA*, *FUL*: *FRUITFUL*, *AP1*: *APETALA 1*, *LFY*: *LEAFY*. Reprinted from Kim et al. (2009).

Insights into the genetic control of flowering in *Arabidopsis* laid a foundation for molecular studies in subtropical tree crops, such as citrus, avocado, mango, longan and litchi. Homologs for *FT*, *SOC1*, *CO*, *AG*, *AP1*, *LFY*, and *TFL1*, among others, were identified in these crops (Acosta-Rangel et al., 2021; Ding et al., 2015, 2018; Jia et al., 2014; Nishikawa, 2013; Sharma et al., 2019;

Zhang et al., 2014, 2016). In litchi, *de novo* RNA sequencing and gene expression analysis of dormant and emerging floral buds revealed that during floral initiation, i.e., in emerging buds, all key floral genes such as *LcSOC1*, *LcFT*, *LcLFY*, *LcAP1*, *LcAP2*, *LcAG*, *LcAGL*, and *LcSEP1* were upregulated, while *LcFLC*, *LcTL1* and *LcEMBRYONIC FLOWER2* (*LcEMF2*) were downregulated compared with dormant buds. Moreover, upregulation of genes related to auxin, cytokinin, jasmonic acid and salicylic acid biosynthesis and downregulation of genes related to abscisic acid biosynthesis corresponded with litchi floral initiation and confirmed findings of previous studies on hormonal regulation of litchi floral development (Zhang et al., 2014).

4.1.1 Molecular events associated with low temperature signaling and shoot maturity

As previously discussed, low temperature plays a crucial role in litchi floral induction and initiation. Molecular studies were able to confirm that low temperature is required for the induction of *LcFT* gene expression and subsequent floral development (Lin et al., 2020; Shen et al., 2016; Xiao et al., 2018; Zhang et al., 2014, 2017). Ding et al. (2015) found that of the two *FT* homolog genes identified in litchi, *LcFT1* and *LcFT2*, only *LcFT1* could be consistently induced by low temperature, while the *LcFT2* homolog was associated with leaf maturity- and reactive oxygen species-promoted flowering in combination with inductive conditions (Lu et al., 2020; Yang et al., 2014). Ding et al. (2015) further suggested that differences in *LcFT1* promoter sequences may be responsible for variation in flowering timing, as well as for different low temperature requirements for floral induction between early-and late-flowering litchi cultivars. In addition, drought or reactive oxygen species-generating treatments can cause an integrative effect together with low temperature and enhance the expression of flower-promoting genes, while suppressing flower-repressing genes (Lu et al., 2017, 2020; Shen et al., 2016).

Similar to other plants, the low temperature stimulus in litchi is perceived in the mature leaves and is transmitted as FT protein in the vascular tissue to the shoot apical meristems through a series of signal transduction mechanisms and complex regulatory networks (Ding et al., 2015; Galvão and Schmidt, 2014; Yang et al., 2014; Zhang et al., 2017). The importance of the presence of leaves as the site of FT protein production was emphasized by Ying and Davenport (2004), who showed that defoliation of terminal shoots of ‘Brewster’ and ‘Mauritius’ litchi could not induce floral development despite low inductive temperatures, whereas all untreated shoots with all leaves remaining, produced flowers. Similar results were found in longan and mango (Fernando et al., 2010; Huang et al., 2021). Further studies on litchi under low temperature conditions confirmed that the *LcFT* gene is expressed significantly higher in leaves than in terminal buds (Ding et al., 2015; Zhang et al., 2017). In addition, the maturity status of the leaves determined the chilling responsiveness and the level of *LcFT* expression, with mature dark green leaves having significantly

higher *LcFT* expression levels compared with immature yellow-red to light-green leaves (Xiao et al., 2018; Yang et al., 2014). Mature leaves were also shown to be essential for the success of potassium chlorate applications to induce off season floral induction in longan (Potchanasin et al., 2009a).

4.1.2 Molecular events associated with bud dormancy and carbohydrate accumulation

Due to the recurrent nature of shoot growth in litchi, a dormancy period prior to floral development is imperative to ensure that the apical buds can respond to the low temperature stimulus, when temperatures have become inductive (Batten and McConchie, 1995; Huang and Chen, 2005; Zhang et al., 2016). In herbaceous and deciduous crops, dormancy was found to be regulated by members of the *DORMANCY ASSOCIATED MADS-BOX* (*DAM*) gene family, such as *SHORT VEGETATIVE PHASE* (*SVP*) genes and *AGAMOUS-LIKE 24* (AGL24) (Horvath, 2015; Liu et al., 2018; Zhang et al., 2016). *SHORT VEGETATIVE PHASE* genes have been well-described in *Arabidopsis*. The *SVP* genes are transcription factors that act as repressors of flowering during the vegetative phase as well as play an important role during floral meristem specification in *Arabidopsis* (Falavigna et al., 2019; Gregis et al., 2013; Horvath, 2015). Furthermore, *SVP* genes are involved in the regulation of flowering time under ambient temperature changes in *Arabidopsis* by interacting with the flowering repressor *FLC* to repress the expression of the *FT* gene (Gregis et al., 2013; Lee et al., 2007). In litchi, three *SVP* genes were identified. Under non-inductive conditions, *LcSVP1* and *LcSVP2* were associated with growth cessation and dormancy maintenance, while *LcSVP3* was related to bud break and shoot growth (Zhang et al., 2016). However, under floral inductive conditions, all three *LcSVP* genes were least expressed during floral initiation and panicle development, and *LcSVP*s are therefore also regarded as repressors in litchi flowering (Hu et al., 2018; Lu et al., 2017).

As mentioned previously, non-structural carbohydrates, such as starch and soluble sugars, accumulate during the dormancy period as an adaptive mechanism in response to cold and/or drought, and as a necessary energy source for floral transition and floral development (Galvão and Schmid, 2014; Rosa et al., 2009; Sami et al., 2016). Particularly, soluble sugars play an important role in signal transduction as well as in metabolic and developmental processes, including flowering, in response to internal and environmental signals by modulating gene expression and enzyme activities (Galvão and Schmid, 2014; Rolland et al., 2002; Rosa et al., 2009). For example, an increase in sugar concentration in leaves, phloem sap or the shoot apex due to different treatments (e.g., changes in photoperiod, exogenous sucrose application or use of transgenic plants) amplified the expression of flowering-related genes, such as *FT* and *CO*, and carbohydrate metabolism-related genes, and promoted flowering in several herbaceous plants (Cho et al., 2018).

Sucrose was also found to modulate genes in hormonal pathways that control bud break, such as those that regulate auxin and cytokinin biosynthesis (Barbier et al., 2015). In litchi, recent studies also showed that soluble sugar and starch accumulation in leaves prior to panicle emergence correlated well with floral gene expression as well as increased floral competence and floral formation (Xiao et al., 2018; Yang et al., 2014; Zhang et al., 2017). Furthermore, Yang et al. (2014) highlighted the importance of leaf maturity for carbohydrate accumulation and flowering-related gene expression in litchi. Their study showed that leaves of mature, terminal shoots had higher carbohydrate (soluble sugars and starch) concentrations as well as higher gene expression of the floral promoters *LcCO* and *LcFT2* than leaves of immature shoots. In a similar study that involved turning, i.e., not yet fully mature, and mature shoots, the leaves of mature shoots displayed accelerated sugar accumulation and intensified *LcFT* expression after exposure to low temperatures compared with leaves of turning shoots (Xiao et al., 2018). The same study also identified several flower-specific *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes belonging to the *miR156/SPL* module, a dominant sugar-mediated flowering regulator. The results revealed that most of these genes showed higher expression levels in fully mature leaves compared with immature leaves (Xiao et al., 2018). However, only the gene expression levels of *LcSPL1* and *LcSPL2* showed a correlation with leaf maturity- and low temperature-promoted flowering (Xiao et al., 2018). Gene expression profiling during bud development under non-inductive conditions further revealed an upregulation of genes in the carbohydrate metabolism pathway during and after bud break, which corresponded with an increased respiration rate and confirmed the importance of carbohydrates to support bud growth and subsequent shoot growth (Zhang et al., 2016).

While good progress has been made to elucidate the mechanisms involved in litchi floral induction and initiation, further research is required to study the effect of horticultural practices, such as PGRs, girdling and water stress, on the association between carbohydrates and flowering-related gene expression, and how to adapt these practices in favor of flowering enhancement.

4.2 Hormonal and biochemical regulation associated with floral induction and initiation in litchi

A number of plant hormones have consistently shown to affect the transition to reproductive growth in horticultural tree crops, either in a positive or negative way (Bangerth, 2006). However, the complex cross-regulatory networks between hormonal and genetic pathways, which regulate flowering in response to endogenous and environmental cues, make it challenging to fully understand flower regulation (Galvão and Schmidt, 2014), particularly in the less studied subtropical tree crops. While some advances have been made regarding hormonal regulation of flowering in mango and citrus (Davenport and Núñez-Eliséa, 1997; Monselise and Goldschmidt,

1982; Wilke et al., 2008), only limited reports are available on the hormonal control of floral formation in litchi, as well as other biochemical constituents that may play a role in floral induction, such as proteins or reactive oxygen species (ROS). These are discussed in the following section.

4.2.1 Cytokinins

Shoot initiation, whether vegetative or generative, is fundamentally linked to cytokinins (CK), which induces cell division and stimulates adventitious bud break (Davenport and Stern, 2005; Letham and Palni, 1983; Skoog and Armstrong, 1970). Increased CK activity was associated with floral bud formation in litchi, longan and mango (Chen, 1987; Chen et al., 1997; Liang et al., 1983; Qiu et al., 2001). In litchi, CK activity was low in the apical meristems of dormant buds, whereas CK activity was found at its maximum during floral bud formation and at full bloom (Chen, 1990, 1991; Naphrom et al., 2001). Exogenous application of kinetin, a synthetic cytokinin-like compound, to dormant buds did not cause bud break in 'Heh Yeh' litchi, but promoted flower bud differentiation when applied after bud dormancy (Chen, 1991). Accumulation of CK in apical meristems during floral transition was also found in both litchi and longan in response to low inductive temperature (Hegele et al., 2010) and in longan in response to potassium chlorate application (Potchanasin et al., 2009a; Winterhagen et al., 2020). It is generally believed that the apices of actively growing roots are the main source of CK synthesis from where they are exported to shoots in the xylem as directed by the transpiration stream (Bangerth, 1994; Chen et al., 1997; Davenport and Stern, 2005; Van Staden and Davey, 1979). In this regard, O'Hare and Turnbull (2004) showed that the zeatin-riboside concentration increased in apical buds of litchi concurrent with an increase in root growth rate, which generally coincides with the dormancy period and the early stages of shoot emergence, and peaked just prior to panicle emergence. However, exogenous application of zeatin-riboside to dormant buds only induced bud break, but did not promote further shoot growth in 'Tai So' litchi (O'Hare and Turnbull, 2004).

Gene expression profiling during vegetative bud development found a down-regulation of the cytokinin pathway genes during bud break and active growth, and it was suggested that down-regulation of CK-related genes at bud break may be a requirement for further growth (Zhang et al., 2016). Furthermore, moderate water stress was shown to increase CK levels in the xylem sap, most likely due to stimulation of root-tip CK production, and promoted bud differentiation in 'Mauritius' litchi (Stern et al., 2003). Severe drought, however, had the opposite effect (Stern et al., 2003). Notwithstanding the clear involvement of CKs in bud development in litchi as well as other tree crops, e.g. mango and longan (Bangerth, 2009), no clear evidence could be found that increased CK levels at specific stages of bud development can replace the low temperature inductive stimulus required for floral induction in litchi, since exogenous application of CKs only resulted in improved

floral bud formation and flowering under cool weather, but caused vegetative shoot growth when applied during warm weather (Chen, 1991; Davenport and Stern, 2005).

4.2.2 Gibberellic acid

Gibberellic acid (GA) plays a critical role in flower differentiation and development as well as in regulating the flowering time of many herbaceous crops (Galvão and Schmidt, 2014). However, studies have shown that GA, in particular $GA_{1/3}$ and $GA_{4/7}$, can inhibit floral initiation in various horticultural fruit crops (Bangerth, 2009; Muñoz-Fambuena et al., 2012; Sharma et al., 2019; Wilke et al., 2008; Zeevaart, 1976). In litchi, GA concentrations were found to be highest in the dormant buds of terminal shoots, while very low endogenous GA levels were linked with floral bud formation (Chen, 1990, 1991; Naphrom et al., 2001). Similar results were found for longan under low temperature treatment (Potchanasin et al., 2009b) as well as for citrus (Koshita et al., 1999). Chen et al. (2014) found that exogenous application of GA_3 during early floral induction of 'Yu Her Pau' litchi promoted the development of leafy inflorescences and decreased the inflorescence length. The same authors suggested that application of GA_3 during floral induction in 'Yu Her Pau' litchi caused the same floral inhibitory effect as GA_3 applied at the same time in other evergreen fruit trees, and therefore, promoted the development of leafy inflorescence instead. Hegele et al. (2010) proposed that low GA concentrations might be necessary to maintain low auxin concentrations, which facilitate a high CK/IAA ratio in buds for the stimulation of floral induction in litchi and longan. Likewise, Qui et al. (2001) found elevated GA levels during floral differentiation suggesting that GA inhibits floral induction, but promotes floral differentiation. This is in accordance with Liu et al. (2011) who found the same trends in pineapple. Furthermore, application of the GA biosynthesis inhibitor paclobutrazol and 2-chloroethyl-trimethylammonium chloride (chlormequat chloride) during autumn could promote litchi flowering by reducing GA levels in leaves and consequently inhibiting vegetative growth in same studies, but did not show consistent results in other studies (Chaitrakulsub et al., 1992; Liang and Yu, 1991; Menzel and Simpson, 1990).

4.2.3 Abscisic acid

The role of abscisic acid (ABA) in flowering is still elusive and its involvement in the flowering process and in deciduous trees was suggested to be associated with dormancy entrance and bud set prior to flowering rather than floral development itself (Galvão and Schmidt, 2014; Horvath, 2009; Olsen, 2010; Zhang et al., 2016). Hence, high levels of ABA are generally correlated with growth cessation and dormancy entrance, particularly when subjected to abiotic stress, such as water stress

or salinity, while the lowest levels were found after reaching the dormant state (Chen et al., 2020; Horvath et al., 2008; Ruttink et al., 2007).

In litchi, ABA-related genes were up-regulated in the hormonal signaling pathways in apical buds during growth cessation and dormancy (Zhang et al., 2016). Severe water stress treatment increased ABA concentrations in the shoot xylem sap of litchi and prevented vegetative shoot growth during autumn and winter, thus, enhancing flower intensity in spring (Stern et al., 2003). Despite its growth inhibiting effects, ABA was found at elevated levels in terminal buds of litchi and longan prior to and during floral bud formation and it was suggested that ABA is involved in floral bud differentiation in these two crops (Chen, 1990; Qiu et al., 2001). However, a study by Zhou et al. (2010), which investigated hormonal levels of vigorously developing panicles and abscising rudimentary leaves, found that ABA levels of abscising rudimentary leaves in litchi were high, while ABA levels in the developing panicle itself were low. Thus, elevated ABA levels at floral initiation may be associated with senescence of the rudimentary leaves rather than floral initiation itself. Furthermore, application of exogenous ABA under low inductive temperatures in litchi enhanced the expression of the floral promoter gene *LcAP1*, which plays a central role in floral initiation and differentiation, while application of the ABA biosynthesis inhibitor naproxen suppressed *LcAP1* expression (Cui et al., 2013). In the same study, the application of exogenous ABA before or during panicle emergence increased the number of flowers per panicle, and enhanced the number of axillary panicles per inflorescence, but reduced the number of leaves per panicle, indicating that the role of ABA in the flowering process may be associated with abiotic stress signaling, rather than direct induction of the floral signal (Cui et al., 2013).

4.2.4 Ethylene

Similar to ABA, ethylene plays an ambiguous role in flowering, and has been associated with both dormancy entrance and dormancy release (Liu and Sherif, 2009a). Ethylene is commonly known for its involvement in the regulation of developmental processes, such as seed germination, leaf development, senescence and fruit ripening (Dubois et al., 2018). Ethylene is also known to inhibit cell division and meristematic growth in shoots and axillary buds (Burg, 1973). The promoting or inhibiting effect of ethylene is organ- and concentration-dependent (Dubois et al., 2018; Pierik et al., 2006). In the context of flowering, ethylene appears to mediate flowering in response to environmental signals, such as temperature and water stress (Galvão and Schmidt, 2014; Yang and Hoffman, 1984).

The exact functional mechanisms of ethylene in the flowering processes of litchi are still unclear. The earliest studies using ethephon for enhancing flower development in litchi were inconclusive (Stern and Gazit, 2003). Defoliation of untimely young vegetative shoots prior to

flowering using foliar ethephon applications stimulated axillary bud growth four to six weeks after treatment, during the inductive period, and thus, successfully improved flowering (Roets et al., 2010). Although leaf abscission and termination of the apical bud in this study could clearly be ascribed to the effect of the ethephon-derived ethylene (Burg, 1973; Dubois et al., 2018), floral bud development itself was more likely attributed to the delayed floral development during a period that was conducive for flowering. Using gene expression profiling during natural bud development in litchi, Zhang et al. (2016) found that major components of the ethylene-signaling pathway, such as *EIN3*, were upregulated during growth cessation and dormancy of terminal buds in litchi, thus, suggesting that ethylene is a key player in inducing growth cessation and dormancy entrance in litchi. Olsen (2010) also referred to the role of ethylene in dormancy-related processes and the formation of dormant buds, but did not implicate the maintenance of dormancy. Ethephon-derived ethylene was also shown to promote panicle primordia development during early inflorescence growth, by causing abscission of the rudimentary leaves which developed during less inductive temperatures, thus, promoting pure panicle formation (Zhou et al., 2013).

4.2.5 Auxin

Auxin generally acts as a growth-promoting agent and regulates bud outgrowth, stem elongation, root growth, fruit development and apical dominance, among others (Davenport and Stern, 2005; Taiz et al., 2015). Similar to ethylene, the effect of auxin is concentration-dependent, with high concentrations acting inhibitory and with low concentrations acting stimulating (Salisbury, 1955). In this regard, the effect of auxins produced inside the apical meristem appears to be different from the effect of auxins produced after bud break due to their interaction with other hormones (Horvath et al., 2003). Auxins also play a role in the initiation of floral primordia and differentiation of floral organs (Cheng and Zhao, 2007).

Few reports are available on the role and use of auxin in litchi floral development. Early studies used synthetic auxins, such as naphthalene acetic acid (NAA), to inhibit autumn vegetative shoot growth to promote litchi flowering, with higher concentrations showing a stronger inhibitory effect than lower concentrations (reviewed by Davenport and Stern, 2005). Experiments that studied hormonal changes during vegetative shoot growth and floral development in litchi found that levels of the auxin indole-3-acetic acid (IAA) were highest in immature leaves during elongation, but lowest in apical buds prior to floral bud formation, similar to GA (Chen 1990; Liang et al., 1987). In line with this observation, Hegele et al. (2010) observed that potassium chlorate or low temperature treatment in longan and litchi, respectively, caused an export of IAA out of leaves accompanied by a reduction of IAA levels in apical buds during the floral induction period, in both species. However, IAA and zeatin riboside were significantly increased in developing litchi panicles

compared with the abscised rudimentary leaves of such panicles (Zhou et al., 2010). Since auxins also regulate root initiation and development, increased root growth activity may promote CK accumulation in shoot tips, which promotes floral initiation under inductive conditions (Davenport and Stern, 2005).

4.2.6 Transcriptomic analysis to elucidate hormonal regulation of flowering

Despite the limitations in past research on hormonal control of floral initiation in litchi, new advances in plant science, such as transcriptomic analysis based on *de novo* RNA sequencing, have revealed new insights into the complexity of hormonal and genetic pathway interactions, in response to external and internal stimuli involved in litchi floral initiation. Transcriptomic analysis of terminal buds at the floral initiation stage showed differential expression of various genes involved in the metabolism and signal transduction of plant hormones. Increased transcripts were found for unigenes of the auxin, cytokinin, jasmonic acid and salicylic acid biosynthesis pathways, while unigenes of the abscisic acid biosynthesis pathway were downregulated at floral initiation (Zhang et al., 2014). Transcriptomes obtained for apical meristems of early and late flowering cultivars that were subjected to low temperature, moderate temperature plus ROS and high temperature treatment, revealed a potential gene network of transcription factors, plant hormones and flowering regulators which controlled the transition from vegetative to floral development (Lu et al., 2017). Among the various unigenes identified to be responsive to both chilling and medium temperature plus ROS treatment, a high number of them were associated with plant hormone biosynthesis and plant hormone signal transduction. Clear differences between treatments were observed, with IAA, GA and ethylene-related genes being downregulated in apical meristems in response to low temperature or medium temperature plus ROS treatment compared with high temperature treatment (Lu et al., 2017). Although transcriptomic analysis brought a greater understanding of the involvement of plant hormones in litchi floral initiation, the regulatory mechanisms are still far from being elucidated.

4.3 Reactive oxygen species associated with floral induction in litchi

Reactive oxygen species are generated in response to environmental stress, such as drought, salinity, chilling, pathogen infection or high doses of chemicals (including plant growth regulators), and act as signaling molecules in response to biotic and abiotic stresses (Neill et al., 2002). In the mixed floral bud of litchi, the rudimentary leaves will abscise and the panicle primordia will develop further, only when temperatures are sufficiently low. Zhou et al. (2008) found an increase in ROS, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO), in the abscising rudimentary leaves in response to chilling. In subsequent experiments, ROS-generating chemicals, such as ethephon,

methyl viologen (MV) and sodium nitroprusside (SNP), promoted the abscission of rudimentary leaves and encouraged panicle development when applied before and at panicle emergence, i.e., floral initiation, while application of ROS scavengers, such as dimethylethiourea (DMTU), inhibited chilling-induced flowering (Zhou et al., 2012, 2013). In addition, it was found that MV and SNP applied under less inductive temperatures promoted leafless panicles as well as increased the number of flowering terminal shoots and flowers. These ROS-generating chemicals also enhanced gene expression of the floral identity genes *LcAP1* and *LcLFY*, which are generally strongly expressed under chilling conditions during the floral initiation stage, whilst decreasing the expression of the floral repressor *LcFLC* (Lu et al., 2017, 2020; Zhou et al., 2012). Hence, it is feasible that not only chilling, but also ROS promote floral transition and initiation.

4.4 Proteomics and metabolomics analysis to elucidate biochemical regulation associated with floral induction in litchi

Thus far, proteomics and metabolomics are fields of study that have not been explored much in litchi flowering research. Proteomics studies of phloem exudates of terminal shoots at the pre-floral induction, floral induction and floral initiation stages found certain proteins specific to each stage. This suggests that these phloem exudate proteins might act as a long-distance signal in litchi floral induction, indicating their involvement in inter-organ communication during litchi floral development (Huang et al., 2020).

Metabolomics analysis of girdled and non-girdled turning shoots following 60 days of cold treatment, revealed several metabolic changes, most of which were mostly related to the metabolism of starch and sucrose, and that of fatty acids and phenylpyruvic acid. Metabolites associated with high flowering rates as found in the leaves of girdled shoots during floral induction included high levels of sucrose, maltose, starch and epinephrine, but low levels of UDP-glucose, D-glucose-6P, D-galactonate and D-fructose (Su et al., 2021). Although epinephrine has been reported for its important role in flowering of other plants, the study by Su et al. (2021) is the first report of its involvement in litchi flowering. In contrast, the level of compounds in galactose, phosphoenolpyruvate and linoleic acid metabolism remained unchanged in leaves of girdled shoots, but changed drastically in the leaves of non-girdled shoots during cold treatment, suggesting that they also play an important role in litchi floral induction (Su et al., 2021). Further studies into the changes of biochemical constituents of leaves, shoots and apical meristems prior to and during floral initiation are necessary to shed more light on the mechanisms involved in floral development in litchi.

C. The role of ethylene in bud development and flowering modulation

1. Use of ethephon in agriculture, with a focus on management of flowering

Ethephon (2-chloroethylphosphonic acid) has been an important and widely used plant growth regulator in horticulture for a range of applications, including fruit ripening and coloration, organ abscission to aid harvesting, bloom delay and suppression of vegetative growth (Nickell, 1994). Ethephon has also been used for bud development and flower stimulation in various fruit crops. In mango, ethephon was believed to play a role in floral induction, but this could not be confirmed reliably (Davenport and Nunez-Elisea, 1991; Davenport and Stern, 2005). However, ethephon consistently induced flowering in pineapple (Batholomew, 1977; Maruthasalam et al., 2010), although the increased ethylene concentrations might not have been the only causal agent, since ethephon application also triggered an increase in ABA and CK, and a decrease in auxin and GA concentrations at the inflorescence induction stage (Liu et al., 2011). In contrast, ethephon application improved flowering in a range of temperate crops, such as stone and pome fruit (Crisosto et al., 1990; Durner and Gianfagna, 1991; Ebel et al., 1999; Liu and Sherif, 2019b; Liu et al., 2021; Mertoğlu et al., 2019), pistachio (Askari et al., 2011) and blueberry (Krewer et al., 2005), by delaying bloom to avoid spring frost. In this context, ethephon treatment triggered a stress response with an increase in ROS levels, which induced bud dormancy, which consequently led to delayed bloom (Islam et al., 2021; Liu and Sherif, 2019b). Similar ethephon effects on delaying flowering were found in cape gooseberry (Yadava, 2012). In pecan, ethephon applications affected vegetative growth and improved the number of female flowers in the following spring via increasing the percentage of terminal shoots when applied to young pecans prior to kernel-filling (Wood, 2011). Likewise, ethephon treatment promoted flowering in litchi by eliminating immature soft vegetative shoots in autumn and early winter to ensure mature shoots during floral induction and initiation (Cronje and Ratlapane, 2018; Cronje et al., 2017b; Roets et al., 2010). Furthermore, ethephon applied to leafy litchi inflorescences caused abscission of rudimentary leaves and promoted axillary panicle primordia development (Zhou et al., 2013). The variety of applications in horticulture demonstrates the contrasting nature of ethephon between growth inhibition and stimulation (Pierik et al., 2006).

2. Ethephon uptake, conversion to ethylene and ethylene signal transduction

Apart from the concentration, the efficacy of ethephon depends on the age and anatomy of the targeted plant organ, as well as environmental conditions, such as air temperature, relative humidity or irrigation status, during and after application (Klein et al., 1978; Olien and Bukovac, 1978; Stover and Greene, 2005; Walters and Lopez, 2018; Walter and Leopold, 1969). Furthermore, different cultivars can vary in their sensitivity to ethephon (Kaur et al., 2021). The effects of ethephon have

generally been attributed to ethylene, the active form of ethephon (Liu et al., 2021). Particularly air temperature and alkalinity of the spray water, which affect both uptake and conversion to ethylene, play an important role in the efficacy of ethephon and may explain the inconsistent results found in literature (Stover and Greene, 2005). Generally, as ethylene evolution from ethephon increases with increasing temperatures between 10°C and 34°C and increasing alkalinity between pH 5 and pH 8, ethephon efficacy decreases (Domir and Foy, 1978; Olien, 1976; Walters and Lopez, 2018; Warner and Leopold, 1969). Relative humidity between 35% and 70% also increased the rate of degradation compared with relative humidity outside this range (Klein et al., 1978).

After absorption by the plant tissue, or in the presence of a base, ethephon rapidly degrades to ethylene, phosphate and chloride (Domir and Foy, 1978; Hartley and Kidd, 1983; Warner and Leopold, 1969). As a gaseous hormone, ethylene moves within the plant across membranes by diffusion (Chang, 2016). Ethylene is perceived by specific ethylene-binding receptors in the membrane of the endoplasmic reticulum, such as ETHYLENE RESISTANT 1 (ETR1) and ETR2 among others. In the absence of ethylene, these receptors activate the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) protein kinase, which negatively regulates the ethylene pathway, i.e., prevents downstream signaling and gene expression. In the presence of ethylene, however, the binding of ethylene to the receptors inactivates CRT1 and results in the activation of the membrane protein ETHYLENE INSENSITIVE 2 (EIN2) in the endoplasmic reticulum. In turn, activation of EIN2 stabilizes the transcription factors *EIN3* and *EIN3-LIKE 1* (*EIL1*), which regulate gene expression of other transcription factors belonging to the *ETHYENE RESPONSE FACTOR* family (*ERF*s) in the nucleus, leading to the transcription of numerous ethylene related genes and eventually to ethylene responses specific to their exogenous or endogenous triggers (Bleecker and Kende, 2000; Chang, 2016; Dubois et al., 2018; Ju and Chang, 2015; Wang et al., 2013).

3. Ethylene functions, biosynthesis and interaction with other pathways

Ethylene is recognized for its involvement in the regulation of various aspects of plant growth and development, such as seed germination, root and shoot development, cellular growth regulation, carbon assimilation, senescence of leaves and flowers, organ abscission and fruit ripening (Burg, 1973; Dubois et al., 2018; Iqbal et al., 2013; Olsen, 2010; Pierik et al., 2006; Wang et al., 2013). Ethylene also acts as a signaling molecule and is involved in responses to biotic and abiotic environmental stresses. These abiotic stresses include temperature extremes (e.g. heat or chilling), drought, flooding, shading, radiation, nutrient deficiency, mechanical damage (e.g. cutting or bruising) and chemical damage (e.g. agrochemicals, ozone or other pollutants). As such, ethylene acts as a link between the changes in the environment and developmental adaptive plant responses (Dubois et al., 2018; Iqbal et al., 2013; Wang et al., 2002; Yang and Hoffman, 1984).

Biosynthesis of endogenous ethylene is activated by internal signal transduction and external stimuli (Müller, 2021). Ethylene biosynthesis occurs in a simple 3-step metabolic pathway, starting with the conversion of the amino acid methionine to S-adenosyl-methionine (SAM) by the enzyme SAM synthetase. In a further, rate-limiting step, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). In the final step, ACC oxidase catalyzes the conversion of ACC to ethylene. Since the conversion from ACC to ethylene is oxygen dependent, no ethylene formation can take place under anaerobic conditions (Dubois et al., 2018; Iqbal et al., 2013; Müller, 2021; Wang et al., 2002; Yang and Hoffman, 1984).

Besides endogenous ethylene biosynthesis and signal transduction, which forms part of normal developmental processes, environmental stressors can trigger ethylene biosynthesis via inducing ROS accumulation caused by oxidative stress inside the plant cells. This leads to the activation of the mitogen-activated protein kinase cascade (MPK3/6 cascade), which regulates ethylene biosynthesis (via ACC synthase activation) and *ETHYLENE RESPONSE FACTOR* (*ERF*) gene family-mediated responses (Dubois et al., 2018; Iqbal et al., 2013). Stimulation of bud growth, which is known to occur in response to external stresses, may be ascribed to the indirect involvement of ethylene, through ethylene inhibiting the polar auxin transport system (Abeles et al. 1992; Hansen and Grossmann 2000; Pierik et al., 2006). Furthermore, stress-induced ethylene was found to activate ABA biosynthesis, leading to growth inhibition. Thus, ethylene and ABA can act in parallel, depending on their endogenous levels (Müller, 2021). These examples hardly cover the extent of ethylene's involvement in plant development and stress response, but demonstrate the complexity of crosstalk between the ethylene-signaling pathway and other hormone signaling and biosynthesis pathways, as well as metabolic pathways and transcription networks (Chang, 2016; Grossman and Hansen, 2001; Pierik et al., 2006).

In the context of flowering, ethylene appears to mediate flowering in response to environmental signals, such as temperature and water stress, via the above-described ethylene-biosynthesis and signaling pathway (Conti, 2017; Galvão and Schmidt, 2014; Yang and Hoffman, 1984). However, the role of ethylene in flowering regulation is rather complex. For example, mechanically-induced stress by bending shoots induced endogenous ethylene production, which in turn reduced polar auxin transport and CK levels in shoots, and consequently increased the percentage of floral buds in apple (Sanyal and Bangerth, 1998). Ethylene was also associated with the release of bud dormancy in grape, following application of dormancy-breaking chemicals (Ophir et al., 2009; Sudawan et al., 2016). By influencing shoot apical meristem activity, ethylene was found to be involved in bud formation and thus floral transition (Vandenbussche and Van der Straeten, 2012). However, as alluded to above, the growth-stimulating effects of ethylene appear to be dependent on interaction with other hormones rather than a direct effect of ethylene itself. It was also proposed

that ethylene might act upstream of the gibberellin biosynthesis pathway and thus, regulate flowering time through *SUPRESSOR OF CONSTANS1* (*SOC1*) and *LFY* gene expression via accumulation of DELLA proteins, which act as central repressors of gibberellin responses (Dubois et al., 2018; Galvão and Schmidt, 2014). Liu et al. (2018) suggested that ethylene, as well as abscisic acid and gibberellins, may regulate genes in the SVP family. Likewise, ethephon-derived ethylene induced bud dormancy and delayed bloom in peach (Liu et al., 2021). A delay in flowering is thus consistent with ethylene's role in growth inhibition (Conti, 2017). Other ROS-generating chemicals, such as MV, were found to upregulate genes involved in ethylene signal transduction (Lu et al., 2014).

Carbohydrate accumulation prior to floral formation plays an important role in floral transition and formation, as previously mentioned (Barbier et al., 2015; Lebon et al., 2008; Marquat et al., 1999; Ortiz-Marchena et al., 2014; Qin et al., 2012). Ethylene exposure was associated with increased stomatal conductance, photosynthetic activity and enhanced nitrogen use efficiency, therefore, contributing to enhanced carbon assimilation in some herbaceous plants. However, the effect of ethylene on carbon assimilation was dependent on ethylene concentration and duration of exposure (Pierik et al., 2006; Iqbal et al., 2013). Moreover, increased C/N ratios in leaves and shoots were found in litchi in response to ethephon application prior to panicle emergence (Mandal et al., 2014). Total sugars and sucrose concentrations also increased in pineapple buds 48 h after ethephon application (Ávila et al., 2005). An upregulated carbohydrate metabolism is possibly related to ethylene-induced ROS accumulation and ABA biosynthesis activation (Chen et al., 2019; Couée et al., 2006; Li et al., 2015; Pierik et al., 2006).

Knowledge of the ethylene-signaling pathway and its interaction with other signaling pathways and transcription networks is essential to understand the effects of exogenous application of ethylene, e.g. in the form of ethephon, in agricultural use and for developing new strategies to manipulate ethylene responses, such as promoting flowering under less favorable conditions. Similar to the successful use of ethephon in stone fruit to prevent spring frost and flower damage (Durner and Gianfagna, 1991; Liu and Sherif, 2019b; Liu et al., 2021), ethephon could also be an important tool for mitigating environmental stressors that affect flowering, based on ethylene's role as a mediator between environmental signals and developmental responses and its involvement in carbohydrate metabolism. Viewed in this context, ethephon may be a useful application in the mitigation of warm winter temperatures to enhance floral initiation in litchi. Thus far, ethephon has only been used in litchi to remove immature shoots prior to panicle emergence (Chaitrakulsub et al., 1992; Cronje et al., 2017b; Huang and Chen, 2014; Mandal et al., 2014; Olesen et al., 1999; Roets et al., 2010; Stern and Gazit, 2003), to facilitate the abortion of rudimentary leaves in leafy panicles to promote pure panicles (Zhou et al., 2013) and to study fruit abscission (Li et al., 2015).

However, the role of ethephon-induced ethylene in litchi flower regulation and floral bud development has not been investigated nor understood yet and requires further research.

D. Horticultural practices to promote floral formation in litchi

Current horticultural practices to promote floral induction and development in litchi are mainly aimed at strategies that manipulate the postharvest flushing cycle to ensure timely shoot maturity, and to eliminate or inhibit any untimely vegetative shoot growth prior to floral induction. At the same time, these practices will also promote carbohydrate accumulation in terminal shoots (Huang and Chen, 2014; Koo-Duang and Subhadrabandhu, 1987). Due to changes in climate over the past few decades, warmer autumns and winters in subtropical regions have become more prevalent. Adequate orchard management strategies to ensure that litchi trees reach the optimal phenological stage, i.e., mature shoots with chilling responsive terminal buds when temperatures become inductive, are therefore becoming increasingly important.

1. Manipulation of flush growth cycles and shoot maturity

1.1 Pruning

Pruning after harvest is an important orchard practice for the management of postharvest shoot growth cycles. The timing of pruning will determine the timing of emergence and maturation of postharvest shoots (Menzel et al., 2002; Pan et al., 2014; Oosthuyse, 2014), while the type of pruning will determine the uniformity of shoot emergence, e.g. hedging by tip pruning of terminal branches up to 30 cm will synchronize shoot emergence (Menzel et al., 2002). Although pruning favors vegetative growth, with adequate grower experience and favorable climatic conditions timely pruning will ensure that the last leaf flush will reach shoot maturity just prior to the inductive period, but without any subsequent vegetative shoot growth that might interfere with floral development (Mitra and Irenus, 2018; Olesen et al., 2013; Stern and Gazit, 2003).

In Australia, researchers have developed a guideline for the optimum pruning time, based on average temperature and solar radiation for the different production regions and cultivars (Menzel et al., 2002). General recommendations in most other countries are to carry out pruning immediately after harvest (for late cultivars) or within one month after harvest (for early to mid-season cultivars) (Cronje et al., 2010; Huang and Chen, 2014; Nagao and Paull, 1998; Singh et al., 2012b; Zhang et al., 1997). In addition, pruning will also improve light interception, which aids good floral development through improved photosynthesis and carbohydrate accumulation (Singh et al., 2012b; Wilke et al., 2008). Permanent shading, as commonly found in dense or overgrown orchards, was shown to significantly inhibit bud break in litchi, thus, suggesting that light is a key factor for the release of bud dormancy (Zhang et al., 2016). Although pruning is an important tool to manipulate

postharvest shoot growth and to ensure optimum light conditions for bud outgrowth, its effects are subject to environmental factors, implicating that pruning does not always provide sufficient control to achieve the desired effect.

1.2 Fertilization practices

Fertilizer application, in particular nitrogen applications, can influence flushing cycles. High nitrogen amounts applied throughout the year or towards autumn can result in increased soil and leaf nitrogen status, leading to untimely vegetative growth during autumn and early winter, and may therefore reduce flowering (Davenport et al., 2001; Li et al., 2001). Australian researchers recommended that leaf nitrogen levels should not exceed 1.8% in autumn to limit flushing during the pre-flowering season (Menzel et al., 1988). However, in Israel, good yields were achieved when fertigation was applied until the start of the water stress period, at the beginning of autumn (Stern and Gazit, 2003). Similarly, research by Cronje et al. (2017a, 2021) showed that applying N fertilizers prior to each postharvest shoot growth cycle advanced postharvest shoot growth. When used in combination with ethephon applications for shoot control during autumn, this strategy limited untimely leaf flushing prior to floral induction, and resulted in good flowering in 'Mauritius' litchi under South African conditions. Multiple foliar applications of potassium nitrate (KNO_3), up to four months prior to panicle emergence, reduced vegetative growth of litchi in India, but these applications were less effective than applications of plant growth regulators or girdling (Mandal et al., 2014; Mitra and Sanyal, 2001). In South Africa, some growers use foliar-applied potassium sulphate (K_2SO_4), at monthly intervals 2-3 months prior to panicle emergence, to suppress vegetative growth in 'Mauritius' litchi (personal observation). However, this practice has not been evaluated scientifically for its effectiveness. Once shoot growth is underway, actions to accelerate shoot maturation processes are considered ineffective as foliar fertilizers have been found to promote shoot growth physiologically, but not phenologically (Huang and Chen, 2014).

2. Elimination or inhibition of new shoot growth prior to floral induction and initiation

2.1 Water stress

Similar to other crops, shoot growth in litchi is strongly linked to root growth (O'Hare and Turnbull, 2004). Root growth occurs during shoot dormancy and in the initial stages of bud growth, and coincides with a peak in CK concentrations in terminal buds, just prior to bud break. Thus, increasing the period between shoot and root growth by limiting root growth, for example through water stress, allows for an extended period of shoot dormancy (O'Hare and Turnbull, 2004). Inducing water stress in autumn is therefore an effective method to suppress autumn and winter shoot growth during dry seasons and has been successfully used in Israel (Stern et al., 1993, 1998),

Australia (Chaikiattiyos et al., 1994) and South Africa (Cronje et al., 2017b). However, unlike with citrus (Southwick and Davenport, 1986), water stress in litchi could not replace the inductive temperatures and induce flowering, but merely allowed the tree to be at the optimum phenological stage when inductive temperatures occured (Chaikiattiyos et al., 1994; Menzel et al., 1989; Stern and Gazit, 2003). Studies under controlled temperature conditions in Australia showed that new shoot growth was suppressed by water stress under high temperatures, but no flowers developed (Chaikiattiyos et al., 1994; Menzel et al., 1989). Similarly, it was noted that 'Sanyuehong' litchi trees that were subjected to water stress treatment under non-inductive conditions in China did not flower, despite 'Sanyuehong' being a cultivar with a low chilling requirement (Zhou et al., 2014). In contrast, well-watered trees with mature shoots produced good flowering, when exposed to low inductive temperatures (Cronje et al., 2017b; Stern and Gazit, 2003). Yet another study showed that water stress in combination with low inductive temperatures caused a higher percentage of flowering shoots as well as higher gene expression of the floral promoter, *LcFT*, compared with plants that were exposed to low inductive temperatures alone, suggesting that water stress possibly enhances chilling responsiveness (Shen et al., 2016). Furthermore, water stress was shown to enhance carbohydrate accumulation in leaves and terminal shoots and induced flowering in *Averrhoa carambola* (Wu et al., 2017). However, in litchi, water stress-induced carbohydrate accumulation and its association with floral formation, other than via restricting vegetative growth, could not be established (Cronje et al., 2017b).

2.2 Girdling

Trunk or branch girdling after maturation of the last desired postharvest flush has been used to suppress shoot growth in litchi with varying success, depending on the time of application, in China (Li and Xiao, 2001; Zhang et al., 1997), India (Kumari et al., 2021; Mandal et al., 2014; Mitra and Sanyal, 2001), Thailand (Koo-Duang and Subhadrabandhu, 1987), Mauritius (Ramburn, 2001), South Africa (Morse and Oosthuizen, 1993) and Australia (Menzel and Simpson, 1987). By inhibiting the assimilate supply to the roots, girdling also promoted carbohydrate accumulation in the terminal shoots, enhanced bud dormancy and promoted flowering (Koo-Duang and Subhadrabandhu, 1987; Liu et al., 2022b; Stern and Gazit, 2003). A recent study showed that even girdling of turning, not yet fully mature shoots, prior to cold exposure increased a range of metabolites, including starch, sucrose and glucose during floral induction, as well as the flowering rate, compared with turning, non-girdled shoots (Su et al., 2021). However, during warm and humid autumns, when the probability for vegetative shoot growth is high, girdling has not always been reliable enough to ensure shoot inhibition and requires additional chemical intervention to achieve effective shoot control (Crane, 2004; Huang and Chen, 2014; Menzel, 1983).

2.3 Plant growth regulators

The application of PGRs is a useful method to eliminate autumn shoots in subtropical crops, particularly where climatic conditions are not always favorable for flowering (Crane, 2004). A range of PGRs, including synthetic auxins, cytokinins and growth retardants were tested in litchi either for their ability to inhibit new vegetative growth or to remove existing vegetative shoots through defoliation. Foliar applications (single or double) or soil drenches with paclobutrazol [(2S,3S)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol] either alone or in combination with potassium dihydrogen phosphate (KH_2PO_4) about two months prior to panicle emergence successfully reduced vegetative growth and increased flowering in China, India, Australia, Thailand and Mauritius (Chaitrakulsub et al., 1992; Liang and Yu, 1991; Mandal et al., 2014; Stern and Gazit, 2003). However, the results of both soil and foliar application of paclobutrazol have not always been consistent, when used in South Africa or Australia (Menzel and Simpson, 1990; Oosthuizen et al., 1995; Oosthuyse, 2004). The use of paclobutrazol has also become controversial due to residues in the fruit at harvest (Kumari et al., 2021; Oosthuizen et al., 1995).

In a recent study, prohexadione-calcium, a gibberellin biosynthesis inhibitor, was used as alternative for paclobutrazol. However, this PGR could not inhibit winter shoot growth as effectively as paclobutrazol or girdling, although it did increase the number of panicles per tree, compared with the control (Kumari et al., 2021). Other growth inhibitors, such as 2-chloroethyl-trimethylammonium chloride, daminozide, maleic hydrazide and 2,3,5-triiodobenzoic acid (TIBA), as well as synthetic auxins, such as sodium naphthalene acetate (SNA) and 1-naphthaleneacetic acid (NAA), were found to deliver inconsistent results (Mitra and Sanyal, 2001; Stern and Gazit, 2003). Defoliation of young vegetative shoots was successfully achieved by single or multiple foliar applications of ethephon alone or in combination with paclobutrazol or 0.5% urea in countries, such as Taiwan, Thailand, India, Australia and South Africa, (Chaitrakulsub et al., 1992; Huang and Chen, 2014; Mandal et al., 2014; Olesen et al., 1999; Roets et al., 2010; Stern and Gazit, 2003). Furthermore, Mandal et al. (2014) found that foliar applications of ethephon at 2 $mL \cdot L^{-1}$ increased the carbon/nitrogen (C/N) ratio in leaves and shoots of 'Bombai' litchi, compared with other shoot control treatments and untreated trees. As the ethephon treatment also resulted in the death of terminal buds and loss of apical dominance, axillary buds resumed growth about 4-6 weeks after application, leading to floral development during the inductive period (Roets et al., 2010).

2.4 Mechanical pruning

Removal of undesirable late autumn flushes can also be achieved by mechanical pruning, as this will result in the release of lateral buds from their apical inhibition on proximal mature shoots,

and will encourage new growth during the inductive period, similar to the effect of some PGRs. This type of pruning has been used in Israel, Australia and Florida with good success (Stern and Gazit, 2003). However, because this practice is labor-intensive and the correct timing is crucial to achieve the desirable effect, most growers prefer to use the more efficient and cost-effective PGRs for shoot control (Campbell, 1994; Stern and Gazit, 2003; Stern et al., 2005).

3. Future approaches

To date, no horticultural practices are available that can directly induce floral induction and initiation in litchi, owing to the close temperature dependence of these processes in litchi. This means that without low inductive temperatures there is little chance for successful floral induction and initiation (Huang and Chen, 2005). However, based on insights obtained from the literature, it is evident that new approaches to improve floral formation in litchi should include strategies that will ensure that the presence of mature terminal shoots with chill-responsive apical buds will coincide with the occurrence of the floral-inducing stimulus, i.e., low temperatures. Considering the higher frequency of above-average temperatures experienced during winter in most subtropical litchi production regions in recent decades, new research approaches should include a focus on mitigating unfavorable seasonal climatic changes. As temperature is the main prerequisite for litchi floral induction and initiation, manipulation techniques should aim at influencing the readiness for growth and chilling responsiveness of trees, under close direction of weather forecasting technology.

So far, application of ROS-generating chemicals, such as ethephon, MV and SNP, showed great potential for promoting litchi panicle development and the development of leafless panicles with a high number of flowers, not only under low inductive temperatures, but also under less inductive conditions (Lu et al., 2020; Zhou et al., 2012, 2013). As these treatments may increase the responsiveness of litchi trees to less inductive temperatures (Lu et al., 2020), further research is needed to elucidate the exact mechanisms and possible interactions with hormones that may influence signaling responses.

One potential approach to promote flowering could be the use of ethephon, which has been successfully used for the control of vegetative growth in autumn and winter. Ethephon application may be perceived as a stress signal through its active component ethylene, and hence, provides specific responses related to dormancy and flowering (Liu et al., 2021). Further research on the role and function of ethephon and ethylene in dormancy and flowering processes of litchi might provide additional clues and treatment options to overcome the erratic flowering problem in litchi, particularly in light of the prospect of increasingly warmer winters in future. Such strategies could

include delaying panicle emergence to a period with more consistent, inductive temperatures for improved floral initiation.

Uniconazole, an inhibitor of GA, ABA and CK, could hold potential for inhibition of vegetative shoot growth in litchi, but has so far only been used to improve flower ratio and fruit set in 'Feizixiao' litchi by controlling litchi inflorescence growth (Wei et al., 2017). Furthermore, hydrogen cyanamide has been successfully used as a dormancy breaking agent under warmer conditions in crops such as grape and kiwi (Sudawan et al., 2016; Hernández and Craig, 2015) and was associated with the accumulation of ROS in grape buds (Sudawan et al., 2016). However, its use has been restricted due to its toxicity and adverse health effects. Alternatives for hydrogen cyanamide, such as alkoxyated fatty alkylamine (Armobreak^{TM}), have shown similar success and may warrant investigation for use in litchi (Hernández and Craig, 2015; Hernández et al., 2015). Applications that directly induce flowering under warmer conditions or even off-season flowering, such as potassium chlorate in longan (Huang et al., 2021; Potchanasin et al., 2009a, 2009b), could not be found for litchi yet. However, with the continuous development and testing of new agrochemicals together with the extended knowledge on genetic and molecular flowering mechanisms, a solution may be within reach in the near future.

More insight is also required to elucidate the role and function of less-studied hormones such as jasmonic acid and salicylic acid in litchi flower regulation (Wei et al., 2017), as both hormones are known to be involved in the control of floral transition in *Arabidopsis* (Conti, 2017). In this regard, endogenous and exogenous hormone studies in combination with gene expression and transcriptomic studies could be particularly helpful to gain deeper insight into the intricate flowering mechanisms of litchi.

Other approaches to address the flowering conundrum of litchi can involve long-term strategies such as molecular and integrated breeding as well as a genomics approach for the development of climate-resilient germplasm and strategies that modify flowering for existing cultivars (Liu et al., 2021). Likewise, already existing cultivars with lower chill requirements, such as lowland cultivars (Sukhvibul et al., 2014), should be evaluated in areas that do not meet the chilling requirements of traditionally planted cultivars anymore.

Finally, the main challenge is to find practical solutions for growers, which can easily be implemented and deliver continuous success, to provide a stable income and healthy food, while being sustainable and environmentally friendly.

E. Conclusion

Litchi production has been a challenge in many subtropical countries due to its erratic flowering and low climatic adaptability. The reproductive failure of litchi has been researched since the

beginning of the 20th century. Despite great research advances, including many molecular studies, the flowering enigma has yet to be fully resolved. It is, however, apparent, that floral induction and initiation in litchi, and that of other subtropical crops subject to similar flowering processes, is not controlled by one factor alone, but by a number of factors that include environmental and developmental signals. Studies that focus on combining physiological, biochemical, molecular and genetic aspects of litchi floral development by using treatment applications (chemical or physical manipulation techniques) in combination with gene expression and transcriptomic studies can potentially deliver a higher output of information and provide a better understanding on the flowering processes in litchi. Such information can then be used to develop new strategies or to adapt existing practices to mitigate environmental and cultivar-inherent factors that currently hamper successful floral induction and initiation. At the same time, it is imperative to provide solutions that are practical and economical for the grower, whilst being cognizant of environmental and human health. By ensuring the success of floral bud development, sustainable productivity and profitability of litchi production, including the improvement of livelihoods, will be secured in many litchi-producing countries. For a country such as South Africa, sustainable production will be key to expand the litchi production and increase its footprint on international markets.

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Paper 2: New approach to shoot growth inhibition with ethephon to improve flowering and yield in 'Mauritius' litchi under South African warm winter conditions

Keywords. Bud dormancy, floral initiation, *Litchi chinensis*, plant growth regulator, shoot maturity, vegetative growth

***Abstract*. Vegetative shoot growth prior to floral induction restricts floral development in litchi (*Litchi chinensis* Sonn.). The conventional shoot control measures used in South Africa, i.e., ethephon spot spray applications to remove young immature vegetative shoots, have become less effective under warm winter conditions. Therefore, the aim of this study was to evaluate a different application approach, i.e., ethephon application to mature terminal shoots, for its effectiveness to inhibit shoot growth and delay bud break until temperatures become conducive for floral induction. Ethephon was applied to 'Mauritius' litchi trees with fully mature terminal shoots at concentrations of 500, 750 and 1000 mg·L^{-1}, at each of three different dates during autumn, i.e., end of March, mid- and end of April, in two climatically different litchi production regions of South Africa. At the cooler site, a whole-canopy application at first signs of shoot growth was included to address uneven shoot growth within trees. At the warmer site, a double whole-canopy application was included to ensure prolonged shoot inhibition under warmer conditions. Spot spray applications at 1000 mg·L^{-1} to soft immature vegetative shoots, as per industry practice, and untreated trees served as controls. Results showed that single whole-canopy ethephon applications to trees with fully mature terminal shoots successfully inhibited leaf flushing prior to floral induction in a dose-dependent manner compared with spot-sprayed trees and untreated trees. Subsequently, this delayed bud break by up to 21 days, reduced the percentage of leafy flower panicles by up to 30%, and increased flowering and yield without delaying fruit maturity. Although the conventional spot sprays effectively removed existing young vegetative shoots, they permitted earlier panicle emergence under less inductive conditions, thus, facilitating the development of leafy flower panicles. A whole-canopy application at first signs of shoot growth performed superior to the conventional spot spray applications in terms of flush control and delaying bud break, but follow-up spot sprays were still necessary. At the warmer experimental site, a double whole-canopy ethephon application extended the dormancy period by an additional five days compared with the highest concentration of a single application. Date of application played an important role in the efficacy of ethephon applications. In this regard, higher**

ethephon concentrations were more effective for early applications (end March, early April) or when temperatures remained high during early winter and a longer dormancy period was required. However, lower concentrations were sufficient for applications at the end of April or later, when temperatures had already dropped.

Introduction

Erratic flowering is a major challenge in litchi (*Litchi chinensis* Sonn.) and is one of the main factors leading to poor productivity (Menzel, 1984; Stern and Gazit, 2003). Litchi undergoes recurrent flush growth cycles that occur more rapidly at higher temperatures than at lower temperatures (Subhadrabandhu and Stern, 2005). These growth cycles are interrupted by rest periods, during which shoot elongation stops and only the meristem of terminal buds enters dormancy, while shoot thickening and darkening of extended leaves continue. Once the leaves have fully matured, bud dormancy is released spontaneously and a new growth cycle can start (Fu et al., 2014; Zhang et al., 2016). However, unlike in temperate tree crops, the release of bud dormancy in litchi is not associated with flower development (Zhang et al., 2016). Only when buds are exposed to floral-inducing chilling temperatures, generally below the daily average of 20°C, can floral development take place (Batten and McConchie, 1995; Chen and Huang, 2005). In this regard, cessation of shoot growth and the ensuing shoot maturation is a prerequisite for successful floral induction under chilling temperatures (Huang and Chen, 2005). In litchi, the final morphological characteristics of the mixed floral bud, which consists of leaf and panicle primordia as well as rudimentary leaves, strongly depend on the prevailing temperature during the differentiation phase. At low temperatures, the floral bud may develop into a pure leafless panicle, while at high temperatures, the rudimentary leaves may fully expand and the axillary panicles shrink (Zhou et al., 2008). These intricate and temperature-specific flowering processes contribute to the low adaptability of litchi to a wider range of climates (Huang and Chen, 2005; Menzel and Simpson, 1988).

When grown outside its native environment or where climatic conditions become less favorable, litchi frequently exhibits continuous vegetative shoot growth into early winter, which can impede successful floral induction and initiation, resulting in unreliable flowering and irregular bearing (Menzel and Simpson, 1994). Various horticultural practices have been developed in the past to address this challenge. Due to the importance of shoot maturation prior to floral induction, many studies focused on manipulation of postharvest flush cycles and suppression or elimination of untimely flush growth prior to the floral induction period. Timely pruning (Olesen et al., 2013; Stern and Gazit, 2003) and nitrogen application (Cronje et al., 2017a; Huang and Chen, 2014; Stern and Gazit, 2003; Zhang et al., 1997) after harvest synchronized flush growth cycles and avoided

flushing prior to the induction period in countries such as China, Israel, Australia, and South Africa. Girdling after maturation of the last desired postharvest flush has also been used to suppress shoot growth in China (Li and Xiao, 2001; Zhang et al., 1997), Mauritius (Ramburn, 2001) and Australia (Menzel and Simpson, 1987). However, girdling was not reliable in ensuring shoot growth inhibition, nor can it substitute for inductive temperatures (Stern and Gazit, 2003). A more targeted approach to suppress autumn and winter shoot growth is autumnal water stress, which has been successfully used in Israel (Stern et al., 1998) Australia (Chaikiattiyos et al., 1994) and South Africa (Cronje et al., 2017b). However, unlike in citrus (Southwick and Davenport, 1986), water stress cannot replace inductive temperatures and induce flowers in litchi, but merely allows the tree to be at the correct phenological stage when inductive temperatures occur (Chaikiattiyos et al., 1994; Menzel et al., 1989; Stern and Gazit, 2003). A range of plant growth regulators (PGRs), including synthetic auxins, cytokinins and growth retardants, have also been evaluated either to inhibit new vegetative growth or to remove existing vegetative shoots by defoliation. Among them, ethephon (2-chloroethylphosphonic acid) alone or in combination with paclobutrazol [(2S,3S)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol] proved to be most efficient in many countries (Chaitrakulsub et al., 1992; Huang and Chen, 2014; Mandal et al., 2014; Olesen et al., 1999; Roets et al., 2010). Nevertheless, environmental conditions during and after application play a major role in the effectiveness of PGRs (Stover and Greene, 2005).

In South Africa, new shoot growth prior to the flower induction period is a common phenomenon in the main commercial cultivar ‘Mauritius’, and if not controlled, reduces flowering and yield. Since the early 2000s, South African litchi growers have been using the PGR ethephon for the chemical removal of untimely vegetative shoots (Roets et al., 2010). Ethephon, an ethylene-releasing compound, is easily absorbed by the small soft litchi leaves, resulting in leaf abscission (Roets et al., 2010; Sexton, 1998). Generally, ethephon is applied as a foliar spot spray, targeting only emerging young soft vegetative shoots during autumn and early winter (between end of March and early May). In most years, a maximum of three applications is sufficient to control all emerging flushes. Four to six weeks later, when temperatures have dropped consistently below the induction threshold, flower panicles appear from the axillary buds (Kift and Roets, 2001). In the past decades, changes in seasonal weather patterns have increasingly influenced phenological growth cycles of subtropical crops, including litchi (Nghi et al., 2020; Mitra, 2018; Sthapit et al., 2012). Late rainfall and delayed onset of chilling temperatures as well as inconsistent low winter temperatures have resulted in prolonged leaf flushing and higher incidences of leafy flower panicles. Under these circumstances, spot spray applications with ethephon were less effective, while being more time- and cost-consuming, due to the increased application frequency. In addition, inconsistent inductive temperatures caused the concomitant emergence of flower panicles (mostly leafy) and vegetative

shoots, making growers hesitant to apply ethephon due to concerns that existing flower panicles could be compromised. As ineffective ethephon treatments result in reduced flowering, yields and grower income, and in view of eminent and persistent climate change, a new approach to pre-flowering shoot control was necessary.

Therefore, this study aimed to investigate whether a single whole-canopy application of ethephon to fully mature terminal shoots is more effective in preventing the emergence of new vegetative shoot growth prior to floral induction than spot spray applications, which are conventionally used to eliminate the progression of already existing young vegetative shoots. We hypothesized that ethephon applied to mature shoots with dormant buds will induce bud dormancy and delay panicle emergence to a time when temperatures are more conducive for flower initiation, thus, leading to improved flowering and yields. The objectives of the study were: 1) to determine the optimal ethephon concentration and application date as related to the maturation time of the last postharvest flush, and 2) to assess the effect of ethephon treatment on vegetative shoot growth incidence prior to floral induction, time of panicle emergence, flower panicle characteristics, flowering, yield, and fruit quality.

Materials and Methods

Experimental sites and plant material. To compare production regions with different climatic conditions during the pre-flowering and flowering period, experiments were conducted in two climatically different regions in the Mpumalanga Province of South Africa. Experimental site 1, near Mbombela (latitude -25.453, longitude 30.945, elevation 680 m), is characterized by moderate autumns and cool winters. Experimental site 2, near Malalane (latitude -25.608, longitude 31.626, elevation 300 m), is considered a region with warm autumns and moderate winters. Daily minimum and maximum temperature and rainfall recordings were obtained from scientific-grade weather stations of the Agricultural Research Council – Institute for Soil, Climate and Water located within a 3 km radius of the Mbombela (station name: ITSC, # 30420) and the Malalane (station name: Mzinti, #30154) experimental site to calculate mean weekly temperatures and mean monthly rainfall between the postharvest shoot growth and flowering period (January to September) for each experimental site and year (Fig. 1).

The experiments were conducted between 2014 and 2017 on 14-year-old and 10-year-old ‘Mauritius’ litchi trees at the Mbombela and Malalane site, respectively. At the Mbombela site, the trees were planted on sandy soil (84% sand, 13% clay and 3% silt) at a planting distance of 6.0 × 6.5 m. At the Malalane site, the trees were planted on clay soil (49% clay, 44% sand and 7% silt) with a planting spacing of 7 × 12 m. At both sites, all experimental trees were selected for uniformity of tree size and phenological growth stage and were subjected to similar conventional

commercial farming practices with respect to fertilization (soil applications), irrigation (micro irrigation) and postharvest pruning. Water stress (~ 50% reduction of soil moisture content) was applied after hardening of the last desired postharvest flush until flower panicles appeared at both experimental sites and in all experimental years.

Treatments and experimental design. A preliminary trial was conducted on April 2, 2013 at Mbombela, about one month prior to flower panicle emergence, to determine the effect of ethephon on either dormant or actively growing terminal buds of less than 15 mm in length (Fig. 2B and 2D). Treatments included untreated trees and two foliar applications with ethephon [(Ethapon SL; Plaaskem (Pty) Ltd, Witfield, South Africa; containing 480 $g \cdot L^{-1}$ a.i.] at concentrations of 750 and 1000 $mg \cdot L^{-1}$, respectively. The foliar sprays were applied with a 2.5 L hand spray bottle (PolySpray P2; Efecto, South Africa) to the point of run-off. No wetting agent was added. Neighboring branches were shielded with a plastic sheet to avoid spray drift. The experiment was designed as a factorial design with two factors (bud stage and ethephon concentration). The treatments were laid out in a completely randomized design with 20 shoots per treatment selected from 12 trees at mid-canopy height.

Based on results and observations from the 2013 trial, treatments for 2014-2017 were adjusted and performed on whole trees at the above-mentioned experimental sites (Tables 1-2). The spray applications were carried out with a motorized sprayer and a hand lance fitted with a 2.5 mm nozzle, at a pressure of 15 bar. A wetting agent [Sanawett 90-940 SL; Dow AgroScience (Pty) Ltd, Bryanston, South Africa; containing 940 $g \cdot L^{-1}$ a.i.] was added to all spray solutions at a concentration of 10 $mL \cdot 100\ L^{-1}$ water. The applications were made during the morning (before 12h00 noon), taking care to avoid hot days. All applications were made to the point of run-off, at an approximate spray volume of 20-25 $L \cdot tree^{-1}$.

To account for seasonal differences in postharvest flush growth cycles, three different application dates were selected for the pre-induction period (T1: end of March or early April; T2: mid-April and T3: end-April or beginning of May). At each experimental site and within each application date, single whole-canopy applications with ethephon were applied at concentrations of 500, 750 and 1000 $mg \cdot L^{-1}$ (Eth500, Eth750 and Eth1000) to trees with fully mature terminal shoots (Fig. 2A) and terminal buds that were at the dormant (Fig. 2B) or at most at the bud swelling stage (Fig. 2C). To compare the whole-canopy treatments with the conventional shoot control practice used in the South African litchi industry, repeated ethephon spot sprays applied to immature soft vegetative shoots (Fig. 2F) at 1000 $mg \cdot L^{-1}$, whenever they emerged during the pre-floral induction period, were included as a Commercial Control. Each application date also included untreated trees (Untreated Control), which had fully hardened shoots and dormant

terminal buds at the start of each application date. Based on previous experience with ethephon under certain environmental conditions, e.g., temperature and soil moisture, additional treatment combinations were added at the individual trial sites. At the Mbombela site, a treatment addressing uneven shoot growth within trees (Whole-canopy+) was included. This entailed applying ethephon as a whole-canopy spray to trees that showed less than 10% new vegetative shoot growth before leaf unfolding (Fig. 2E) within the crown of the tree. As shoots at this early stage are not affected by ethephon, follow-up sprays after leaf unfolding were applied as targeted spot sprays to remove the immature vegetative shoots (Fig. 2F) (Table 1). At the Malalane experimental site, two whole-canopy ethephon applications, the first one at 1000 $mg \cdot L^{-1}$ followed by another one at 750 $mg \cdot L^{-1}$ two weeks later (Eth1000+750), were included to evaluate the effectiveness of a double application on trees grown in climates with higher winter temperatures (Table 2).

All experiments conducted between 2014 and 2017 were laid out in a randomized complete block design with five trees per treatment and five block replicates. Each experimental unit consisted of three trees, using the middle tree for data collection. Each experimental unit was separated by buffer trees. New experimental trees were chosen each successive year, to ensure that all experimental trees were at the same phenological growth stage for all application dates. As the experiments were conducted under open-field conditions, enough experimental trees at the designated phenological stage were not always available for each planned treatment and application date and therefore, an unbalanced trial design could not be avoided.

Phenological observations. Ten representative terminal shoot per tree were selected at mid-canopy height (approximately 1.5–1.8 m above the ground) and tagged. Tree response between spray applications and full bloom, i.e., female flower opening, was monitored at a 7-14-day interval. The time interval between treatment application and bud break and floral initiation stage, respectively, was calculated as the number of days between application and the visible appearance of at least 50% of shoots at the respective stage. The days to bud break (DTBB) and days to floral initiation stage (DTFI) served as demarcation points for the duration of the dormancy period and definite floral initiation, respectively. The bud break stage was defined as terminal buds starting to swell and turn color from brown to green (Zhang et al., 2016). The floral initiation stage, later referred to as panicle emergence, was defined as inflorescence primordia becoming visible in the leaf axils in the form of millet-like whitish hairy structures, following a period of inductive temperatures (Huang and Chen, 2005). The percentage of vegetative shoots, flowering shoots, and leafy panicles was calculated from the total number of tagged shoots. During the second season (2015), it was observed that treated trees had a higher number of panicles per terminal shoot, and visibly shorter panicles compared with untreated trees. Therefore, the number of flower panicles

per tagged shoot (including those arising from axillary buds) was counted and the length of the longest panicle per shoot was measured during full bloom in 2015 to 2017, at both sites.

Yield and fruit quality. Fruit harvest was generally carried out over two harvest dates. At each harvest date, the weight of the harvested fruit was recorded for each experimental tree and total yield per tree calculated at the end of the harvesting period. A representative sample of 50 fruit per tree was collected at the main harvest for fruit quality assessments. Fruit mass and size were determined for each individual fruit. Fruit diameter was measured at the widest part of the fruit (shoulder) to determine fruit size using a generic digital caliper. The total soluble solids concentration (TSS) of unfiltered juice from 10 peeled and de-seeded litchi fruit was measured using a digital refractometer (PAL-1; ATAGO, Tokyo, Japan). The same juice sample was used to determine titratable acid content (TA) by titration with 0.1562 N sodium hydroxide. Fruit maturity was calculated as the ratio of TSS to TA.

Statistical analysis. All data was subjected to analysis of variance (ANOVA) using the General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4, SAS Institute Inc, Cary, USA). Data collected over time was subjected to repeated measures analysis using SAS software with application date, treatment, and application date × treatment as fixed factors. Where ANOVA indicated significance at $P < 0.05$, a comparison of treatment means was done using Fisher's least significant difference (LSD) test at a significance level of 5%, unless stated otherwise. The Shapiro-Wilk and Levene's tests confirmed normality of the data and homogeneity of variances, respectively, except for percentage of flush growth due to a high number of zero values. ARCsine transformation of flush data did not improve homogeneity and therefore, flush data is presented untransformed. Because kurtosis values, i.e., symmetrical distribution, were larger than skewness values, normality was accepted. Means across the four experimental years were calculated according to the available data, within application dates and per year, to gain insight into trends that emerged over time for the various treatments. Agglomerative hierarchical cluster analysis (AHC) with Ward's minimum variances method on principal component analysis (PCA) factor scores was performed on the 4-year mean data to investigate possible differences between experimental sites using XLSTAT (Version 2020.5.1.1047, Paris, France).

Results

2013 Pilot Study

Application of 750 and 1000 mg·L^{-1} ethephon to shoots with dormant terminal buds inhibited bud growth for up to 28 days following application, only allowing up to 15% new bud growth. However, untreated buds commenced growth immediately, with 60% and 95% of the buds having

initiated growth by day 28 and 36, respectively (Fig. 3A). Ethephon treatment of shoots with actively growing, green buds arrested most bud growth (Fig. 3B), visible as treated buds reverting to a dormant state with brown appearance (Fig. 2B). After about two weeks, however, bud growth in ethephon-treated buds resumed, with more than 60% of buds actively growing by day 36, and 100% of buds growing by day 52 following treatment. Furthermore, it was observed that actively growing buds with slightly elongated rudimentary leaves (Fig. 2E) were not affected by ethephon sprays and continued to grow uninterrupted. New vegetative shoot growth was only observed in the group with actively growing buds and resulted in 20% and 5% flush growth for the control and the 750 $mg \cdot L^{-1}$ ethephon treatment, respectively (data not shown). Flowering rates ranged between 90% and 100%, with no significant differences between treatments (data not shown). Overall, ethephon applications did not cause any drop of older leaves on terminal or proximal shoots.

2014-17 Experiments

Weather conditions at experimental sites during the trial period. At both sites, the weather conditions between the pre-floral induction and flowering period (March to September) varied between years, with distinct differences during certain periods (Fig. 1A and 1B). Overall, trends observed in temperature fluctuations and rainfall incidences were similar for both sites, but with the Malalane site being on average 3 to 4°C warmer and having higher rainfall than the Mbombela site. The temperature drop below the 20°C-threshold for floral induction in 'Mauritius' (Batten and McConchie, 1995) occurred about one month earlier at the Mbombela site (May) than at the Malalane site (June). Temperatures prior to floral induction were lowest in 2014, and highest in 2016 and 2015 for April and May, respectively. The lowest winter temperatures for June were recorded in 2014 and 2015, and for July in 2014 and 2016. In addition, 2015 and 2017 were characterized by below-average rainfall during the summer months, but with above-average rainfall in May, at both sites.

Effect of ethephon and weather conditions on shoot growth pattern. In 2014, shoot growth of untreated trees (Untreated Control) at the Mbombela site depended on the prevailing temperatures at the start of each application date and therefore, was high for the treatment period starting at the end of March (T1) (Fig. 4A; Table 3). Shoot growth was reduced for the application date in mid-April (T2) (Fig. 4B) and negligible for the treatment period starting at the end of April (T3) (Fig. 4C). In contrast, no shoot growth was observed in the Untreated Control during April and May at the warmer Malalane site (Fig. 4D). The commercial ethephon treatment applied as a spot spray (Commercial Control) was effective at both sites, provided several applications were made to ensure successful shoot elimination (Tables 1 and 2; Fig. 4). The Whole-canopy+ treatment at the Mbombela site successfully eliminated existing immature vegetative shoots and prevented further

new flush development prior to or during the flower induction period (Fig. 4A). However, where active buds displayed elongated rudimentary leaves (Fig. 2E) similar to observations made in the pilot study, the ethephon sprays were ineffective and required a re-application when the leaves had fully unfolded. Yet, all single whole-canopy ethephon applications (Eth500, Eth750, Eth1000) effectively suppressed new flush growth during the induction period, for all application dates and both experimental sites (Fig. 4). At the warmer Malalane site, the double application of ethephon at 1000 mg·L^{-1} followed by an application at 750 mg·L^{-1} two weeks later (Eth1000+750) showed the best inhibitory effect compared with other whole-canopy treatments (Fig. 4D). However, at the cooler Mbombela site, it was observed that ethephon applied at 750 and 1000 mg·L^{-1} at the end of April (T3), when temperatures had already dropped below the average of 20°C, resulted in new shoot growth after the induction period (July) compared with the other ethephon treatments (Fig. 4C).

In 2015 at the Mbombela site, initial flush growth during April was considerably lower compared with flushing for the same period in 2014, due to drought conditions in combination with overall higher temperatures (Fig. 1A). The impact of temperature differences on tree phenology between sites was noticeable in the flushing intensity of the Untreated Controls at the mid and late application dates, which was more vigorous during May and June at the Malalane site compared with the Mbombela site (Fig. 5B-E). Spot spray applications in the Commercial Control had to be repeated several times at both sites, yet flushing still occurred during the induction period (May and June), as spot spraying was discontinued in early May, so as not to compromise early emerging flower panicles. At the Mbombela site, the Whole-canopy+ treatment proved to be more effective in terms of shoot inhibition than the Commercial Control (Fig. 5A). Likewise, all the single whole-canopy applications at all application dates at Mbombela almost completely inhibited vegetative shoot growth prior to and during the induction period. However, at the Malalane site, the lower ethephon concentrations (500 and 750 mg·L^{-1}) could not suppress new flush growth during the floral induction period as effectively as the higher concentrations (1000 mg·L^{-1} and 1000 mg·L^{-1} followed by 750 mg·L^{-1}).

In 2016, high temperatures during April caused 70-95% flush growth in the Untreated Control during the early treatment period (T1) at both experimental sites (Fig. 1, Fig. 6A and 6C). Similarly, high initial flush growth was recorded in the Commercial Control before the spot sprays were applied. However, repeated spot spray applications (as much as four times) in the Commercial Control as well as in the Whole-canopy+ treatment successfully reduced and inhibited new flush growth. In contrast, the lowest ethephon concentration of 500 mg·L^{-1} (Eth500) could not inhibit flush growth under these weather conditions when applied early (end of March; T1) and still resulted in up to 50% new flush growth during the pre-induction period at both sites. Even the

Eth750 and Eth1000 treatments at both sites, and the Eth1000+750 treatment at Malalane were not efficient enough to inhibit flush growth entirely at the early application date (Fig. 6A and 6C). Yet, single whole-canopy applications at the end of April (T3) fully suppressed any new flush growth, at both sites (Fig. 6B and 6D). Likewise, untreated trees did not exhibit any shoot growth during the treatment period starting at the end of April.

In 2017, initial vegetative shoot growth in the Commercial Control and Whole-canopy+ treatments at the end of March (T1) was similar to that observed in trees in 2016 at the Mbombela site, where both treatments successfully reduced existing flush growth during April (Fig. 7A). However, after the last spot spray application at the end of April, both treatments could not prevent new flush growth during May and June, when above-average temperatures and rainfall prevailed (Fig. 1A). Under these weather conditions, the Whole-canopy+ treatment proved to be more effective than the Commercial Control (Fig. 7A). In contrast, the single whole-canopy applications inhibited flush growth almost completely for the entire observation period (Fig. 7A). Extremely late whole-canopy ethephon applications towards the end of May (T3) at the Mbombela site inhibited new flush growth during the warm period of June, but then allowed new flush growth from mid-July onwards (Fig. 7B), similar to tree responses seen during the late application date in 2014. A high flushing rate was also observed in untreated trees from June onwards. At the Malalane site, the Commercial Control as well as the single whole-canopy applications reduced existing flush growth and inhibited new growth to less than 6%, despite the persistent high temperatures for both the mid-April and early-May application periods. The Eth1000 and Eth1000+750 treatments prevented new flush growth entirely, whereas 30% of new shoot development was observed in untreated trees for these periods (Fig. 7C and 7D).

Effect of ethephon on bud dormancy and panicle emergence. The inhibitory effect of whole-canopy applications (including the Whole-canopy+ treatment) caused prolonged bud dormancy and resulted in delayed bud break and panicle emergence, i.e., floral initiation, although only significant in certain years (Tables 4 and 5). In general, the number of days to bud break following application increased in a dose-dependent manner within each application date and was generally higher in whole-canopy treatments than in the Commercial Control at both sites. Hence, frequent spot spray applications of the Commercial Control did not effectively delay bud break. Likewise, untreated trees showed early bud break for all application dates, except in 2016 at the earliest application date (T1), when persistent high temperatures allowed untreated trees at both sites to complete their shoot growth cycle during April and early May (Fig. 6A and 6C). Consequently, this permitted floral induction and initiation at a later stage when temperatures had dropped sufficiently (Table 6 and 7). A less obvious dose-dependency was observed for panicle emergence. The time interval

between application and panicle emergence in the whole-canopy ethephon treatments was similar to the Commercial and the Untreated Control, despite later bud break. This can be attributed to ethephon whole-canopy applications reducing the interval between bud break and panicle emergence, also in a dose-dependent manner (Tables 4 and 5). In general, panicle emergence took longer at the warmer Malalane site compared with the cooler Mbombela site.

Ethephon effects on flower panicle characteristics and flowering. Flower panicle characteristics were influenced by treatment and application date effects (Tables 6 and 7). Whole-canopy ethephon applications (including the Whole-canopy+ treatment) reduced the percentage of leafy panicles compared with the Commercial Control at the Mbombela site. However, at the Malalane site, the spot spray applications of the Commercial Control started later (mid-April) for two out of the four years (Table 2), and therefore, the trees in this treatment were subject to different temperature regimes compared with trees at the Mbombela site. At both sites, the percentage of leafy panicles tended to decrease with increasing ethephon concentrations and as the application date advanced, with some exceptions. A similar dose-time response was observed for panicle length at both sites (Tables 6 and 7). Overall, the longest panicles at both sites were observed in the Commercial Control, in the Untreated Control and in the whole-canopy treatments applied at low ethephon concentrations. Panicle length was generally longer at the warmer Malalane site compared with the cooler Mbombela site. Furthermore, it was observed that the number of panicles, including those developing from axillary buds, was generally higher in ethephon-treated trees (including the Commercial Control) compared with untreated trees, at both sites (Tables 6 and 7). The number of panicles per shoot also tended to increase in a dose-dependent manner in most years, particularly at the cooler Mbombela site.

The flowering rate at both sites depended greatly on the flushing rate during the induction period as well as the application date, and was generally lowest for the Untreated Controls, except in 2014 and 2016 at the latest application dates (Tables 6 and 7). Significant differences between treatments were more apparent at the warmer Malalane site compared with the cooler Mbombela site. It was also noted that during colder winters, e.g. at the Mbombela site in 2014 and 2016, late whole-canopy applications at high ethephon concentrations reduced the flowering rate compared with treatments at low ethephon concentrations. In contrast, during warmer winters (2015 and 2017) late applications at higher ethephon concentrations led to better flowering rates than lower concentrations, particularly at the Malalane site. The Commercial Control showed similar flowering rates compared with the whole-canopy applications, at both sites.

Ethephon effects on yield and fruit quality characteristics. Yield corresponded with trends in flowering rates, particularly for the Malalane site (Tables 8 and 9). For the earliest application date

(T1), yields tended to increase with increasing ethephon concentrations, at both sites. However, for the mid- and late application dates (T2 and T3), lower ethephon concentrations appeared to produce higher yields compared with trees that received higher concentrations, except during warm winters (e.g., 2015 and 2017), where treatments with higher ethephon concentrations produced better yields. This was particularly evident for the warmer Malalane site. At both sites, the Commercial Control performed weaker in terms of yield during years with warmer winters (2015 and 2017), but showed comparable yields in years with cooler winters (2014 and 2016) when compared with the whole-canopy applications. Untreated trees showed the lowest yields overall within each application date, year and experimental sites.

Whole-canopy ethephon applications did not influence fruit mass and size in individual years compared with the Commercial Control (Tables 8 and 9). However, untreated trees tended to bear fruit with lower fruit mass and size compared with all ethephon treatments (including the Commercial Control) within each application date, year and experimental site. Likewise, fruit maturity (TSS/TA ratio) did not differ between all ethephon treatments, but untreated trees showed consistently lower TSS/TA ratios compared with ethephon treatments.

Main treatment and application date effects. An evaluation of the consolidated data to elucidate general treatment and application date effects across years was attempted, considering that the datasets for both sites were statistically unbalanced. The combined data revealed that despite climatic differences between the sites, the treatment effects were still comparable. Across application dates and years, single whole-canopy applications performed equally or better than the Commercial Control at both sites, particularly in inhibiting vegetative shoot growth during flower induction, in delaying panicle emergence, in reducing the percentage of leafy panicles and in increasing yield (Table 10). Likewise, the Whole-canopy+ treatment at the Mbombela site was comparable to the single whole-canopy applications. The double ethephon application at the Malalane site (Eth1000+750 treatment) achieved the same or better results than single whole-canopy applications, particularly in terms of delaying bud break and floral initiation. No clear treatment differences between spot spray and whole-canopy applications were observed for flowering rate at both sites (Table 10). However, untreated trees had significantly lower flowering rates compared with ethephon-treated trees, which was associated with their significantly higher flushing rates.

When comparing the two sites in terms of application date, irrespective of treatment and year, it was evident that application date exerted its own effect, which may be attributed to temperature differences between application dates (Tables 3 and 11). Significant differences were particularly evident at the Mbombela experimental site. Different trends in the percentage of leafy panicles

between the two experimental sites were observed and may be related to temperature differences between sites. While application date did not appear to influence flowering rate and yield at the warmer Malalane site, early (T1) and mid (T2) application dates favored flowering and yield at the cooler Mbombela site.

Agglomerative hierarchical clustering (AHC) identified two main clusters at the Mbombela site, one cluster consisting of all untreated trees (black-colored cluster) and one cluster consisting of all ethephon treatments (red-colored cluster) (Fig. 8A). Within the ethephon-cluster, two further sub-clusters were identified according to application dates, whereby the whole-canopy applications at the early (T1) and mid (T2) application dates and the Commercial Control were separated from the late (T3) whole-canopy applications. In general, the clusters were listed according to effectiveness with the least effective treatments (Untreated Controls) at the far left and the most effective treatments (early whole-canopy treatments) at the far right of the dendrogram. At the Malalane site, AHC identified the same two main clusters, one with the Untreated Controls (black-colored group) and one with the whole-canopy treatments and Commercial Control (red-colored cluster). However, dissimilarities between treatments within the ethephon-cluster were more homogenous than at the Mbombela site (Fig. 8B). The same ranking according to efficacy of treatments was detected as for the Mbombela site. However, the Commercial Control was grouped together with the whole-canopy treatments sprayed at the late (T3) application date, while at the Mbombela site, this treatment was grouped together with the whole-canopy treatments sprayed at the early (T1) application date.

Discussion

Environmental influence on shoot growth and ethephon efficacy. In general, the effects of ethephon are attributed to ethylene, the active compound of ethephon (Liu et al., 2021). Apart from the concentration, the efficacy of ethephon depends on environmental conditions during and after application (Klein et al., 1978; Olien and Bukovac, 1978; Stover and Greene, 2005; Walter and Leopold, 1969; Walters and Lopez, 2018). Particularly, temperature increases within the temperature range of 10-34°C accelerate the rate of ethephon degradation and ethylene evolution (Olien and Bukovac, 1978; Stover and Greene, 2005; Walters and Lopez, 2018). In our study, above-average temperatures during April and May, particularly in 2016, may have increased ethylene evolution and thus, reduced the inhibitory effect of ethylene. This was evident by increased flushing incidence and earlier bud break compared with the same treatment concentrations applied during periods with moderate temperatures (Tables 3-5; Fig. 1, Figs. 3-7). In this regard, a double application (Eth1000+750) prolonged the inhibitory effect of ethephon and avoided bud break and shoot growth under high temperature conditions. In contrast, under

decreasing temperatures, such as experienced during the late application dates in 2014 and 2017 at Mbombela, it is likely that slower ethephon degradation extended the inhibitory effect on bud growth beyond the inductive period, which resulted in new flush growth during early spring instead of panicle development, thereby reducing flowering and yield (Figs. 4C and 7B). Therefore, our study showed that ethephon applications are not required, and can even be disadvantageous, when temperatures are already sufficiently low for floral induction. Considering the prevailing temperatures at the time of application as well as forecasted temperatures will be key in choosing the appropriate concentration for the desired effect on shoot inhibition and bud break delay. Additional studies on ethylene evolution in leaves and terminal buds following ethephon application will provide more insight into these temperature-driven interactions in litchi.

Furthermore, severe drought and chilling can prevent litchi floral bud development regardless of the success of floral induction and lead to crop failure (Huang and Chen, 2005). The influences of drought conditions on shoot growth were also observed during the current study, in particular during 2015 and 2017 (Figs. 1, 5 and 7). The drought conditions noticeably reduced the flushing rate in the Untreated Control, particularly at the Mbombela site, which is characterized by sandy soils with low water-holding capacity. These effects were less pronounced on the heavy clay soil at the Malalane site, which has a higher water-holding capacity. Furthermore, autumnal water stress applied as part of standard orchard practices may also have exacerbated the inhibitory effect of ethephon in the treated trees in those years. Similarly, Klein et al. (1978) found that withholding irrigation prior to applying ethephon increased the effect of ethephon to aid the harvesting of olive fruit. The effect of soil moisture on the efficacy of ethephon spot sprays for eliminating immature vegetative shoots has been known in litchi (Roets et al., 2010). Therefore, specific recommendations have been added to the product label. However, further studies are required to elucidate the effect of various levels of soil moisture on the duration of the growth-inhibiting effect of ethephon when applied to mature shoots with dormant buds, both under warmer and cooler conditions. Such knowledge will be useful to provide comprehensive guidelines for growers.

Effect of ethephon treatment on bud dormancy. Ethephon exhibited a strong inhibitory effect on bud development in a dose-dependent manner, with higher concentrations generally exerting a stronger inhibitory effect than lower concentrations. A similar concentration effect of ethephon was found when used to delay bloom in peaches (Liu et al., 2021). Results from the 2013 pilot study and subsequent experiments revealed that the application of ethephon to mature shoots only affected the apical bud meristem, subsequently preventing bud break or arresting initial bud growth, respectively, for several weeks compared with untreated shoots of the Untreated and the Commercial Control (Figs. 3-7). Conversely, none of the ethephon concentrations used in the

current study (500-1000 mg·L^{-1}) caused any drop of mature leaves on terminal or proximal shoots. Ethylene is known to inhibit cell division and meristematic growth in shoots and axillary buds (Burg, 1973). Both ethylene and ethephon treatments have been associated with growth inhibition and delay of bud growth in several other crops (Abeles et al., 1992; Durner and Gianfagna, 1991; Hansen and Grossmann 2000; Liu et al., 2021). As ethephon application to mature shoots with dormant buds inhibited the untimely emergence of new vegetative shoots when the appropriate concentration was used, particularly during warm winters, it proved to be a more efficient use of ethephon in litchi compared with the conventional spot spray applications (Commercial Control), which only targeted immature shoots, but allowed the untreated mature shoots within the same tree to commence growth at any time. Likewise, bud growth in the mature shoots of Commercial Control trees progressed earlier in comparison with the mature shoots of untreated trees, particularly at early application dates (T1 and T2), as the trees in the Commercial Control were overall more advanced in shoot maturity and readiness to flush compared with untreated trees that had just reached shoot maturity when observations started (Tables 4 and 5).

Effective whole-canopy applications require uniform orchards and therefore good orchard management that ensures synchronized shoot growth (Huang and Chen, 2014). When bud development at the time of application was too far advanced (extended rudimentary leaves before leaf unfolding; Fig. 2E), as observed in the Whole-canopy+ treatment at the Mbombela site, application of ethephon could not prevent further growth. The insensitivity of these small emerging shoots to ethylene may possibly be associated with a shift in hormone ratios in favor of growth promoters, e.g., gibberellic acid (Davenport and Stern, 2005; Pierik et al., 2006). It is also known from other crops that the efficacy of ethephon depends on the age and anatomy of the targeted plant organ (Dubois et al., 2018; Stover and Greene, 2005). In our study, a re-application of ethephon to these shoots was then necessary to completely remove the vegetative shoots once the leaves had fully unfolded (Fig. 2F and 2G), as soft immature litchi leaves are most sensitive to ethylene and will abscise after exposure to ethylene (Roets et al., 2010). Nevertheless, by inhibiting bud break of dormant buds on lateral branches, the Whole-canopy+ treatment achieved equal or better shoot inhibition during warm years compared with the Commercial Control and whole-canopy applications at low ethephon concentration, respectively, despite the need for re-applying ethephon to some of the shoots.

Effect of ethephon treatment on panicle emergence. In general, buds emerging during an earlier period were more likely to be affected by intermittent warm temperatures. As mentioned above, early bud development was associated with the Commercial and the Untreated Control, as untreated mature shoots within the same tree could start their growth unrestricted, hence, leading to new

vegetative growth when emerging early under warm temperature conditions, or leading to early flower panicle development when emerging later under intermittent low temperatures. In contrast, whole-canopy ethephon applications delayed bud break and panicle emergence in a dose-dependent manner within each application date and when compared with the Commercial and the Untreated Control (Tables 4, 5 and 10). The later application dates, however, decreased the time interval between application and bud break or panicle emergence when compared with earlier application dates, despite the trees being at the same phenological stage at the time of application (Tables 4, 5 and 11). This was evident for both treated and untreated trees and may indicate that terminal buds either were inherently more inclined to start growing closer to the induction period, and/or were more responsive to the lower inductive temperatures that prevailed with later application dates. Ethephon treatment has been used in various deciduous fruit and nut trees to delay bud break and flowering to increase cold hardiness and avoid frost damage in spring (Askari et al., 2011; Crisosto et al., 1990; Ebel et al., 1999; Liu et al., 2021; Proebsting and Mills, 1973). When applied at the dormant bud stage, ethephon also successfully delayed bud break and bloom in cherry (Gianfagna et al., 1992), peach (Liu et al., 2021) and pistachio (Askari et al., 2011), and reduced bud sensitivity to elevated temperatures and the associated de-acclimation conditions after mid-winter thaws. This effectively increased the chill requirement of ethephon-treated trees. Similarly, by delaying bud break to a period with lower temperatures, whole-canopy ethephon treatments applied to mature shoots with dormant buds may have increased chill hour accumulation in our study and thus possibly amplified the inductive stimulus in emerging litchi buds, resulting in a higher percentage of pure flower panicles (Tables 6, 7 and 10). In addition, the shorter time interval between bud break and panicle emergence caused by the whole-canopy ethephon applications (Tables 4 and 5) suggests that ethylene might promote the transition from vegetative to floral bud, i.e., floral morphological differentiation.

Apart from delayed panicle emergence, our study found that ethephon treatment to dormant buds caused axillary bud break and a significant increase in the number of flower panicles per shoot (arising from both terminal and axillary buds) several weeks after treatment, when compared with untreated shoots. To our knowledge, no other reports of ethylene affecting axillary bud break in litchi are known. According to Huang and Chen (2005), axillary buds in litchi also have the ability to induce flower panicles when exposed to inductive temperatures. However, natural axillary bud break occurring in litchi is generally attributed to the retardation of the terminal bud caused by strong chilling, leading to the reduction or loss of apical dominance (Lynch 1958; Menzel and Simpson 1988; O'Hare, 2002). It is possible that ethephon application to dormant buds in our study occasionally resulted in the termination of the apical bud with subsequent axillary bud break. However, our general observations could not confirm this. Other mechanisms may have been at

play, such as those described by Burg (1973) who reported for petunia plants and pea seedlings that, despite ethylene inhibiting bud growth of both apical and axillary buds while present, all axillary buds were released from apical dominance once ethylene was removed. This effect may be ascribed to ethylene restricting the polar auxin transport system or possibly affecting auxin synthesis via increased abscisic acid levels (Abeles et al., 1992; Hansen and Grossmann 2000; Pierik et al., 2006). In contrast, the increased number of panicles arising from axillary buds observed in the Commercial Control was caused by the chemical removal of the entire immature terminal shoot and therefore the removal of apical dominance (Davenport and Stern, 2005; Roets et al., 2010; Shimizu-Sato and Mori, 2001; Taiz et al., 2015).

Effect of ethephon on panicle and fruit quality. Delayed bud break and panicle emergence did not result in delayed full bloom date (data not shown) and few significant differences in flowering rate were observed between the whole-canopy treatments and the Commercial Control. However, whole-canopy applications, in particular the higher concentrations, reduced the percentage of leafy panicles (at the Mbombela site) as well as panicle length (at both sites) compared with the Commercial Control. This can be attributed to the delayed bud break in whole-canopy-treated trees, which generally occurred during a period of lower temperatures (Tables 6, 7 and 10). The temperature effect on length and leafiness of panicles was also evident when comparing the overall effect of application date on these parameters (Tables 3 and 11). Both ethephon treatment (Subhadrabandhu and Koo-Duang, 1987) and temperature (Chaikiattiyos et al., 1994; Chen et al., 2013, 2016; Menzel and Simpson, 1992) were also previously found to reduce panicle length and leafiness in litchi. Buds emerging under lower temperatures were shown to abort the rudimentary leaves in the mixed bud, giving rise to pure leafless panicles (Huang and Chen, 2005; Zhou et al., 2008). Late-emerging panicles tend to be shorter as their development is initiated closer to spring, compared with early emerging panicles that develop under lower temperatures and over a longer period (Huang and Chen, 2005; Menzel and Simpson, 1992). Reduced leafiness and/or panicle length has great relevance for litchi production. Both shorter and leafless flower panicles were found to produce more female flowers and fruit than leafy panicles (Huang and Chen, 2014; Lee and Chang, 2019; Menzel and Simpson, 1992). Delayed bloom following ethephon application also increased flower bud density in blueberry (Krewer et al., 2005). Other studies also reported increased yields in various fruit crops because of ethephon-mediated bloom delay (Askari et al., 2022; Proebsting and Mills, 1973). Shorter, leafless panicles together with a higher number of panicles per shoot could also have been the main contributing factor to the overall higher yields in the whole-canopy ethephon treatments in the current study. In contrast, untreated trees could not achieve good flowering or yield under the less favorable climatic conditions experienced during

the trial period (Tables 8-10). This was mainly due to high flushing rates during autumn and early winter (Figs. 4-7).

Delayed panicle emergence in whole-canopy treatments did not affect fruit quality (fruit maturity, mass and size) compared with the Commercial Control (Tables 8 and 9), because of accelerated panicle development once emerged (Tables 4 and 5). Although only significant for the 4-year means across application dates and years (Table 10), all ethephon treatments tended to advance fruit maturity compared with the Untreated Control. Several studies that used ethephon for bloom delay also did not record differences in fruit quality, although some recorded a delay in fruit maturity (Crisosto et al., 1990; Ebel et al., 1999; Krewer et al., 2005; Liu et al., 2021).

Agglomerative hierarchical cluster analysis confirmed the trends seen in the absolute data and was able to highlight differences between both sites, based on the measured parameters and climatic differences. Untreated Controls were clearly separated from the ethephon treatments at both sites, thus, highlighting the inability of untreated trees to maintain productivity under warmer climates (Fig. 8). Furthermore, AHC categorized the most suitable treatment concentrations and application dates for each site, which may be used as a general guideline, despite the variation in yearly weather patterns. The sub-clustering within the ethephon-cluster at the cooler Mbombela site also confirmed that early ethephon applications in this production region are more beneficial for timely shoot inhibition and production improvement than late applications (Fig. 8A), as floral induction occurs about one month earlier compared with the warmer Malalane area (Cronje et al., 2017b). In contrast, no obvious sub-clusters within the ethephon-cluster were found at the warmer Malalane site, which suggests that the efficacy of whole-canopy ethephon treatments may be more variable under warmer climates and requires high concentrations for both early and late applications dates (Fig. 8B).

Conclusion

The current study revealed that ethephon applied as whole-canopy spray to trees with mature shoots provided a more reliable response in terms of bud dormancy and shoot growth inhibition prior to floral induction compared with the conventional spot spray applications that only target existing immature vegetative shoots. Furthermore, whole-canopy applications delayed panicle emergence to a period with consistently lower temperatures. Although this did not significantly increase flowering rates compared with conventional spot spray applications, it did reduce the percentage of leafy panicles and contributed to improved yields without affecting harvesting time and fruit maturity. In contrast, untreated trees were unable to maintain productivity under warm climates. Furthermore, ethephon concentration, prevailing temperatures during and after application, as well as application date, i.e., time interval prior to the occurrence of floral inductive

temperatures, affected treatment responses, and therefore need to be considered to ensure optimum efficacy. In this regard, early applications and warm weather conditions required higher ethephon concentrations (1000 mg·L^{-1}) to achieve the same level of shoot inhibition and panicle delay compared with lower ethephon concentrations (500-750 mg·L^{-1}) used at later application dates or under moderate weather conditions. A double application provided an extended dormancy period to mitigate the higher temperatures at the warmer production region. Late ethephon applications at high concentrations under already prevailing low temperatures should be avoided as they cause an excessive inhibitory effect and prevent floral induction, thus, reducing flowering. Sound general orchard management practices that will ensure synchronized shoot growth for whole-canopy applications, knowledge of floral induction and initiation periods for each specific production region, as well as close monitoring of weather conditions and forecasts will therefore greatly assist growers in making the most appropriate choice on the optimum application timing and ethephon concentration. Further research is required to elucidate the subsequent effects of ethephon-mediated shoot growth inhibition on flowering-related physiological responses, such as potential carbohydrate accumulation, as well as the effect of ethephon on molecular mechanism involved in floral induction and initiation.

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Table 1. Treatments applied at an experimental site with cool winters near Mbombela, South Africa, during autumn and/or early winter of 2014 to 2017. Treatments consisting of ethephon applied as spot spray to immature vegetative shoots or as whole-canopy sprays to mature shoot at different concentrations and application dates, to evaluate its efficacy for pre-induction shoot inhibition in 'Mauritius' litchi trees. Untreated trees served as control and were included on all application dates.

Treatment code	Treatment description	Phenological growth stage at time of application	Application frequency / date			
			2014[i]	2015[ii]	2016[iii]	2017[iv]
Commercial Control	1000 mg·L^{-1} ethephon as repeated spot sprays	Soft immature vegetative shoots	3[v]	3	4	2
Whole-canopy+	1000 mg·L^{-1} ethephon as whole-canopy spray at first signs of vegetative growth, followed by 1000 mg·L^{-1} as spot sprays whenever new vegetative shoots emerged	<10% newly emerging vegetative shoots, the remainder comprising mature shoots with dormant buds, with follow-up applications on soft vegetative shoots	3	3	4	2
Untreated Control	No ethephon application	Mature dark green shoots with dormant buds	T1, T2, T3 [vi]	T1, T2, T3	T1, T3	T1, T3
Eth500	500 mg·L^{-1} ethephon as single whole-canopy spray	Mature dark green shoots with dormant buds	T1, T2, T3	T1, T2, T3	T1, T3	T1, T3
Eth750	750 mg·L^{-1} ethephon as single whole-canopy spray	Mature dark green shoots with dormant buds	T1, T2, T3	T1, T2, T3	T1	T1
Eth1000	1000 mg·L^{-1} ethephon as single whole-canopy spray	Mature dark green shoots with dormant buds	T1, T2, T3	T1, T2, T3	T1, T3	T1, T3

[i]2014 spray dates: 25 March (T1), 8 April (T2) and 25 April (T3).
[ii]2015 spray dates: 30 March (T1), 13 April (T2) and 28 April (T3).
[iii]2016 spray dates: 23 March (T1) and 4 May (T3).
[iv]2017 spray dates: 30 March (T1) and 23 May (T3).
[v]Number of spot sprays applied to the same trees during the application period of March to May.
[vi]Date of application (ethephon treatments) or monitoring start (untreated trees) within experimental year, i.e., T1: early, T2: mid and T3: late application date (as indicated in i-iv).

Table 2. Treatments applied at an experimental site with moderate winters near Malalane, South Africa, during autumn and/or early winter of 2014 to 2017. Treatments consisting of ethephon applied as spot spray to immature vegetative shoots or as whole-canopy spray to mature shoots at different concentrations and application dates, to evaluate its efficacy for pre-induction shoot inhibition in 'Mauritius' litchi trees. Untreated trees served as control and were included on all application dates.

Treatment code	Treatment description	Phenological growth stage at time of application	Application frequency / date			
			2014[i]	2015[ii]	2016[iii]	2017[iv]
Commercial Control	1000 mg·L^{-1} ethephon as repeated spot sprays	Soft immature vegetative shoots	2[v]	2	3	2
Untreated Control	No ethephon application	Mature dark green shoots with dormant buds	T1[vi]	T2, T3	T1, T3	T2, T3
Eth500	500 mg·L^{-1} ethephon as single whole-canopy	Mature dark green shoots with dormant buds	Not applied	T2, T3	T3	T2, T3
Eth750	750 mg·L^{-1} ethephon as single whole-canopy	Mature dark green shoots with dormant buds	T1	T2, T3	T1	T2, T3
Eth1000	1000 mg·L^{-1} ethephon as single whole-canopy spray	Mature dark green shoots with dormant buds	T1	T2, T3	T1, T3	T2, T3
Eth1000+750	1000 mg·L^{-1} ethephon as whole-canopy spray, followed by 750 mg·L^{-1} as whole-canopy spray two weeks later	Mature dark green shoots with dormant buds	T1	T2	T1	T2

[i]2014 application date: 3 April (T1).
[ii]2015 application dates: 15 April (T2) and 30 April (T3).
[iii]2016 application dates: 31 March (T1) and 5 May (T3).
[iv]2017 application dates: 18 April (T2) and 3 May (T3).
[v]Number of spot sprays applied to the same trees during the application period of March to May.
[vi]Date of application (ethephon treatments) or monitoring start (untreated trees) within experimental year, i.e., T1: early, T2: mid and T3: late application date (as indicated in i-iv).

Table 3. Mean daily temperature on the day of application of different ethephon and untreated control treatments during the years 2014 to 2017. Treatments were applied during early (T1; end-March or early-April), mid (T2; mid-April) or late (T3; end-April or early-May) autumn and early winter, respectively.

	Mean daily temperature on day of application (°C)			
Application period	2014	2015	2016	2017
	Mbombela experimental site			
Early (T1)	23.1 (25 Mar)[i]	25.3 (30 Mar)	23.1 (23 Mar)	24.8 (30 Mar)
Mid (T2)	18.7 (08 Apr)	22.2 (13 Apr)	n/a[ii]	n/a
Late (T3)	18.9 (25 Apr)	20.6 (28 Apr)	18.8 (04 May)	20.4 (23 May)
	Malalane experimental site			
Early (T1)	27.7 (03 Apr)	n/a	29.9 (31 Mar)	n/a
Mid (T2)	n/a	23.9 (15 Apr)	n/a	25.8 (18 Apr)
Late (T3)	n/a	24.8 (30 Apr)	22.8 (05 May)	24.6 (03 May)

[i]Date of application. T1: end-March or early-April; T2: mid-April; T3: end-April or early-May.
[ii]n/a means treatments were not applied.

Table 4. Bud break and floral initiation reaction time in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi at the Mbombela experimental site (cool winter area). Ethephon was applied during the period of March to May as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), as a single whole-canopy application to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Values represent means (n = 5).

		Days between application and bud break				Days between application and panicle emergence				Days between bud break and panicle emergence			
Application date (AD)	Treatment (Trt)	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
March to May	Commercial Control	32.6[i]	16.0 h[ii]	33.0 c	41.8	62.8 e	44.4	79.0 a	68.0	30.2	28.4 a	46.0 a	26.2
	Whole-canopy+	41.8	36.4 bc	40.0 bc	44.6	73.6 b	48.0	79.0 a	73.8	31.8	11.6 efg	39.0 a	29.2
End March / early April (T1)	Untreated Control	33.8	35.0 bcd	50.2 b	36.2	64.6 de	46.8	84.8 a	64.8	30.8	11.8 defg	34.6 ab	28.6
	Eth500	45.0	35.0 bcd	65.4 a	39.0	71.8 bc	48.0	81.8 a	68.0	26.8	13.0 def	16.4 c	29.0
	Eth750	48.4	39.2 ab	62.0 a	47.4	71.8 bc	48.0	79.0 a	70.6	23.4	8.8 ef	17.0 c	23.2
	Eth1000	56.8	45.2 a	62.0 a	50.2	83.4 a	52.4	79.0 a	68.0	26.6	7.2 g	17.0 c	17.8
Mid April (T2)	Untreated Control	32.8	21.0 gh	n/a[iii]	n/a	62.4 e	43.0	n/a	n/a	29.6	22.0 b	n/a	n/a
	Eth500	50.4	25.2 fg	n/a	n/a	70.4 bcd	44.6	n/a	n/a	20	19.4 bc	n/a	n/a
	Eth750	50.4	32.8 cde	n/a	n/a	72.6 bc	49.4	n/a	n/a	22.2	16.6 cd	n/a	n/a
	Eth1000	57.2	37.4 bc	n/a	n/a	74.8 b	52.0	n/a	n/a	17.6	14.6 cde	n/a	n/a
End April / early May (T3)	Untreated Control	12.0	6.0 i	45.8 b	42.5	50.4 f	29.6	67.8 b	69.5	38.4	23.6 ab	22.0 c	27.0
	Eth500	41.5	16.0 h	48.0 b	46.5	67.5 cde	31.2	70.2 b	76.5	26	15.2 cde	22.2 bc	30.0
	Eth750	45.8	26.8 efg	n/a	n/a	74.0 b	36.0	n/a	n/a	28.2	9.2 fg	n/a	n/a
	Eth1000	52.4	29.6 def	67.6 a	70.0	74.0 b	41.6	84.0 a	112.0	21.6	12.0 defg	16.4 c	42.0
P values[iv]													
AD		*0.0069*	*< 0.0001*	*0.5305*	*0.3656*	*0.0002*	*< 0.0001*	*0.0055*	*0.1702*	*0.0543*	*0.0005*	*0.0016*	*0.4304*
Trt		< 0.0001	< 0.0001	< 0.0001	*0.1729*	*< 0.0001*	*< 0.0001*	*0.2566*	*0.4599*	*0.0064*	*< 0.0001*	*0.0003*	*0.0995*
AD × Trt		*0.3573*	*0.0297*	*0.0172*	*0.7712*	*0.0027*	*0.4904*	*0.0009*	*0.4962*	*0.5749*	*0.0491*	*0.0704*[v]	*0.3506*

[i]Means within a column without letters do not differ significantly.

[ii]Means within a column followed by different letters indicate statistical significance of AD × Trt interaction according to Fisher's LSD test (p = 0.05).

[iii]n/a means treatment was not applied.

[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA (P < 0.05).

[v]AD × Trt interaction was accepted as significant at ANOVA P < 0.10.

Table 5. Bud break and floral initiation reaction time in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi at the Malalane experimental site (moderate winter area). Ethephon was applied during the period of March to May as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), or as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750). Untreated Control represents untreated trees. Values represent means (n = 5).

		Days between application and bud break				Days between application and panicle emergence				Days between bud break and panicle emergence			
Application date (AD)	Treatment (Trt)	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
March to May	Commercial Control	49.4 b[i]	12.6[ii]	37.2 b	33.8	68.8	42.0	75.0 b	49.8	19.4	29.4	37.8	16.0
End March / early April (T1)	Untreated Control	46.4 b	n/a[iii]	63.6 a	n/a	65.8	n/a	84.6 a	n/a	22.0	n/a	21.0	n/a
	Eth750	47.2 b	n/a	57.6 a	n/a	59.6	n/a	75.0 b	n/a	12.4	n/a	17.4	n/a
	Eth1000	51.6 ab	n/a	60.0 a	n/a	67.4	n/a	75.0 b	n/a	15.8	n/a	15.0	n/a
	Eth1000+750	60.6 a	n/a	60.0 a	n/a	76.6	n/a	75.0 b	n/a	16.0	n/a	15.0	n/a
Mid April (T2)	Untreated Control	n/a	39.5	n/a	31.4	n/a	57.8	n/a	57.2	n/a	18.3	n/a	25.8
	Eth500	n/a	52.0	n/a	38.2	n/a	71.0	n/a	62.2	n/a	16.0	n/a	24.0
	Eth750	n/a	37.8	n/a	45.0	n/a	55.6	n/a	70.0	n/a	17.8	n/a	25.0
	Eth1000	n/a	45.8	n/a	45.0	n/a	64.8	n/a	70.0	n/a	19.0	n/a	25.0
	Eth1000+750	n/a	47.0	n/a	47.4	n/a	61.6	n/a	73.2	n/a	14.6	n/a	25.8
End April / early May (T3)	Untreated Control	n/a	18.0	34.0 b	34.8	n/a	38.8	56.0 c	45.2	n/a	20.8	22.0	10.4
	Eth500	n/a	47.2	40.0 b	45.0	n/a	59.2	56.0 c	53.0	n/a	12.0	16.0	8.0
	Eth750	n/a	47.0	n/a	47.4	n/a	60.4	n/a	56.2	n/a	13.4	n/a	8.8
	Eth1000	n/a	45.6	40.0 b	47.4	n/a	60.4	58.6 c	59.4	n/a	14.8	18.6	12.0
P values[iv]													
AD		-	*0.0017*	*< 0.0001*	*0.0283*	-	*0.0102*	*< 0.0001*	*< 0.0001*	-	*0.0014*	*0.1867*	*< 0.0001*
Trt		*0.0288*	*0.1342*	*< 0.0001*	*0.0003*	*0.2393*	*0.0973*	*0.0226*	*< 0.0001*	*0.5389*	*0.5245*	*< 0.0001*	*0.8244*
AD × Trt		-	*0.2034*	*0.0739*[v]	*0.8814*	-	*0.1622*	*0.0008*	*0.8743*	-	*0.7151*	*0.5924*	*0.8996*

[i]Means within a column followed by different letters indicate statistical significance of AD × Trt interaction according to Fisher's LSD test (p = 0.05).
[ii]Means within a column without letters do not differ significantly.
[iii]n/a means treatment was not applied.
[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA (P < 0.05).
[v]AD × Trt interaction was accepted as significant at ANOVA P < 0.10.

Table 6. Panicle characteristics and flowering rate in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi at the Mbombela experimental site (cool winter area). Ethephon was applied during the period of March to May as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), as a single whole-canopy application to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Values represent means (n = 5).

		Leafy panicle rate (%)				Panicle length (cm)				Panicles per shoot (incl. axilary) (no.)				Flowering rate (%)			
Application date (AD)	Treatment (Trt)	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
March to May	Commercial Control	56.0[i]	52.0	42.0 a[ii]	64.0	n/a	26.2	25.1 bc	25.1	n/a	2.6	3.0	3.3	88.0	90.0	98.0 a	80.0
	Whole-canopy+	22.0	36.0	28.0 a	68.0	n/a	25.5	27.9 b	22.4	n/a	2.7	3.5	3.3	62.0	88.0	98.0 a	92.0
End March / early April (T1)	Untreated Control	26.0	56.0	26.0 a	76.0	n/a	24.0	24.8 bc	24.8	n/a	1.6	1.8	2.5	56.0	94.0	74.0 b	88.0
	Eth500	50.0	48.0	30.0 a	48.0	n/a	25.0	28.3 b	25.6	n/a	2.6	2.2	3.1	84.0	100.0	78.0 ab	98.0
	Eth750	28.0	32.0	38.0 a	56.0	n/a	23.1	26.9 b	24.8	n/a	2.5	2.8	3.1	74.0	94.0	90.0 ab	98.0
	Eth1000	32.0	28.0	42.0 a	36.0	n/a	22.2	32.4 a	26.2	n/a	2.9	2.3	3.5	92.0	96.0	96.0 ab	98.0
Mid April (T2)	Untreated Control	22.0	36.0	n/a[iii]	n/a	n/a	26.7	n/a	n/a	n/a	1.9	n/a	n/a	62.0	96.0	n/a	n/a
	Eth500	34.0	40.0	n/a	n/a	n/a	23.8	n/a	n/a	n/a	2.4	n/a	n/a	90.0	96.0	n/a	n/a
	Eth750	32.0	40.0	n/a	n/a	n/a	22.0	n/a	n/a	n/a	2.7	n/a	n/a	80.0	98.0	n/a	n/a
	Eth1000	14.0	36.0	n/a	n/a	n/a	22.6	n/a	n/a	n/a	2.9	n/a	n/a	84.0	98.0	n/a	n/a
End April / early May (T3)	Untreated Control	28.0	32.0	34.0 a	15.0	n/a	25.0	22.8 c	8.3	n/a	1.7	2.3	2.5	54.0	84.0	96.0 ab	6.0
	Eth500	16.0	36.0	4.0 b	20.0	n/a	22.1	16.9 d	10.1	n/a	2.7	3.1	1.5	52.0	98.0	100.0 a	16.0
	Eth750	14.0	40.0	n/a	n/a	n/a	21.7	n/a	n/a	n/a	2.5	n/a	n/a	40.0	92.0	n/a	n/a
	Eth1000	16.0	24.0	2.0 b	0.0	n/a	19.7	13.3 d	3.8	n/a	2.6	2.7	2.7	40.0	88.0	74.0 b	8.0
P values[iv]																	
AD		*0.0101*	*0.0868*	*< 0.0001*	*< 0.0001*	-	*0.0833*	*< 0.0001*	*< 0.0001*	-	*0.7795*	*0.4684*	*<0.0001*	*< 0.0001*	*0.0885*	*0.8551*	*< 0.0001*
Trt		*0.0531*	*0.0628*	*0.1529*	*0.0208*	-	*0.0287*	*0.3837*	*0.8388*	-	*< 0.0001*	*< 0.0001*	*0.5821*	*0.1303*	*0.1701*	*0.4153*	*0.1475*
AD × Trt		*0.3698*	*0.1351*	*0.0008*	*0.5413*	-	*0.7951*	*< 0.0001*	*0.3463*	-	*0.8928*	*0.4501*	*0.1974*	*0.3104*	*0.6703*	*0.0091*	*0.7339*

[i]Means within a column without letters do not differ significantly.

[ii]Means within a column followed by different letters indicate statistical significance of AD × Trt interaction according to Fisher's LSD test (p = 0.05).

[iii]n/a means treatment was not applied.

[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA (P < 0.05).

Table 7. Panicle characteristics and flowering rate in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi at the Malalane experimental site (moderate winter area). Ethephon was applied during the period of March to May as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), or as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750). Untreated Control represents untreated trees. Values represent means (n = 5).

		Leafy panicle rate (%)				Panicle length (cm)				Panicles per shoot (incl. axilary) (no.)				Flowering rate (%)			
Application date (AD)	Treatment (Trt)	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
March to May	Commercial Control	24.0 b[i]	20.0[ii]	56.0	22.0	n/a	37.7 a	31.0 ab	30.0	n/a	1.9	2.4	3.2	72.0 ab	86.0	98.0 a	94.0
	Untreated Control	16.0 b	n/a[iii]	18.0	n/a	n/a	n/a	28.3 abc	n/a	n/a	n/a	1.6	n/a	58.0 b	n/a	54.0 b	n/a
End March / early April (T1)	Eth750	48.0 a	n/a	38.0	n/a	n/a	n/a	33.5 a	n/a	n/a	n/a	1.9	n/a	96.0 a	n/a	96.0 a	n/a
	Eth1000	30.0 ab	n/a	30.0	n/a	n/a	n/a	32.8 a	n/a	n/a	n/a	2.2	n/a	88.0 a	n/a	98.0 a	n/a
	Eth1000+750	24.0 b	n/a	26.0	n/a	n/a	n/a	30.7 ab	n/a	n/a	n/a	2.2	n/a	92.0 a	n/a	92.0 a	n/a
	Untreated Control	n/a	40.0	n/a	46.0	n/a	31.4 ab	n/a	30.1	n/a	1.7	n/a	1.2	n/a	56.0	n/a	82.0
	Eth500	n/a	52.5	n/a	42.0	n/a	30.8 b	n/a	28.7	n/a	2.5	n/a	3.0	n/a	72.0	n/a	94.0
Mid April (T2)	Eth750	n/a	40.0	n/a	38.0	n/a	32.9 ab	n/a	26.8	n/a	2.5	n/a	3.2	n/a	94.0	n/a	94.0
	Eth1000	n/a	48.0	n/a	34.0	n/a	30.1 b	n/a	24.3	n/a	2.3	n/a	2.8	n/a	78.0	n/a	98.0
	Eth1000+750	n/a	38.0	n/a	34.0	n/a	29.9 b	n/a	24.2	n/a	3.8	n/a	3.8	n/a	92.0	n/a	80.0
	Untreated Control	n/a	15.0	28.0	44.0	n/a	19.9 c	25.9 bcd	31.5	n/a	1.3	1.3	1.8	n/a	26.0	100.0 a	88.0
End April / early May	Eth500	n/a	44.0	6.0	36.0	n/a	33.9 ab	20.7 d	28.3	n/a	2.3	1.7	3.3	n/a	62.0	92.0 a	96.0
(T3)	Eth750	n/a	44.0	n/a	26.0	n/a	31.4 ab	n/a	27.5	n/a	2.9	n/a	3.5	n/a	74.0	n/a	88.0
	Eth1000	n/a	32.0	14.0	28.0	n/a	31.0 ab	23.7 cd	22.7	n/a	3.3	2.0	3.8	n/a	70.0	94.0 a	88.0
P values[iv]																	
AD		-	*0.0117*	*0.0149*	*0.1141*	-	*0.0178*	*< 0.0001*	*0.0194*	-	*0.0327*	*0.0087*	*0.1988*	-	*0.0217*	*0.0982*	*0.6734*
Trt		*0.0688*	*0.4455*	*0.0926*	*0.2811*	-	*0.3259*	*0.3494*	*< 0.0001*	-	*< 0.0001*	*0.0051*	*< 0.0001*	*0.0384*	*0.0077*	*0.0044*	*0.0419*
AD × Trt		-	*0.2571*	*0.1726*	*0.9246*	-	*0.0688*[v]	*0.0765*[v]	*0.4521*	-	*0.1921*	*0.7021*	*0.5585*	-	*0.7730*	*0.0004*	*0.2893*

[i]Means within a column followed by different letters indicate statistical significance of AD × Trt interaction according to Fisher's LSD test (p = 0.05).
[ii]Means within a column without letters do not differ significantly.
[iii]n/a means treatment was not applied.
[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA ($P < 0.05$).
[v]AD × Trt interactions were accepted as significant at ANOVA $P < 0.10$.

Table 8. Yield and fruit quality characteristics in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi at the Mbombela experimental site (cool winter area). Ethephon was applied during the period of March to May as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), as a single whole-canopy application to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Values represent means (n = 5).

		Yield (kg·tree^{-1})				Fruit mass (g)				Fruit size (mm)				Fruit maturity (TSS/TA ratio)			
Application date (AD)	Treatment (Trt)	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
March to May	Commercial Control	78.3[i]	36.9	33.7	30.7 b[ii]	23.2	25.1	24.2	24.2	34.0	35.0	34.5	34.4	36.3	43.7	34.5	34.1 abc
	Whole-canopy+	77.7	53.7	37.8	46.0 ab	22.7	26.3	25.6	25.1	33.8	35.5	35.1	34.9	36.5	47.8	38.1	38.2 ab
End March / early April (T1)	Untreated Control	43.6	51.0	39.6	30.1 b	22.6	24.3	24.2	22.8	33.8	34.5	34.5	33.4	21.5	42.1	27.8	32.4 bcd
	Eth500	73.8	58.7	19.4	48.2 a	23.1	25.0	24.0	25.0	34.1	34.9	35.4	34.9	32.7	44.6	32.8	35.8 ab
	Eth750	68.9	65.2	31.9	60.5 a	23.4	24.5	24.7	24.4	34.4	34.5	35.0	34.4	30.3	50.8	36.8	37.8 ab
	Eth1000	89.7	64.3	43.8	60.7 a	23.1	25.2	24.8	24.6	34.0	35.0	35.0	34.5	34.8	46.2	36.9	38.5 a
Mid April (T2)	Untreated Control	68.3	55.4	n/a[iii]	n/a	21.4	24.2	n/a	n/a	32.7	34.4	n/a	n/a	27.4	42.7	n/a	n/a
	Eth500	102.5	74.0	n/a	n/a	23.4	24.8	n/a	n/a	34.2	34.8	n/a	n/a	29.0	49.9	n/a	n/a
	Eth750	94.4	74.3	n/a	n/a	23.1	24.8	n/a	n/a	34.1	34.8	n/a	n/a	32.7	48.1	n/a	n/a
	Eth1000	80.9	68.7	n/a	n/a	22.1	25.9	n/a	n/a	33.4	35.4	n/a	n/a	36.8	48.5	n/a	n/a
End April / early May (T3)	Untreated Control	46.4	59.7	53.5	3.5 c	23.0	24.3	23.3	23.8	33.8	34.6	34.2	33.9	21.1	44.6	31.5	26.6 d
	Eth500	45.3	69.3	48.9	3.0 c	23.8	24.7	24.7	23.2	34.5	34.9	34.9	34.2	27.9	45.8	33.2	29.3 cd
	Eth750	47.6	78.2	n/a	n/a	25.0	24.7	n/a	n/a	35.5	34.9	n/a	n/a	29.0	45.3	n/a	n/a
	Eth1000	40.1	71.9	63.3	2.4 c	24.0	24.8	25.3	26.6	34.7	35.0	35.2	36.1	28.4	50.0	37.6	21.3 e
P values[iv]																	
AD		*< 0.0001*	*0.0045*	*0.0016*	*< 0.0001*	*0.0122*	*0.2153*	*0.8057*	*0.5182*	*0.0119*	*0.9474*	*0.6876*	*0.8443*	*0.0037*	*0.5855*	*0.7434*	*< 0.0001*
Trt		*0.1949*	*0.0127*	*0.4248*	*0.0060*	*0.1657*	*0.0016*	*0.2609*	*0.1579*	*0.0403*	*0.0123*	*0.5117*	*0.0882*	*< 0.0001*	*0.036*	*0.0006*	*0.1067*
AD × Trt		*0.2367*	*0.9884*	*0.6300*	*0.0320*	*0.8489*	*0.7668*	*0.4259*	*0.1123*	*0.7726*	*0.8498*	*0.7460*	*0.1511*	*0.4845*	*0.2490*	*0.5959*	*0.0511*[v]

[i]Means within a column without letters do not differ significantly.
[ii]Means within a column followed by different letters indicate statistical significance of AD × Trt interaction according to Fisher's LSD test (p = 0.05).
[iii]n/a means treatment was not applied.
[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA (P < 0.05).
[v]AD × Trt interaction was accepted as significant at ANOVA P < 0.10.

Table 9. Yield and fruit quality characteristics in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi at the Malalane experimental site (moderate winter area). Ethephon was applied during the period of March to May as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), or as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750). Untreated Control represents untreated trees. Values represent means (n = 5).

		Yield (kg·tree^{-1})				Fruit mass (g)				Fruit size (mm)				Fruit maturity (TSS/TA ratio)			
Application date (AD)	Treatment (Trt)	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
March to May	Commercial Control	73.1 ab[i]	40.1[ii]	96.3	104.9	23.2	22.7	24.1	23.8	33.7	33.9	33.9	34.1	44.3	54.5	45.1	43.6
	Untreated Control	49.0 b	n/a[iii]	67.3	n/a	22.7	n/a	24.4	n/a	33.9	n/a	34.2	n/a	37.4	n/a	36.3	n/a
End March / early April (T1)	Eth750	86.4 a	n/a	92.6	n/a	22.2	n/a	23.8	n/a	33.3	n/a	33.8	n/a	45.3	n/a	45.0	n/a
	Eth1000	99.7 a	n/a	105.6	n/a	22.5	n/a	24.7	n/a	33.4	n/a	34.7	n/a	49.7	n/a	45.3	n/a
	Eth1000+750	104.9 a	n/a	101.6	n/a	22.7	n/a	24.8	n/a	33.5	n/a	34.4	n/a	48.2	n/a	41.6	n/a
	Untreated Control	n/a	33.7	n/a	55.6	n/a	21.0	n/a	23.6	n/a	32.9	n/a	34.3	n/a	44.5	n/a	33.8
	Eth500	n/a	64.2	n/a	122.4	n/a	22.0	n/a	24.0	n/a	33.2	n/a	34.4	n/a	55.4	n/a	40.8
Mid April (T2)	Eth750	n/a	67.0	n/a	107.8	n/a	20.9	n/a	24.3	n/a	32.7	n/a	34.5	n/a	48.8	n/a	39.8
	Eth1000	n/a	55.0	n/a	95.1	n/a	21.3	n/a	24.8	n/a	33.1	n/a	34.6	n/a	49.3	n/a	42.0
	Eth1000+750	n/a	76.0	n/a	97.3	n/a	21.5	n/a	24.6	n/a	33.0	n/a	34.5	n/a	51.6	n/a	41.8
	Untreated Control	n/a	18.2	89.9	98.8	n/a	20.7	23.2	23.4	n/a	32.7	33.5	34.0	n/a	40.0	33.3	38.9
End April / early May (T3)	Eth500	n/a	35.1	102.6	110.4	n/a	21.9	24.1	23.7	n/a	33.2	34.0	34.0	n/a	45.7	39.6	42.1
	Eth750	n/a	59.3	n/a	121.3	n/a	22.1	n/a	24.6	n/a	33.4	n/a	34.7	n/a	49.3	n/a	41.5
	Eth1000	n/a	62.9	124.5	121.4	n/a	23.3	23.6	24.0	n/a	33.9	33.5	34.3	n/a	50.3	40.4	43.2
P values[iv]																	
AD		-	*0.2046*	*0.2448*	*0.1067*	-	*0.027*	*0.1674*	*0.4102*	-	*0.0361*	*0.1486*	*0.2474*	-	*0.1936*	*0.0686*	*0.0324*
Trt		*0.0284*	*0.0561*	*0.2741*	*0.0152*	*0.8654*	*0.2296*	*0.832*	*0.1293*	*0.8733*	*0.4701*	*0.7515*	*0.2811*	*0.1926*	*0.5608*	*0.2280*	*0.0010*
AD × Trt		-	*0.6173*	*0.9041*	*0.1433*	-	*0.1439*	*0.9009*	*0.6563*	-	*0.4325*	*0.7014*	*0.5763*	-	*0.6535*	*0.8182*	*0.5075*

[i]Means within a column followed by different letters indicate statistical significance of AD × Trt interaction according to Fisher's LSD test (p = 0.05).
[ii]Means within a column without letters do not differ significantly.
[iii]n/a means treatment was not applied.
[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA (P < 0.05).

Table 10. Various growth parameters in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi at the Mbombela and Malalane experimental sites, respectively. Ethephon was applied during the period of March to May as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), or as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Values represent means across all application dates and years (n = 5-15).

Treatment	Flushing rate during FInd[i] (%)	Days between application and bud break	Days between application and panicle emergence	Leafy panicle rate (%)	Panicle length (cm)	Panicles per shoot (incl. axilary) (no.)	Flowering rate (%)	Yield (kg·tree^{-1})	Fruit mass (g)	Fruit size (mm)	Fruit maturity (TSS/TA ratio)
					Mbombela experimental site (cool winter area)						
Commercial Control	7.5 b[ii]	30.9 c	63.4 b	53.5 a	25.5[iii]	3.0 ab	89.0 a	44.9 c	24.2 b	34.4 ab	37.1 b
Whole-canopy+	3.0 bc	40.7 b	68.6 a	38.5 b	25.3	3.2 a	85.0 ab	53.8 bc	24.9 a	34.8 a	40.2 a
Untreated Control	15.2 a	29.7 c	60.4 c	34.7 b	24.5	2.0 d	72.3 c	47.9 c	23.3 c	34.0 b	32.6 c
Eth500	4.8 bc	39.9 b	63.1 b	33.1 b	22.7	2.6 c	83.2 ab	60.1 ab	24.3 ab	34.7 a	37.3 ab
Eth750	2.3 bc	41.6 b	64.0 b	33.8 b	22.9	2.7 bc	81.3 ab	68.0 a	24.3 ab	34.7 a	39.4 ab
Eth1000	1.5 c	50.4 a	68.6 a	24.1 c	21.7	2.8 bc	79.7 bc	61.9 ab	24.4 ab	34.7 a	39.8 ab
P value[iv]	*< 0.0001*	*< 0.0001*	*< 0.0001*	*0.0003*	*0.1856*	*< 0.0001*	*0.0335*	*0.0006*	*0.0009*	*0.0010*	*< 0.0001*
					Malalane experimental site (moderate winter area)						
Commercial Control	7.0 b	33.3 d	58.9 c	30.5	32.9	2.5 b	87.5 a	78.6 a	23.4	33.9	46.9 a
Untreated Control	28.8 a	39.8 cd	60.9 bc	30.2	28.8	1.5 c	65.4 b	57.3 b	23.0	33.8	37.1 b
Eth500	2.8 b	44.6 bc	60.6 bc	37.8	28.6	2.6 b	83.2 a	88.0 a	23.2	33.8	44.6 a
Eth750	3.7 b	47.0 b	62.8 bc	39.0	30.9	2.6 b	90.3 a	89.1 a	23.0	33.7	44.7 a
Eth1000	2.4 b	48.5 ab	66.0 ab	31.9	28.6	2.6 b	88.3 a	93.5 a	23.4	33.9	45.7 a
Eth1000+750	2.5 b	53.8 a	71.6 a	30.5	28.8	3.0 a	89.0 a	94.9 a	23.4	33.8	45.8 a
P value	*< 0.0001*	*< 0.0001*	*0.0011*	*0.2945*	*0.2085*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*0.6123*	*0.9243*	*< 0.0001*

[i]FInd means floral induction period.
[ii]Means within a column followed by different letters indicate statistical significance according to Fisher's LSD test (p = 0.05).
[iii]Means within a column without letters do not differ significantly.
[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA (P < 0.05).

Table 11. Various growth parameters in response to different ethephon treatments or in untreated trees of 'Mauritius' litchi according to application dates, at the Mbombela and Malalane experimental site, respectively. Ethephon was applied between March and May as repeated spot spray applications to immature vegetative shoots, as single or double whole-canopy treatment to mature shoots, or as a combination of whole-canopy and spot spray application. Untreated Control represents untreated trees. Values represent means across treatments and years (n = 20-30).

Application date[i]	Flushing rate during FInd[ii] (%)	Days between application and bud break	Days between application and panicle emergence	Leafy panicle rate (%)	Panicle length (cm)	Panicles per shoot (incl. axilary) (no.)	Flowering rate (%)	Yield (kg·tree^{-1})	Fruit mass (g)	Fruit size (mm)	Fruit maturity (TSS/TA ratio)
	Mbombela experimental site (cool winter area)										
Early application (T1)	11.0 a[iii]	43.2 a	66.7 a	42.5 a	25.6 a	2.8 a	87.8 a	52.2 b	24.3 a	34.6 a	37.1 b
Mid application (T2)	2.8 b	38.4 b	58.7 b	31.8 b	23.8 b	2.5 b	88.0 a	77.3 a	23.7 b	34.3 b	39.8 a
Late application (T3)	1.3 b	35.8 b	56.5 b	21.8 c	19.4 c	2.5 b	61.3 b	47.5 b	24.4 a	34.8 a	35.7 b
P value[iv]	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*0.0182*	*< 0.0001*	*< 0.0001*	*0.0116*	*0.0071*	*0.0003*
	Malalane experimental site (moderate winter area)										
Early application (T1)	8.6[v]	51.4 a	70.2 a	29.1 b	31.6 a	2.1 b	84.9	86.4	23.5	33.9	44.2
Mid application (T2)	8.6	42.7 b	64.0 b	40.8 a	28.9 b	2.7 a	84.0	77.4	22.9	33.8	44.3
Late application (T3)	9.1	41.4 b	55.4 b	30.3 b	27.6 b	2.5 a	79.9	86.2	23.3	33.8	41.9
P value	*0.9824*	*< 0.0001*	*< 0.0001*	*0.0024*	*0.0004*	*< 0.0001*	*0.2932*	*0.1974*	*0.1011*	*0.6906*	*0.1106*

[i]Early applications (T1) were made between end of March and early April. Mid applications (T2) were made during mid-April. Late applications (T3) were made between end of April and beginning of May.
[ii]FInd means floral induction period.
[iii]Means within a column followed by different letters indicate statistical significance according to Fisher's LSD test (p = 0.05).
[iv]Statistical analysis of data was assessed according to a randomized block design ANOVA ($P < 0.05$).
[v]Means within a column without letters do not differ significantly.

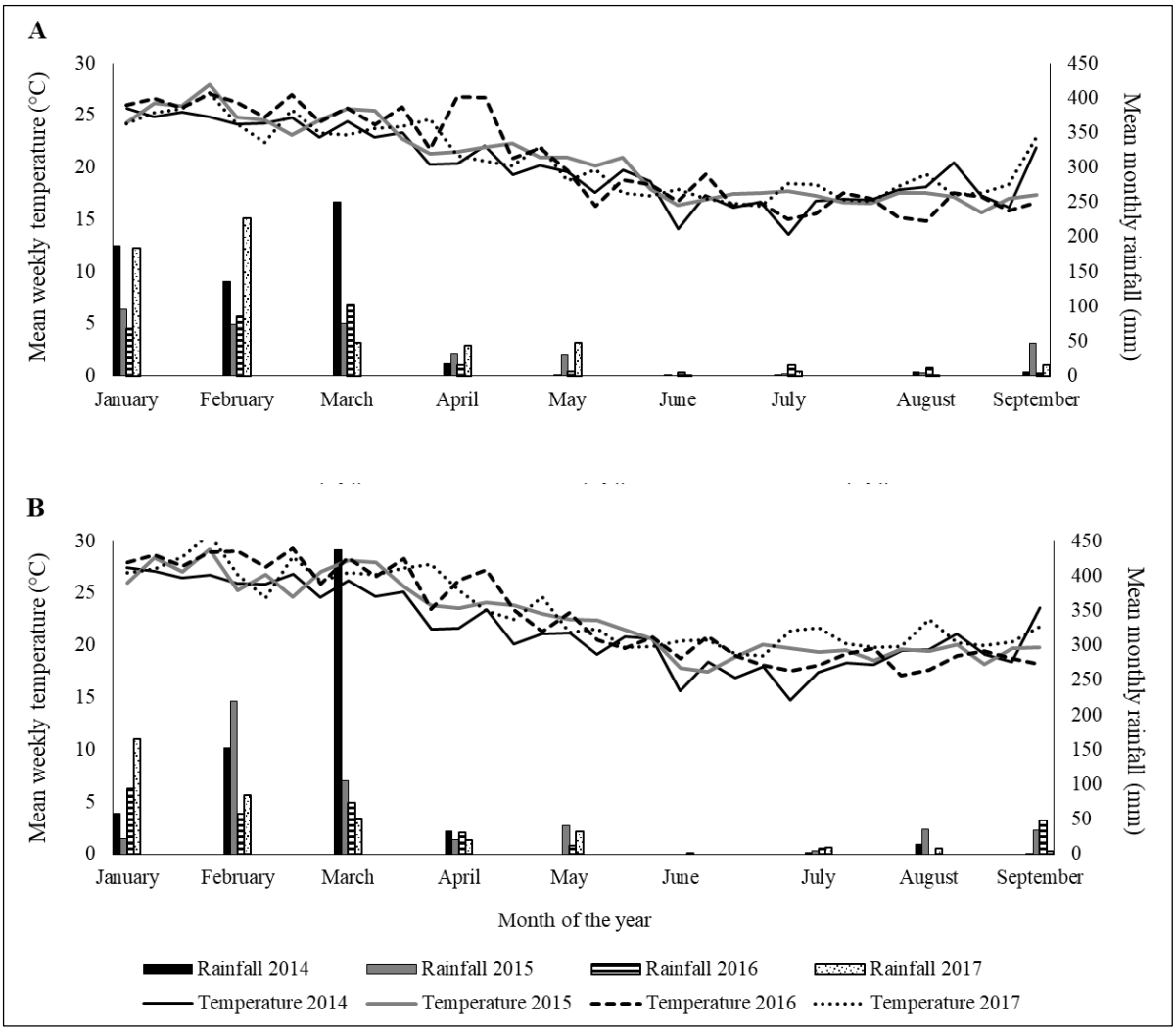

Fig. 1. Mean weekly temperature and mean monthly rainfall between the postharvest shoot growth and flowering period (January to September) at the Mbombela (A) and Malalane (B) experimental sites, for the years 2014 to 2017.

Fig. 2. Developmental stages of terminal shoots and apical buds of 'Mauritius' litchi trees used in the experiment: A) fully hardened mature shoot with dormant terminal buds, B) dormant bud, C) swelling bud, D) actively growing bud (< 15 mm length), E) emerging shoot with early elongation of rudimentary leaves, F) immature soft vegetative shoot, and G) vegetative shoot after leaf drop following ethephon application. Ethephon spot spray applications of the Commercial Control and follow-up sprays for the Whole-canopy+ treatment were applied to immature soft vegetative shoots (F). Untreated trees or trees treated with whole-canopy ethephon applications were applied to fully mature shoots (A).

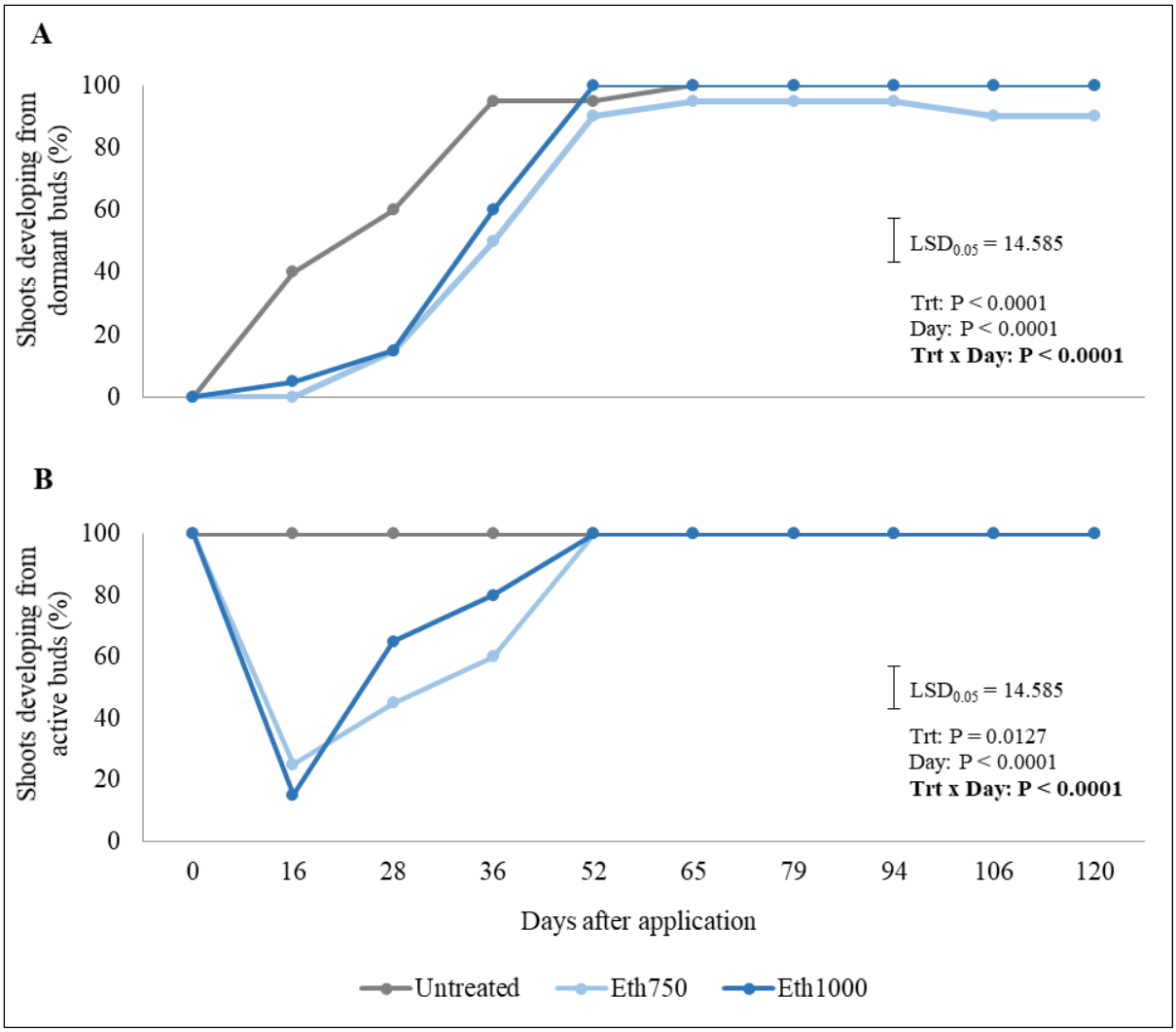

Fig. 3. Percentage shoots (out of total number of tagged terminal shoots) developing from dormant (A) and active (B) apical buds following treatment with ethephon (Eth750, Eth1000) or no treatment (Untreated) of terminal shoots in 'Mauritius' litchi. Ethephon was applied to mature terminal shoots at concentrations of 750 mg·L^{-1} (Eth750) or 1000 mg·L^{-1} (Eth1000) during April 2013 at the experimental site near Mbombela. Data was collected on the day of application and over a 120-day period after ethephon application. Values represent means (n = 20). Statistical analysis of data was assessed with repeated measures ANOVA (P < 0.05), for dormant and actively growing bud data, respectively. Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

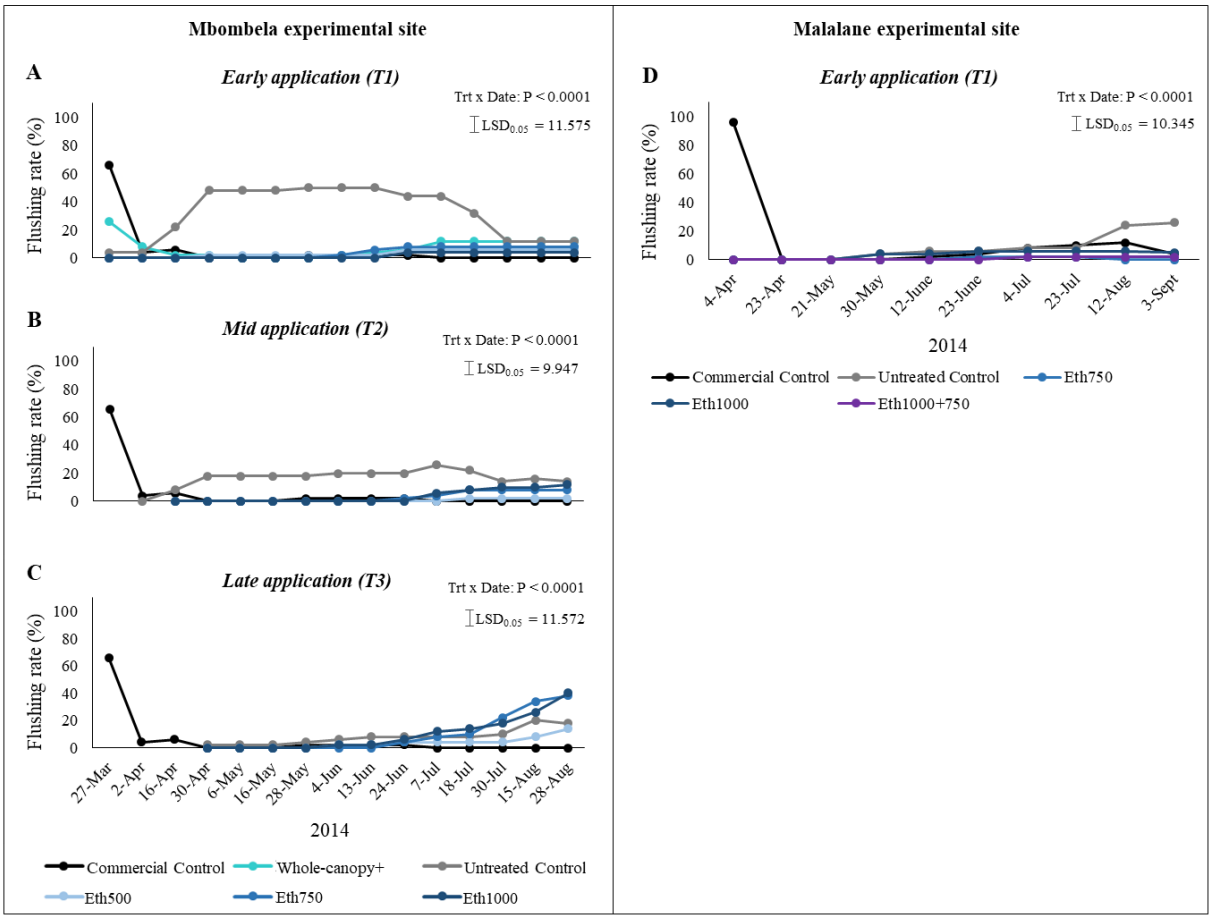

Fig. 4. Flushing rate of terminal shoots in 'Mauritius' litchi in response to different ethephon applications during the pre-flowering period of 2014 at the Mbombela (A-C) and the Malalane experimental site (D). Ethephon was applied as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Applications at the Mbombela site were made on 25 March (A), 8 April (B) and 25 April (C). Applications at the Malalane site were made on 3 April (D). Data points represent means (n = 50). Statistical analysis of data was assessed with repeated measures ANOVA (P < 0.05). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

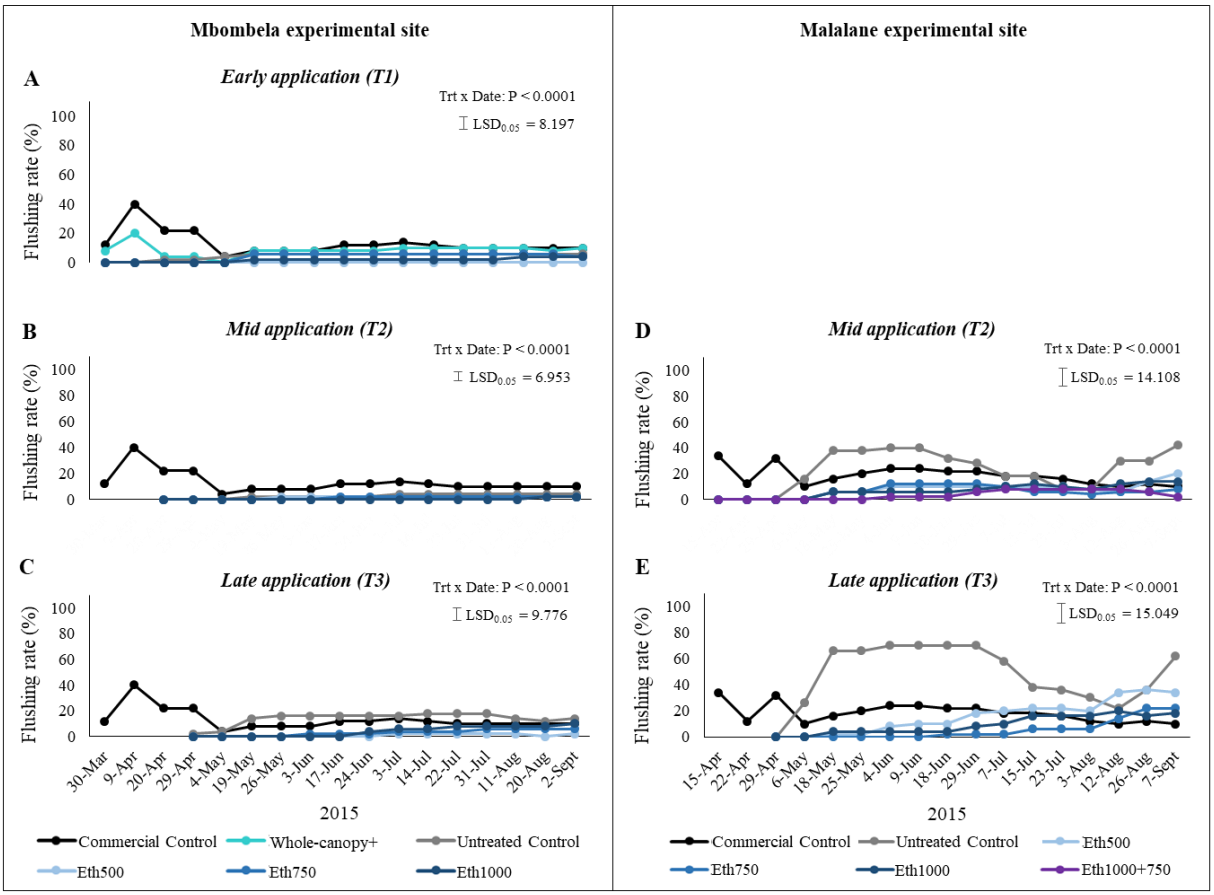

Fig. 5. Flushing rate of terminal shoots in 'Mauritius' litchi in response to different ethephon applications during the pre-flowering period of 2015 at the Mbombela (A-C) and the Malalane experimental site (D-E). Ethephon was applied as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Applications at the Mbombela site were made on 20 March (A), 13 April (B) and 28 April (C). Applications at the Malalane site were made on 15 April (D) and 30 April (E). Data points represent means (n = 50). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

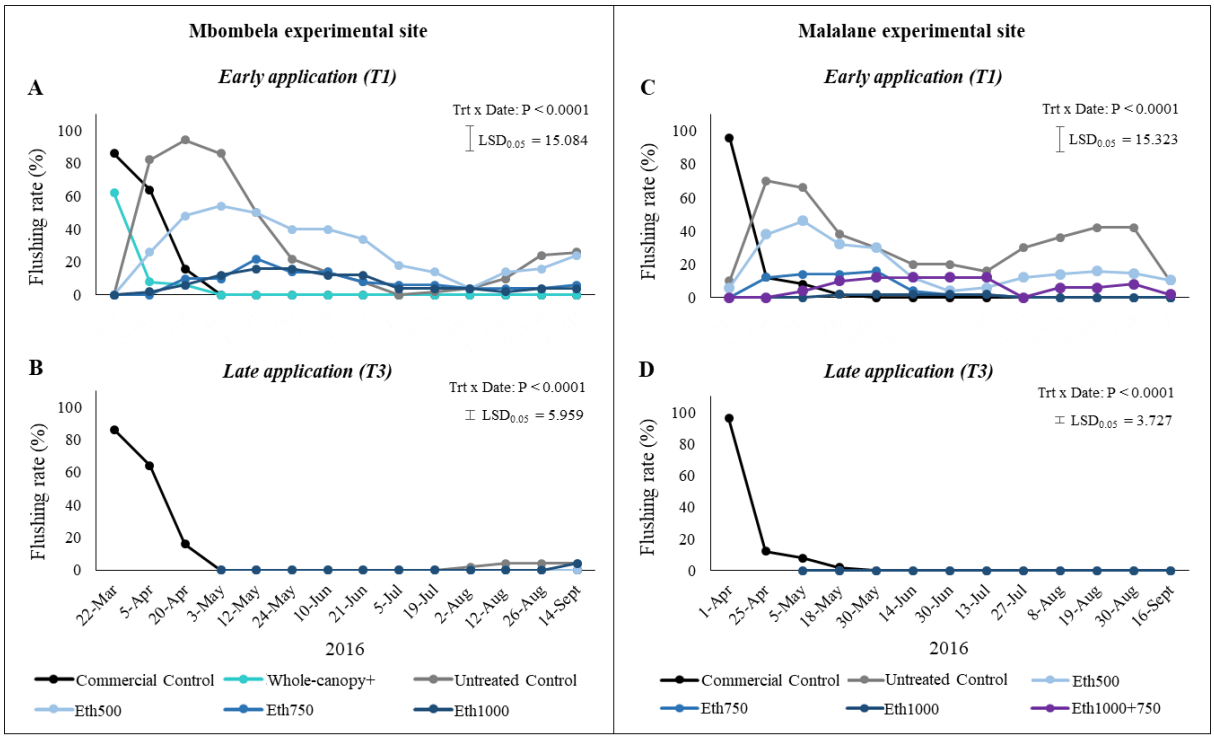

Fig. 6. Flushing rate of terminal shoots in 'Mauritius' litchi in response to different ethephon applications during the pre-flowering period of 2016 at the Mbombela (A-B) and the Malalane experimental site (C-D). Ethephon was applied as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Applications at the Mbombela site were made on 23 March (A) and 4 May (B). Applications at the Malalane site were made on 31 March (C) and 5 May (D). Data points represent means (n = 50). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

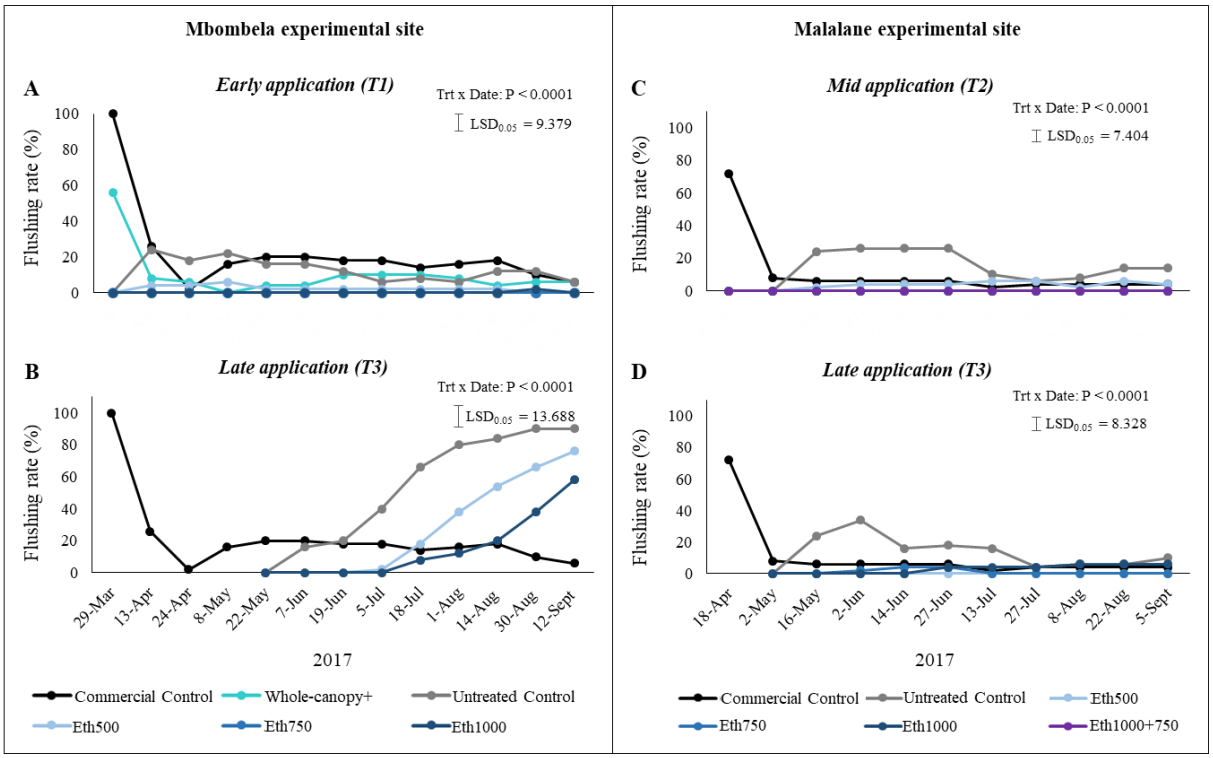

Fig. 7. Flushing rate of terminal shoots in 'Mauritius' litchi in response to different ethephon applications during the pre-flowering period of 2017 at the Mbombela (A-B) and the Malalane experimental site (C-D). Ethephon was applied as repeated spot spray applications to immature vegetative shoots at a concentration of 1000 mg·L^{-1} (Commercial Control), as single or double whole-canopy treatment to mature shoots at concentrations between 500 mg·L^{-1} and 1000 mg·L^{-1} (Eth500, Eth750, Eth1000, Eth1000+750), or as a single whole-canopy application followed by repeated spot sprays (Whole-canopy+). Untreated Control represents untreated trees. Applications at the Mbombela site were made on 30 March (A) and 23 May (B). Applications at the Malalane site were made on 18 April (C) and 2 May (D). Data points represent means (n = 50). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

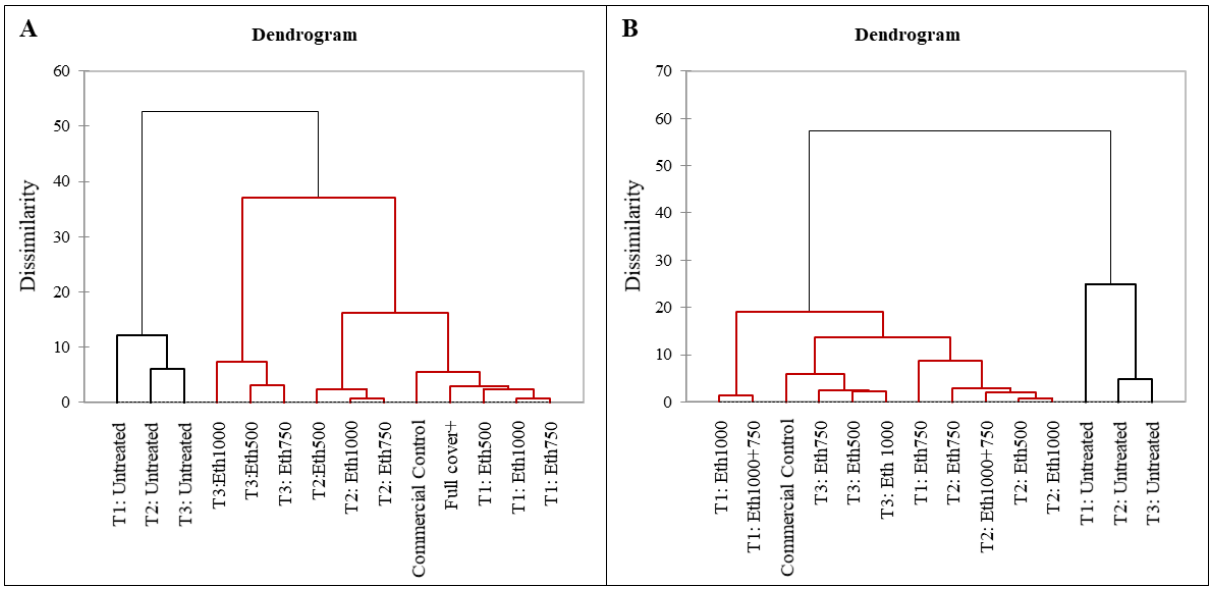

Fig. 8. Agglomerative hierarchical clustering dendrograms for different ethephon treatments applied during autumn and/or early winter of 2014 and 2017 at the Mbombela (A) and Malalane (B) experimental sites indicate dissimilarities between treatment groups. Black-colored clusters, representing untreated trees, were highly dissimilar from the ethephon treatments (red-colored clusters) at both sites. Sub-clustering of ethephon groups was according to application times and/or ethephon concentrations. Treatment points represent data combined for all application dates and years. Agglomerative hierarchical cluster analysis was performed on PCA factor scores. Eth: ethephon. T1: early application date (end-March/early April), T2: mid application date (mid-April), T3: late application date (end-April/early May).

Paper 3: Ethephon-mediated phenology modifications are associated with alterations in carbohydrate reserves of small branches and enhancement of flowering and fruiting in litchi, cv. 'Mauritius'

Keywords. Carbohydrate accumulation, flowering rate, *Litchi chinensis*, panicle emergence, shoot inhibition, starch, soluble sugars

Abstract. **Successful floral initiation in litchi requires mature shoots with adequate carbohydrate reserves to coincide with low inductive temperatures. Any new vegetative growth prior to floral induction will impede flower formation in litchi. Therefore, this study investigated the effect of three contrasting shoot control measures on the carbohydrate reserves of small branches in the litchi cultivar 'Mauritius'. These treatments included: 1) ethephon applied as repeated spot sprays at 1000 mg·L^{-1} to remove already developing small immature shoots, 2) ethephon applied as a single whole-canopy spray to trees with fully mature shoots at 1000 mg·L^{-1} to inhibit the emergence of any new vegetative shoots prior to panicle emergence, and 3) untreated trees to allow unrestricted shoot growth. The treatments were applied at three different dates during autumn and early winter (end-March to mid-May) of 2014 to 2017. Non-structural carbohydrates in small branches were determined between maturation of the last postharvest shoots and the following harvest, and were correlated with phenological events, such as flushing rate, flowering rate, time of panicle emergence and chilling degree hour accumulation. Both ethephon treatments reduced pre-flowering shoot growth, and subsequently promoted carbohydrate accumulation and flowering. In contrast, untreated trees allowed new pre-flowering vegetative shoot growth in most years, which restricted carbohydrate accumulation and reduced flowering. The carbohydrates found in the highest concentrations were starch, surose and quebrachitol. Irrespective of treatment, year and application date, starch concentrations accumulated during the pre-flowering period according to the level of flushing rate, but declined during panicle and/or fruit development of high yielding trees. Sucrose concentrations were negatively influenced by strong vegetative shoot growth and panicle development. Quebrachitol remained relatively stable throughout the observation periods, but declined at the start of panicle development in heavy flowering trees, suggesting that quebrachitol may also play a role as reserve carbohydrate to support flower development in litchi. Close associations between the whole-canopy ethephon treatment, the time interval to panicle emergence, carbohydrate levels prior to panicle emergence and flowering highlighted the**

important role of ethephon in promoting pre-flowering carbohydrate accumulation and flowering in litchi.

Introduction

The role of carbohydrate reserves for crop productivity has been studied in many fruit crops grown under subtropical conditions, such as citrus (Garcia-Louis et al., 1995; Goldschmidt, 1999; Goldschmidt and Golomb, 1982; Stander et al., 2018), avocado (Scholefield et al., 1985; Whiley and Wolstenholme, 1990; Wolstenholme and Whiley, 1997), mango (Davie et al., 2000; Prasad et al., 2014) and papaya (Allan, 2001). The annual carbohydrate dynamics in litchi concurred with the findings in the above-mentioned crops and were found to be highly dependent on the phenological growth phases. Carbohydrate accumulation in litchi occurred primarily during the winter dormancy period, a time when carbohydrate demand is generally low. However, carbohydrate levels were shown to decline during flowering, fruit set and fruit development, indicating that reproductive growth has a higher carbohydrate demand than vegetative growth (Cronje and Mostert, 2009; Cronje et al., 2021; Menzel et al., 1995; Yuan et al., 2009). In addition, several studies correlated high pre-flowering carbohydrate concentrations in shoots and branches with high flowering rates suggesting that carbohydrates play an important role in the success of flowering in litchi. (Chen et al., 2004; Jiang et al., 2012; Yang et al., 2014; Yuan et al., 2009). The two main reserve carbohydrates in litchi are starch and sucrose. The highest starch concentrations were reported in the small to medium branches (Chen et al., 2004; Hieke et al., 2002b; Menzel et al., 1995; Yuan et al., 2009), while soluble sugars were dominant in the terminal shoots (Chen et al., 2004). In a more recent study, the methylated cyclitol, quebrachitol, was found in many plant parts of litchi and in similar quantities to sucrose, particularly in the leaves (Wu et al., 2018). As an inert source of reserve energy, it has the potential to be converted to other sugars under low sucrose conditions (Wu et al., 2018).

Carbohydrate accumulation in litchi is dependent on shoot maturity (Fu et al., 2022; Hieke et al., 2002d; Menzel et al., 1995). Mature shoots are an important prerequisite for successful floral induction and initiation as it ensures that apical buds are chilling responsive when low inductive temperatures occur (Batten and McConchie, 1995; Chen and Huang, 2005; Davenport and Stern, 2005; O'Hare, 2002). Moreover, the low temperature stimulus for floral induction is perceived in mature leaves (Yang et al., 2014; Zhang et al., 2017). In addition, Yang et al. (2014) showed that mature leaves of terminal shoots had higher carbohydrate levels as well as higher expression levels of the flowering gene *LcFT* under low temperature conditions compared with immature leaves. Research by Xiao et al. (2018) corroborated these findings and suggested that floral competence in litchi is associated with the accumulation of soluble sugars in mature leaves of terminal shoots.

Therefore, the presence of immature shoots prior to and during the floral induction period is undesirable in litchi, as they will impede floral development. Orchard practices that reduce, remove or inhibit such undesirable shoot growth during autumn and early winter include the induction of autumn water stress, girdling and the use of plant growth regulators (Carr and Menzel, 2014; Chaitrakulsub et al., 1992; Cronje et al., 2017; Huang and Chen, 2014; Mandal et al., 2014; Menzel and Simpson, 1991; Roets et al., 2010). However, the efficacy of these manipulation techniques can vary depending on the prevailing weather conditions (Cronje et al., 2017; Roets et al., 2010).

In South Africa, unwanted autumn shoots are conventionally removed by foliar applications of ethephon (2-chloroethylphosphonic acid), which specifically target the small immature vegetative shoots that emerge prior to floral induction. However, in the past decades, increasingly warmer autumns and winters in the subtropical regions of the country led to continuous flushing, necessitating more frequent foliar applications with ethephon to eliminate such young vegetative shoots. Developing vegetative shoots in litchi have a high water and nutrient demand (Huang and Chen, 2014). Initially, newly emerging shoots are consumers of assimilates and are only able to contribute to carbohydrate reserves upon reaching 50% of their maximum photosynthetic capacity, when the leaves have fully expanded and develop a dark green color (Fu et al., 2022; Hieke et al., 2002d; Menzel et al., 1995). We hypothesized that complete prevention of new shoot growth prior to panicle emergence, as opposed to elimination of already emerged shoots, will further promote carbohydrate accumulation in small branches and thus enhance flowering in litchi. Therefore, we studied the changes in carbohydrate reserves in small branches in response to the level of shoot inhibition achieved by different ethephon applications for the period between maturation of the last postharvest flush and harvesting. Furthermore, the relative contribution of carbohydrate reserves to flowering and yield, in association with chilling degree hour accumulation, was investigated.

Materials and Methods

Experimental site and plant material. The experiment was conducted on a commercial farm near Mbombela in the Mpumalanga Province of South Africa (latitude -25.453, longitude 30.945, elevation 680 m). The experimental site is characterized by a warm subtropical climate with moderate autumns and cool winters. Daily minimum/maximum and hourly temperature and rainfall recordings were obtained from a scientific-grade weather station of the Agricultural Research Council – Institute for Soil, Climate and Water located 2.6 km from the Mbombela experimental site (station name: ITSC, # 30420). Mean weekly temperatures and mean monthly rainfall between January and September (postharvest shoot growth to flowering) were calculated from the daily minimum and maximum temperatures and rainfall, respectively, for the experimental years 2014 to 2017 (Fig. 1). Chilling degree hours (CDH) and CDH accumulation were calculated based on

the formula provided by Chen et al. (2016), but using 20°C as the base temperature for 'Mauritius', as determined by Batten and McConchie (1995), and incorporating 12-h day and 12-h night mean temperatures instead of hourly temperatures. Chilling degree hour accumulation between ethephon application and bud break or panicle emergence was obtained by means of the equation:

$$CDH_d = \begin{cases} 0, & 12(T_{bi} - T_d) < 0 \\ 12(T_{bi} - T_d), & 12(T_{bi} - T_d) \geq 0 \end{cases}$$

$$CDH_n = \begin{cases} 0, & 12(T_{bi} - T_n) < 0 \\ 12(T_{bi} - T_n), & 12(T_{bi} - T_n) \geq 0 \end{cases}$$

$$CDH_i = CDH_d + CDH_n$$

$$CDH_t = \sum_{i=start\ of\ observation}^{t=bud\ break\ or\ panicle\ emergence} CDH_i$$

where CDH_d is the floral induction quantity for the 12-h day period, CDH_n is the floral induction quantity for the 12-h night period, T_d is the mean air temperature over the 12-h day period (°C), T_n is the mean air temperature over the 12-h night period (°C), T_{bi} is the base temperature for floral induction (20°C), CDH_i is the floral induction quantity for the 24-h cycle, CDH_t is the sum of chilling degree hours from predetermined start (i.e., ethephon application) to end of observation (i.e., bud break or panicle emergence).

All experiments were conducted on 14-year-old 'Mauritius' litchi trees planted on sandy soil (84% sand, 13% clay and 3% silt) at a planting distance of 6.0 × 6.5 m. The experimental trees were selected for uniformity of tree size and vigor and were subjected to the same conventional farming practices with respect to fertilization, irrigation (micro irrigation) and postharvest pruning. In all experimental years, water stress (~50% reduction of soil moisture content) was applied after hardening of the last desired postharvest flush until flower panicles appeared.

Treatments and experimental design. The study was conducted over a four-year period (2014 to 2017). Based on previous experience, three different treatments were selected according to their contrasting phenological responses. These treatments included: 1) ethephon [Ethapon SL; Plaaskem (Pty) Ltd, Witfield, South Africa; containing 480 $g \cdot L^{-1}$ a.i.] applied as a spot spray

application to immature vegetative shoots at 1000 mg·L^{-1}, which represents the current standard practice in South Africa (Spot Spray), 2) ethephon applied as a single whole-canopy spray to trees with fully mature terminal shoots and dormant buds at 1000 mg·L^{-1} (Whole-Canopy), 3) untreated trees with fully mature terminal shoots and dormant buds (Control). The Spot Spray treatment entailed repeated applications to the same experimental trees as new leaf flushes emerged between end of March and early-May. The Whole-Canopy treatment consisted of a single application on each of three respective application dates, i.e., at the end of March (T1), in mid-April (T2) and at the beginning of May (T3) to account for seasonal differences in postharvest flush growth cycles (Table 1). Each application date included untreated trees (Control) to compare the natural shoot development of untreated trees with the ethephon-mediated shoot growth in the Spot Spray and th Whole-Canopy treatment. All ethephon applications were carried out with a motorized sprayer and a hand lance fitted with a 2.5 mm nozzle, at a pressure of 15 bar. A wetting agent [Sanawett 90-940 SL; Dow AgroScience (Pty) Ltd, Bryanston, South Africa; containing 940 g·L^{-1} a.i.] was added to all spray solutions at a concentration of 10 mL·100 L^{-1} water. The applications were made during the morning (before 12h00 noon), taking care to avoid hot days. All applications were made to the point of run-off, at an approximate spray volume of 20-25 L·tree^{-1}.

All experiments were laid out in a randomized complete block design with five trees per treatment and five block replicates. Each experimental unit consisted of three trees, using the middle tree for data collection. Each experimental unit was separated by buffer trees. New experimental trees were chosen each successive year, to ensure that all experimental trees were at the same phenological growth stage on all application dates. As the experiments were conducted under open-field conditions, enough experimental trees at the designated phenological stage were not always available for each scheduled treatment and/or application date and therefore, an unbalanced trial design could not be avoided.

Phenological observations. Ten representative terminal shoot per tree were selected at mid-canopy height (approximately 1.5–1.8 m above the ground) and tagged. Tree response between spray applications and full bloom, i.e., female flower opening, was monitored at a 7-14-day interval. The time interval between treatment application and bud break and floral initiation stage, respectively, was calculated as the number of days between application and the visible appearance of at least 50% of shoots at the respective stage. The number of days to bud break and days to floral initiation stage served as demarcation points for the duration of the dormancy period and definite floral initiation, respectively. The bud break stage was defined as terminal buds starting to swell and turn color from brown to green (Zhang et al., 2016). The floral initiation stage, later referred to as panicle emergence, was defined as inflorescence primordia becoming visible in the leaf axils

in the form of millet-like whitish hairy structures, following a period of inductive temperatures (Huang and Chen, 2005). Flushing and flowering rates were calculated as the percentage of vegetative and flowering shoots, respectively, of the total number of tagged shoots. The yield of each experimental tree was recorded at harvesting.

Carbohydrate determination. Wood (excluding the bark) of four small branches (30-40 mm diameter), randomly selected from all four quarters within the canopy of the experimental trees, were sampled with a hand brace and flat bit, and pooled to one sample per tree. Sampling was conducted on the day of application, during the pre-induction period (May), at flower panicle emergence (May/June), during rapid flower panicle development (June/July), at full bloom (September) and at harvesting (December/January). After collection, the samples were oven-dried at 60°C for 72 h and thereafter ground to a fine powder using a laboratory grinder (Fritsch, Idar-Oberstein, Germany) fitted with a 0.5 mm mesh.

Soluble sugars were extracted according to the method described by Chow and Landhäusser (2004), with slight modifications. Briefly, 100 mg sample was suspended in 3 mL of 80% (v/v) ethanol and total sugars extracted at 80°C for 30 min. The extraction process was repeated twice following the first extraction, and the respective supernatants pooled. Thereafter, the ethanol fraction in the pooled supernatants was evaporated at 80°C until dry. Prior to analysis, the sample extracts were re-suspended in 2 mL distilled water and treated with 150 mg of aluminium oxide (activated, acidic, Brockman 1) (Sigma-Aldrich, Johannesburg, South Africa) to remove organic acids and salts, which can co-elute with sugars and interfere with the analysis, based on recommendations by Waters (Waters Corporation, 2020). The samples were analyzed using a HP 1100 LC system (Agilent Technologies, Santa Clara, CA, USA) with refractive index detection and equipped with a Rezex RCM Monosaccharide Ca^{+2} column (Phenomenex, Torrance, CA, USA). The analyses were performed on 20 µl injection volume with deionized water as eluent, at a flow rate of 0.5 $mL \cdot min^{-1}$. The column temperature was kept at 80°C. Quantification was achieved based on external standard calibration derived from a standard curve with analytical-grade sucrose, fructose, glucose, raffinose, myo-inositol and quebrachitol (Sigma-Aldrich, Johannesburg, South Africa). The sugar concentrations were expressed as percentage of sample dry weight (DW).

Starch was extracted and analyzed according to the iodine colorimetric method as described by Xu et al. (1998). Fifty milligram sample was ground in 5 mL of 1% PVP-40 (w/v). The homogenate was then centrifuged at 5000 rpm (~ 4700 × *g*) for 10 min at room temperature using an Allegra X-22 centrifuge (Beckman Coulter, Fullerton, CA, USA). After discarding the pigmented supernatant, starch was extracted by re-suspending the pellet in 10 mL of 80% calcium nitrate ($Ca(NO_3)_2$) at 100°C for 20 min and then centrifuged at 5000 rpm for 10 min at room temperature. The extraction

process was repeated once following the first extraction, whereafter the respective supernatants were filtered through cellulose filter papers (Advantec No. 2, Advantec Toyo Kaisha, Tokyo, Japan) pooled and adjusted to 25 mL with 80% $Ca(NO_3)_2$ solution. For analyses, 0.1 mL of 0.01 N I_2-KI solution was added to 2.9 mL of the pooled sample supernatant, blanks (distilled water) and starch standard solutions to a final volume of 3.0 mL. The starch concentration was determined from the changes in absorbance of the starch-iodine complex at 620 nm using a UV-1800 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of starch was calculated based on external standard calibration derived from a standard curve with analytical-grade soluble starch (Sigma-Aldrich, Johannesburg, South Africa). The starch concentration was expressed as percentage of sample dry weight (DW).

Statistical analysis. All data was subjected to analysis of variance (ANOVA) using the General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Data collected over time was subjected to repeated measures analysis using SAS software with application date, treatment, and application date × treatment as fixed factors. Where ANOVA indicated significance at $P < 0.05$, comparison of treatment means was done using Fisher's least significant difference (LSD) test at a significance level of 5%, unless stated otherwise. A weighted ANOVA was performed for the data on percentage of flushing and flowering shoots over time and across years. The Shapiro-Wilk and Levene's tests confirmed normality of data and homogeneity of variances, respectively, except for data on the percentages of flushing shoots due to a high number of zero values. ARCsine transformation of flush data did not improve homogeneity and therefore, flush data is presented untransformed. Means across the four experimental years were calculated for specific phenological events for the respective application dates to gain more insight into general trends that emerged for the various treatments. Principal component analysis (PCA) with a correlation matrix was performed to investigate the relationship between treatment factors (application date × treatment) and selected variables using XLSTAT (Version 2015.1.03.15485, Paris, France).

Results

For a more comprehensive interpretation, results for non-structural carbohydrate concentrations detected in small branches are presented together with vegetative shoot and panicle growth patterns as well as yield (Figs. 2-5). The Spot Spray treatment, which was repeatedly applied between March and May, is added in the figures to all applications dates to facilitate comparisons with the other treatments. In general, the most abundant carbohydrate present in the small branches was starch (Fig. 2-5). Sucrose and quebrachitol accounted for more than 50% and

35% of the total soluble sugars, respectively. Starch concentrations were overall higher during the colder years of 2014 and 2015, and lower during the warmer years of 2016 and 2017, while this trend was inverse for sucrose and quebrachitol concentrations. Glucose, fructose, raffinose, inositol and bornesitol were found in very small concentrations in the small branches, and therefore, only the results for starch, sucrose and quebrachitol will be discussed further on.

Effect of ethephon-mediated phenology modulation on reserve carbohydrate dynamics

Weather conditions during autumn and early winter impacted on pre-induction vegetative shoot growth, particularly during the early application periods (T1), where higher temperature and rainfall prevalence promoted vegetative shoot growth in untreated trees (Control), compared with later application dates (T2 and T3) (Fig. 1). However, under these growth-promoting conditions, both the Spot Spray and the Whole-Canopy treatment reduced and completely prevented pre-induction vegetative shoot growth, respectively, and increased flowering rate and yield (Figs. 2-5). In addition, the Whole-Canopy treatment delayed bud break and panicle emergence almost across all application dates and years and consequently accumulated more chilling degree hours (CDH) compared with the Spot Spray and the Control treatment (Table 2 and 3). In general, flowering rate and yield were positively correlated (Fig. 2E, 2J and 2O; Fig. 3E, 3J and 3O; Fig. 4E and 4J; Fig. 5E and 4J).

2014 experimental year. In 2014, the initial shoot growth in the Spot Spray treatment resulted in the lowest sucrose and quebrachitol concentrations in April compared with the other two treatments. Nevertheless, sucrose concentrations increased in the Spot Spray treatment prior to panicle development since no further shoot growth took place following treatment (Fig. 2B and 2D). In general, starch concentrations in the Spot Spray treatment were at a higher level during April and May, compared with the other treatments, and appeared to be unaffected by the initial shoot growth (Fig. 2A and 2D). In the Control treatment, high flushing rates between April and June (Fig. 2D) delayed starch accumulation and reduced sucrose concentrations during May compared with the Spot Spray treatment (Fig. 2A and 2B). The lack of vegetative shoot growth in the Whole-Canopy treatment resulted in the highest sucrose concentrations in May as well as the highest increase in starch accumulation in June (Fig. 2A and 2E). Concurrently, the Whole-Canopy treatment significantly delayed bud break and panicle emergence compared with the other two treatments (Table 2 and 3). Rapid panicle development in June significantly reduced sucrose and quebrachitol concentrations in the Whole-Canopy treatment, but not in the Spot Spray treatment, which had an equally high flowering rate (Fig. 2A-C and 2E). Instead, panicle development in the Spot Spray treatment concurred with a decline in starch concentrations in June. During full bloom

and harvest, no treatment differences were observed for any of the carbohydrates, despite the lower flowering rate and yield in the Control treatment compared with the two ethephon treatments (Fig. 2E).

For the application date in mid-April (T2), similar trends in carbohydrate dynamics were observed compared with the early application date, although less pronounced due to the lower flushing rates in the untreated trees (Fig. 2F-J). No treatment differences were observed at full bloom or harvest. However, the increased yields in both ethephon treatments at the mid application date (T2) were associated with decreased starch and quebrachitol concentrations at harvest, compared with the Control treatment (Fig. 2J).

At the late application date (T3), limited or no shoot growth was recorded for all treatments between mid-April and end of June, which resulted in limited treatment differences for the respective carbohydrate types (Fig. 2K-N). Late vegetative growth in the Whole-Canopy and the Control treatment during July and August reduced flowering rate, but did not appear to influence sucrose and quebrachitol concentrations during the same period. Starch concentrations in the Whole-Canopy treatment accumulated until September and were highest at full bloom, which was associated with the delayed and decreased panicle development compared with the other two treatments (Table 2 and 3). Similar to the mid application date, the starch and quebrachitol concentrations were inversely associated with crop load (Fig. 2K, 2M and 2O).

2015 experimental year. Initial flushing incidence in the Spot Spray treatment was lower than in 2014 and minimal in the Control and the Whole-Canopy treatment across all application dates (Fig. 3D, 3I and 3N). For the early application date (T1), no distinct treatment differences were observed for starch and quebrachitol concentrations over the entire observation period (Fig. 3A and 3C). However, at trial start, sucrose concentrations were lowest in the Spot Spray treatment and highest in the Whole-Canopy treatment (Fig. 3B). Sucrose concentrations increased thereafter in all treatments towards early panicle emergence, which was about 2 weeks earlier in 2015 than in 2014 (Fig. 3B and 3E; Table 3). The Whole-Canopy treatment exhibited distinctly higher sucrose concentrations during panicle development in May compared with the Spot Spray and the Control treatment. Between May and September (panicle development to full bloom period), sucrose concentrations declined again in all treatments, concomitant with their high flowering rates (Fig. 3E). No treatment differences for the respective carbohydrate types were observed at harvest despite significant differences in yield between the Whole-Canopy and Spot Spray treatments.

Carbohydrate dynamics in trees of treatments applied at the mid and late application dates (T2 and T3) were comparable to those of trees at the early application date due to similar trends in

flushing rate, flowering rate and yield (Fig. 3F-O). No significant treatment differences were observed for the various parameters.

2016 experimental year. During the early application period (T1), late summer rainfall together with above-average temperatures during March and April caused high flushing rates in the Spot Spray and the Control treatment, but only limited shoot growth in the Whole-Canopy treatment (Figs. 1 and 4D). The Spot Spray treatment successfully removed the initial and any recurring leaf flush. Vegetative shoots in the Control treatment completed maturation in time to achieve successful floral induction and initiation, which occurred almost a month later compared with 2014 and 2015 (Table 3). Starch accumulated steadily in all treatments prior to and during panicle growth, with no significant differences at full bloom (Fig 4A). However, similar to 2014, high flushing rate in the Control treatment reduced sucrose levels during the pre-induction period, particularly during May, while sucrose concentrations in the Spot Spray and the Whole-Canopy treatment increased or remained stable, respectively (Fig. 4B). Quebrachitol concentrations remained stable throughout the reproductive phase, with a slight, but non-significant, reduction during rapid panicle development in July in the Spot Spray and the Whole-Canopy treatment, which both had the highest flowering rates (Fig. 4C and 4E). Despite high flowering rates, crop load was low for all treatments, which might be associated with the low starch concentrations at harvest across all treatments.

For the late application time (T3), no shoot growth was recorded between May and September for any of the treatments (Fig. 4I). All treatments showed a small, but steady, increase in starch concentrations up until full bloom (Fig. 4F). Unlike 2014 and 2015, sucrose concentrations of all treatments applied late in 2016, particularly in the Whole-Canopy treatment, increased until July in accordance with their delayed panicle emergence compared with 2014 and 2015 (Table 3), before decreasing again at full bloom and harvest (Fig. 4G). Concurrent with the lower flowering rates, sucrose concentrations were significantly higher in the Whole-Canopy treatment in July and September compared with the Spot Spray and the Control treatment (Fig. 4G and 4J). Quebrachitol concentrations remained stable in the Control treatment, but slightly declined in the Spot Spray treatment during rapid panicle development in July and increased in the Whole-Canopy treatment towards full bloom (Fig. 4H). At harvesting, no treatment differences were observed for the respective carbohydrate types or yield (Fig. 4F-J).

2017 experimental year. At the early application date (T1), low vegetative shoot growth was observed for the Spot Spray and the Control treatment, and no flushing was noted in the Whole-Canopy treatment (Fig. 5D). This led to starch accumulation in all treatments during the pre-induction and panicle growth period, followed by a decline at full bloom in the ethephon treatments,

and a subsequent recovery in starch concentrations for each treatment at harvest (Fig. 5A). Sucrose concentrations increased in all treatments until panicle emergence, and thereafter declined until full bloom and harvesting (Fig. 5B). No distinct treatment differences were observed for quebrachitol concentrations throughout the observation period (Fig. 5C). Yield was significantly higher in the Whole-Canopy treatment compared with the Spot Spray and the Control treatment. However, this was not reflected in significant lower starch concentrations as observed in some of the previous seasons (Fig. 5E).

Treatments applied at the late application date (T3) were subject to late rainfall and warm winter temperatures, which caused vegetative shoot growth after May in the Control treatment and to a lesser extent in the Whole-Canopy treatment (Figs. 1 and 5I). Nevertheless, starch concentrations accumulated continuously in these two treatments until September. Starch concentrations in the Spot Spray treatment increased until July, but declined in September at full bloom in line with its high flowering rate (Fig. 5F and 5J). Likewise, sucrose concentrations accumulated between April and June in all treatments, but declined in the Spot Spray and the Control treatment until full bloom, concurrent with their respective panicle or vegetative shoot growth (Fig. 5G, 5I and 5J). In contrast, lack of panicle development in the Whole-Canopy treatment allowed sucrose accumulation until July, which was then followed by a decline in September, corresponding with this treatment's high flushing rate. Quebrachitol concentrations fluctuated slightly throughout the observation period and were overall lowest in the Control treatment (Fig. 5H). Flowering and yield were significantly higher in the Spot Spray treatment compared with the other two treatments (Fig. 5J), but no treatment differences were observed for starch, sucrose and quebrachitol at harvest (Fig. 5F-H).

For the evaluation of general trends across years, the four-year data was consolidated according to specific phenological stages, taking into account that there were only two years of data available for the mid application date. The combined data confirmed and emphasized certain trends observed in the individual years, although few treatment effects were significant (Tables 4 and 5). Noticeable trends included that the initial flush development in the Spot Spray treatment in April reduced sucrose concentrations at all application dates and quebrachitol concentrations at the mid application date (T2), but did not appear to affect starch concentrations negatively (Table 4). Likewise, complete flush development in the Control treatment in May reduced sucrose as well as starch concentrations at all application dates, while suppression of vegetative shoot growth (Spot Spray and Whole-Canopy treatments) allowed carbohydrate accumulation in May (Table 4). Furthermore, vegetative shoots developing concurrent with panicle development tended to reduce sucrose and quebrachitol concentrations, while starch concentrations remained unaffected (Table

5). Treatments resulting in the highest flowering rates were associated with higher starch, sucrose and quebrachitol concentrations at flowering, while trees with the highest yields tended to have lower starch and sucrose concentrations at harvest (Table 5).

Relationship between phenological growth events, CDH accumulation and reserve carbohydrate status

Principal component analysis (PCA) was performed with factors that influence carbohydrate dynamics prior to flowering, i.e., flushing rate, days to panicle emergence (DTPE), CDH accumulation, flowering rate, as well as starch and sucrose concentrations between May and July (as the main period for carbohydrate accumulation relevant for successful flowering). Quebrachitol was omitted due to limited treatment differences and seasonal fluctuations. The PCA results revealed similar trends for 2014 and 2015, which had cooler winters and earlier panicle emergence, compared with 2016 and 2017, which had warmer winters and later panicle emergence (Figs. 1 and 6; Tables 2 and 3). Overall, pre-induction flushing rate was negatively correlated with flowering rate and sucrose concentration (May, June and/or July) (Fig. 6; Table 6). Treatments associated with flushing rate were mostly the Control treatments, particularly at the early application date. In contrast, flowering rate was positively related with sucrose and starch concentrations prior to panicle emergence, i.e., in May, and was primarily associated with the Whole-Canopy treatment at the early and mid application dates (T1 and T2) (Table 6). Although not consistently related to flowering rate, DTPE was positively correlated with sucrose in May during the years with early panicle emergence (2014, 2015), and with sucrose in June or July during years with late panicle emergence (2016, 2017) (Fig. 6; Tables 3 and 6). Interestingly, the Pearson's correlation matrices (from Principal Component Analysis) further revealed strong positive correlations between CDH accumulation and DTPE in 2014 and 2017, as well as with sucrose in May (for 2014/2015) or June/July (for 2016/2017), respectively, depending on the time of panicle emergence in the respective years (Tables 3 and 6). Chilling degree hour accumulation was also mainly associated with the Whole-Canopy treatment (Fig. 6).

Discussion

The role of ethephon-mediated vegetative shoot growth regulation in carbohydrate accumulation prior to panicle development. In our study, the two different shoot control approaches with ethephon and no treatment, created contrasting effects in terms of pre-induction vegetative shoot growth (Figs. 2-5; Tables 4 and 5). These effects manifested in either unrestricted, partial, or no vegetative shoot growth prior to panicle emergence, in the Control, the Spot Spray and the Whole-Canopy treatment, respectively. The reserve carbohydrate dynamics associated with these

differences in vegetative or floral shoot development, and its relevance for litchi flowering, was subsequently considered.

Flushing rates below 30%, irrespective of time of emergence or treatment application, did not cause significant changes in starch, sucrose or quebrachitol concentrations. However, high flushing rates in the Spot Spray treatment at the beginning of the study in April, only reduced sucrose concentrations slightly, but did not affect starch and quebrachitol as the ethephon spot sprays removed the soft vegetative shoots before it could complete its growth cycle, compared with vegetative shoots in the untreated trees that were allowed to finish their development, e.g., at the early application date in 2014 (Figs. 2-5). These effects on sucrose concentrations were more evident in 2014 and 2015 than in 2016 and 2017. The four-year consolidated data confirmed these trends and further revealed higher sucrose accumulation increments in May following flush elimination compared with the Whole-Canopy and the Control treatment (Table 4). Nevertheless, an additional sampling point prior to shoot emergence in the Spot Spray treatment could have provided more clarity on the true impact of initial shoot growth and early elimination of young shoots on the sucrose reserve status.

In contrast, vegetative shoot growth rates above 40% reduced sucrose, and in some years quebrachitol concentrations, irrespective whether the shoot growth occurred prior to floral induction, e.g., during April/May of 2014 and 2016 (Fig. 2B-D and 4B-D; Table 4), or commenced its growth past the induction period, e.g., during July and August of 2017 (Fig. 5G-I). This was also reflected in negative correlations between flushing rate and sucrose concentrations in May or July of the respective years (Fig. 6; Table 6) and indicates that the intensity of vegetative shoot growth influences the sink strength. Nevertheless, starch concentrations were not negatively affected by vegetative shoot growth, neither in its initial stage (Spot Spray treatment) nor when allowed to complete its full development (Control treatment), but continued to accumulate until panicle development and in some instances even until full bloom (Fig. 2A-5A). However, high flushing rates restricted the extent of starch accumulation, as could be observed in the Control treatment, e.g., in 2014 at the early application date. This was in contrast to Menzel et al. (1995) who found that shoot growth reduced starch concentration in small and medium branches. Previous studies on litchi reported that vegetative shoots are strong sinks for assimilates and rely on reserve carbohydrates for their initial growth (Cronje and Mostert, 2009; Fu et al., 2022; Hieke et al., 2002d; Menzel et al., 1995). Young shoots are only able to contribute to the carbohydrate reserve pool when reaching about 50% of their maximum photosynthetic capacity. This sink-to-source transition occurs when the leaves have fully expanded and start turning dark green in color (Fu et al., 2022; Hieke et al., 2002d; Menzel et al., 1995).

In contrast to the Control and the Spot Spray treatment, the ethephon application to fully mature shoots with dormant buds (Whole-Canopy treatment) prevented pre-induction vegetative shoot emergence almost entirely. This promoted almost immediate sucrose and starch accumulation prior to flower panicle emergence (Fig. 3), except in 2015, when starch levels remained stable, possibly due to drought conditions during the summer and autumn months (Fig. 1), which may have restricted photosynthetic activity (Menzel, 2005; Rosa et al., 2009). However, quebrachitol concentrations remained unaffected (Figs. 2-5; Table 4). In addition, the Whole-Canopy treatment delayed bud break and panicle emergence, subsequently providing additional time for carbohydrate accumulation compared with the Spot Spray treatment (Table 3). Whole-canopy ethephon application to mature shoots therefore proved to be more beneficial for carbohydrate accumulation prior to panicle development by preventing new vegetative shoot growth entirely compared with spot spray applications which removed the vegetative shoots only once emerged. Similarly, an Indian study found that ethephon application at a concentration of 960 $mg \cdot L^{-1}$ increased the C/N ratio in leaves and shoots of the litchi cultivar 'Bombai' prior to panicle emergence (Mandal et al., 2014). However, this study did not mention at which phenological stage ethephon was applied.

Ethephon-mediated carbohydrate accumulation may possibly be related to ethylene-induced reactive oxygen species accumulation and abscisic biosynthesis activation, which are both known to influence sugar metabolism (Chen et al., 2019; Couée et al., 2006; Li et al., 2015; Pierik et al., 2006). However, the effect of ethylene depends on the concentration and duration of exposure (Pierik et al., 2006). Studies on some herbaceous plants, such as mustard, showed that short-term exposure and low ethylene concentration (< 200 $\mu L \cdot L^{-1}$) increased stomatal conductance, photosynthetic activity and enhanced nitrogen use efficiency, therefore, contributing to enhanced carbon assimilation. High ethylene concentrations, however, reduced stomatal conductance and subsequently lowered photosynthetic activity (Pierik et al., 2006; Iqbal et al., 2013). Similarly, low concentration of ethephon (150 $mg \cdot L^{-1}$) increased total sugars and sucrose concentrations in pineapple buds 48 h after application (Ávila et al., 2005). Whether the carbohydrate accumulation in our study was directly related to the effect of ethephon-derived ethylene on a potential modification of photosynthesis and carbon assimilation and what role the cultivar and plant organ played in the sensitivity to the different ethylene concentrations in this regards, requires further research.

The role of carbohydrate accumulation in panicle development, flowering rate and yield. In the current study, ethephon applications did not only promote carbohydrate accumulation by suppressing vegetative growth, but also facilitated the maintenance of shoot maturity for an extended period (Figs. 2-5). Many studies have highlighted the important role of shoot maturity as

essential prerequisite for successful litchi flower induction (Batten and McConchie, 1995; Chen and Huang, 2005; Davenport and Stern, 2005; O'Hare, 2002; Xiao et al., 2018; Yang et al., 2014). In our study, the relationship between shoot maturity, carbohydrate accumulation and flowering rate was emphasized by positive correlations between sucrose concentrations in May, i.e., prior to panicle emergence, and flowering rate, which were both associated particularly with the Whole-Canopy treatment at the various application dates (Fig. 6; Table 6). In most years and application dates, the Whole-Canopy treatment resulted in higher carbohydrate accumulation increments of particularly sucrose compared with the Spot Spray and the Control treatment.

Similar to vegetative shoot growth, early panicle development is a strong sink that requires readily available sugars to meet the high carbohydrate demand for developmental processes (Burnett, 2019; Zhang et al., 2016). Growing in warmer climates, litchi buds do not require protective scales like those of temperate tree crops and are therefore naked with only rudimentary leaves enclosing the apical meristem (Zhang et al., 2016). As such, breaking litchi buds have very high respiration rates, even higher than during active growth, thus, creating a high energy demand (Yang et al., 2014; Zhang et al., 2016). However, floral initiation and panicle development in litchi takes place in winter, when photosynthetic activity is generally low due to low temperatures and cold- or drought-induced photoinhibition and supply of current assimilates for growth processes is restricted (Cronje and Mostert, 2009; Menzel, 2005; Rosa et al., 2009).

The high carbohydrate demand for panicle development in our study was demonstrated by a decline in sucrose concentrations during rapid panicle development up until full bloom, particularly in heavily flowering trees (Figs. 2-5; Table 5), and confirmed similar trends observed by Chen et al. (2004) in the litchi cultivars 'Guiwei', 'Feizixiao' and 'Nuomici'. A reduction of sucrose concentrations during floral induction and initiation were also found in mature leaves of terminal shoots of the litchi cultivars 'Guiwei', 'Feizixiao' and 'Huaizhi' (Yang et al., 2014). Our study also found a decrease in quebrachitol concentrations during early flower formation in treatments with high flowering rates, e.g., in the Whole-Canopy treatments in 2014 and 2016 (Figs. 2 and 4; Table 5). This decline may indicate a shortage of readily available assimilate supply that required mobilization of quebrachitol to meet the high carbon demand. Although quebrachitol is considered an inert source of reserve energy, it was suggested that it may be converted to other sugars under conditions of high sugar demand, and as such may play a unique role in litchi as important osmolyte to maximize carbon use efficiency and growth (Wu et al., 2018).

The role of quebrachitol has not been deliberated for litchi floral development. Yet, Wu et al. (2018) found that mature autumn leaves had higher quebrachitol concentrations compared with mature summer leaves, indicating that quebrachitol is being accumulated prior to flowering and may therefore play a role as a reserve carbohydrate to support flowering, similar to other sugar

alcohols, e.g., sorbitol in many Rosaceae species (Moing, 2000). Some degree of quebrachitol accumulation prior to panicle emergence was also observed in the ethephon-treated trees in our study. Although fluctuations of quebrachitol concentrations were generally less apparent than those of sucrose, as was also observed by Wu et al. (2018), the accumulation of quebrachitol may have contributed to the high flowering rates of ethephon-treated trees, particularly in 2015 and 2016 (Figs. 3 and 4). Studying quebrachitol concentrations in leaves and terminal shoots may show stronger associations between quebrachitol concentrations and certain phenological stages and may contribute to a better understanding of its function in floral development in litchi. Overall, the results of our study suggest that similar to vegetative growth, panicle development strongly relies on previously accumulated soluble sugars.

In contrast, starch concentrations increased progressively between application and panicle development, but decreased toward full bloom and fruit development, which suggests that blooming, fruit set and possibly early stages of fruit development were supported by starch reserves, particularly in high yielding trees (Figs. 2-5; Table 5). These results concur with other reports on starch dynamics during panicle and fruit development in litchi (Chen et al., 2004; Cronje and Mostert, 2009; Cronje et al., 2021; Menzel et al., 1995; Yuan et al., 2009). Other studies on litchi found that early fruit development was more reliant on stored reserves than later fruit development stages, which in turn depended more on current assimilates (Yuan et al., 2009; Hieke et al., 2002a). Therefore, promoting the accumulation of carbohydrates prior to floral development is crucial to support bud break, panicle development and initial fruit growth until a time when fruit growth can be supported by current photoassimilates under more favorable temperatures that will promote photosynthetic activity (Hieke et al., 2002c; Roe et al., 1997).

The role of ethephon-mediated dormancy extension and CDH in carbohydrate accumulation. Both ethylene and ethephon treatments are known to inhibit cell division and growth, promote senescence and delay bud growth in various crops (Abeles et al., 1992; Burg, 1973; Durner and Gianfagna, 1991; Hansen and Grossmann 2000). Ethephon treatment has been used in various deciduous fruit trees to delay bud break and flowering to increase cold hardiness (via increased carbohydrate accumulation), as well as to reduce bud sensitivity to elevated temperatures and the associated de-acclimation conditions after mid-winter thaws to avoid frost damage in spring (Crisosto et al., 1990; Ebel et al., 1999; Gianfagna et al., 1992; Liu et al., 2021). Likewise, the Whole-Canopy treatment in the current study delayed bud break and panicle emergence on average by two to three weeks compared with the Spot Spray and the Control treatment (Tables 2 and 3).

Maintaining or prolonging shoot maturity is particularly important during years with warm winters, such as experienced in 2016 and 2017 in our study (Fig. 1), where the likelihood of new

vegetative shoot growth and the presence of immature shoots prior to floral induction increases. By suppressing vegetative growth during warm winters and delaying panicle emergence, the Whole-Canopy treatment not only extended the period for carbohydrate accumulation, but also provided more time for CDH accumulation, and promoted panicle emergence at a time when temperatures were conducive for floral induction and initiation, compared with panicle emergence in the Control (Tables 2 and 3). The multifaceted interactions between ethephon application date, flushing rate, carbohydrate reserves, time of panicle emergence, CDH, as well as flowering rate were highlighted by the PCA. Principal Component Analysis showed strong associations between the Whole-Canopy treatment and the time intervals between application and panicle emergence (DTPE) (except in 2016; Fig. 6C), CDH, sucrose and starch concentrations. Furthermore, positive correlations between DTPE or CDH and sucrose or starch concentrations clearly depict the role of the Whole-Canopy treatment in promoting pre-flower carbohydrate accumulation and flowering in litchi (Fig. 6; Table 6). No consistent associations between application date and carbohydrate levels at certain months were found. However, CDH accumulation during the years with cooler winters (2014 and 2015) was positively correlated with sucrose concentrations in May, while CDH accumulation during the years with warmer winter (2016 and 2017) was positively correlated with sucrose concentrations in June and July (Fig. 6), according to the time of panicle emergence in the respective years (Tables 2 and 3). While flowering rate was positively correlated with sucrose concentrations in May in all four years, as well as with starch concentrations in May/June/July in 2016 and 2017, it could not be consistently correlated with CDH, possibly due to data pooling. Therefore, our study was not able to establish whether high pre-flowering carbohydrate levels can also contribute to the chilling responsiveness of trees, as proposed by Huang and Chen (2014), or whether merely delaying bud break to a time with consistently low temperatures provided sufficient favorable conditions for successful floral induction and initiation in the Whole-Canopy treatment.

Conclusion

The current study provided insight into the changes of reserve carbohydrates of small branches of litchi depending on phenological growth events. The results showed that pre-induction vegetative shoot growth reduced or arrested carbohydrate accumulation prior to flowering, while prevention of vegetative shoot growth promoted carbohydrate accumulation. In this regard, complete prevention of shoot growth by applying ethephon to mature vegetative shoots proved to be more beneficial compared with successive elimination of existing immature shoots by spot spray applications. Furthermore, the data suggests that vegetative shoot growth and panicle development depend more on sucrose reserves rather than starch reserves. Although generally very stable, quebrachitol concentrations were affected in trees with high flushing or flowering rates and

therefore, also appear to play a role as easily transportable carbohydrate that supports flowering processes in litchi. Limited knowledge on the function of quebrachitol in litchi flowering warrants further research.

The current study only investigated ethephon-mediated changes in phenology and its effects on carbohydrate dynamics in small branches. However, the effect of ethephon on various physiological and molecular mechanisms involved in regulating shoot maturity, bud dormancy and carbohydrate accumulation in leaves and terminal shoots remains unclear.

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Table 1. Treatments applied to 'Mauritius' litchi trees to study carbohydrate reserve dynamics in small bearing branches associated with treatment-mediated phenological changes. Treatments were conducted at an experimental site near Mbombela, South Africa, during autumn and/or early winter of 2014 to 2017.

Treatment code	Treatment description	Phenological growth stage at time of application	Application date / frequency			
			2014[i]	2015[ii]	2016[iii]	2017[iv]
Control	Untreated	Mature dark green shoots with dormant buds	T1, T2, T3[v]	T1, T2, T3	T1, T3	T1, T3
Spot Spray	1000 mg·L^{-1} ethephon as repeated spot sprays	Soft immature vegetative shoots	3[vi]	3	4	2
Whole-Canopy	1000 mg·L^{-1} ethephon as single whole-canopy spray	Mature dark green shoots with dormant buds	T1, T2, T3	T1, T2, T3	T1, T3	T1, T3

[i]2014 spray dates: 25 March (T1), 8 April (T2) and 25 April (T3).
[ii]2015 spray dates: 30 March (T1), 13 April (T2) and 28 April (T3).
[iii]2016 spray dates: 23 March (T1) and 4 May (T3).
[iv]2017 spray dates: 30 March (T1) and 23 May (T3).
[v]Date of application (ethephon treatments) or monitoring start (untreated trees) within an experimental year, i.e., T1: early, T2: mid and T3: late application date (as indicated in i-iv)
[vi]Number of spot sprays applied to the same trees during the application period of March to May.

Table 2. Bud break or floral initiation reaction time, as well as accumulated chilling degree hours (CDH) between application and panicle emergence, in response to different ethephon treatments (Spot Spray, Whole-canopy) or in untreated trees (Control) of 'Mauritius' litchi. Ethephon was applied at the end of March (early, T1), mid-April (mid, T2) and/or early-May (late, T3) as repeated spot spray applications to small immature shoots at a concentration of 1000 mg·L^{-1} (Spot Spray) or as a single whole-canopy treatment to fully mature shoots at a concentration of 1000 mg·L^{-1} (Whole-Canopy). Control represents untreated trees. Values represent means (n = 5).

	Time interval between application and bud break (days)				Time interval between application and panicle emergence (days)				Accumulated CDH between application and panicle emergence			
	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
Treatment	*Early application (T1)*											
Control	33.8 b[i]	35.0 a	50.2 a	36.2 b	64.6 b	46.8[ii]	84.8	64.8	3778.7 b	2440.3	4843.9	4747.0
Spot Spray[iii]	32.6 b	16.0 b	33.0 b	41.8 ab	62.8 b	44.4	79.0	68.0	3608.9 b	2221.8	4268.3	5175.3
Whole-Canopy	56.8 a	45.2 a	62.0 a	50.2 a	83.4 a	52.4	79.0	68.0	6166.2 a	3058.4	4268.3	5175.3
P value[iv]	*<0.001*	*0.0017*	*0.0073*	*0.0181*	*< 0.0001*	*0.1794*	*0.0893*	*0.4334*	*< 0.0001*	*0.2180*	*0.1137*	*0.4334*
	Mid application (T2)											
Control	32.8 b	21.0 b	n/a[v]	n/a	62.4 b	43.0 b	n/a	n/a	4854.1 b	2902.2 b	n/a	n/a
Spot Spray	32.6 b	16.0 b	n/a	n/a	62.8 b	44.4 b	n/a	n/a	3608.9 c	2221.8 c	n/a	n/a
Whole-Canopy	57.2 a	37.4 a	n/a	n/a	74.8 a	52.0 a	n/a	n/a	6562.8 a	3927.3 a	n/a	n/a
P value	*0.0046*	*0.0004*			*0.0007*	*0.0010*			*< 0.0001*	*< 0.0001*		
	Late application (T3)											
Control	12.0 c	6.0 c	45.8 b	42.5	50.4 c	29.6 b	67.8 b	69.5	4848.3 b	2408.9 b	6918.0 b	8937.7
Spot Spray	32.6 b	16.0 b	33.0 c	41.8	62.8 b	44.4 a	79.0 a	68.0	3608.9 c	2221.8 b	4268.3 c	5175.3
Whole-Canopy	52.4 a	29.6 a	67.6 a	70.0	74.0 a	41.6 a	84.0 a	112.0	7819.7 a	4031.5 a	8949.7 a	13814.0
P value	*0.0008*	*0.0003*	*0.0004*	*0.9101*	*< 0.0001*	*0.0013*	*0.0101*	*0.9296*	*< 0.0001*	*0.0062*	*< 0.0001*	*0.2645*

[i]Treatment means within a column and application period followed by different letters indicate statistical significance according to Fisher's LSD test (p = 0.05).
[ii]Means within a column without letters do not differ significantly.
[iii]Data for the Spot Spray treatment was added to each application date to aid comparisons and therefore is the same for all application times within a year.
[iv]Statistical analysis of data was assessed according to randomized block design ANOVA (P < 0.05).
[v]n/a means treatment was not applied.

Table 3. Average dates for bud break and panicle emergence in 'Mauritius' litchi trees in response to different ethephon treatments (Spot Spray, Whole-Canopy) or in untreated trees (Control) for application dates at the end of March (early, T1), mid-April (mid, T2) and/or early/mid-May (late, T3) between 2014 and 2017. Ethephon was applied as repeated spot spray applications to small immature shoots at a concentration of 1000 mg·L^{-1} (Spot Spray) or as a single whole-canopy treatment to fully mature shoots at a concentration of 1000 mg·L^{-1} (Whole-Canopy). Control represents untreated trees.

	Average bud break date				Average panicle emergence date			
Treatment	2014	2015	2016	2017	2014	2015	2016	2017
	Early application (T1)							
Control	27-Apr	3-May	11-May	4-May	29-May	15-May	15-Jun	6-Jun
Spot Spray[i]	26-Apr	14-Apr	24-Apr	10-May	26-May	12-May	9-Jun	6-Jun
Whole-Canopy	20-May	13-May	25-May	18-May	15-Jun	19-May	9-Jun	6-Jun
	Mid application (T2)							
Control	10-May	3-May	n/a[ii]	n/a	9-Jun	22-May	n/a	n/a
Spot Spray	26-Apr	14-Apr	n/a	n/a	26-May	12-May	n/a	n/a
Whole-Canopy	3-Jun	19-May	n/a	n/a	22-Jun	3-Jun	n/a	n/a
	Late application (T3)							
Control	6-May	3-May	18-Jun	4-Jul	14-Jun	27-May	10-Jul	29-Jul
Spot Spray	26-Apr	14-Apr	24-Apr	10-May	26-May	12-May	9-Jun	6-Jun
Whole-Canopy	15-Jun	27-May	10-Jul	31-Jul	7-Jul	7-Jun	26-Jul	11-Sep

[i]Data for the Spot Spray treatment was added to each application date to aid comparisons and therefore is the same for all application times within a year.
[ii]n/a means treatment was not applied.

Table 4. Mean starch, sucrose and quebrachitol concentrations (expressed as percentage of wood dry weight (DW) in small branches) and flushing rate of 'Mauritius' litchi trees during the pre-induction period (April and May), in response to different ethephon treatments (Spot Spray and Whole-Canopy) or no treatment (Control) across the four-year period of 2014-2017. Treatments were applied at the end of March (early, T1), mid-April (mid, T2) and end-April (late, T3). Values represent means (n = 20 (for T1 and T3); n = 10 (for T2)).

	During pre-induction period							
Treatment	Starch Apr (%DW)	Sucrose Apr (%DW)	Quebrachitol Apr (%DW)	Flushing rate Apr (%)	Starch May (%DW)	Sucrose May (%DW)	Quebrachitol May (%DW)	Flushing rate May (%)
	Early application (T1)							
Control	4.24[i]	1.05 b[ii]	0.70	1.0 b	4.94 b	1.11 b	0.74	40.0 a
Spot Spray[iii]	6.19	0.98 b	0.70	73.0 a	7.95 a	1.46 a	0.76	5.0 b
Whole-Canopy	3.74	1.21 a	0.71	0.0 b	5.48 b	1.59 a	0.76	3.0 b
P value[iv]	*0.1300*	*0.0143*	*0.9694*	*<0.0001*	*<0.0001*	*0.0005*	*0.9772*	*<0.0001*
	Mid application (T2)							
Control	6.38 b	1.10 b	0.73 a	0.0 b	7.33 b	1.27	0.68	9.0
Spot Spray	10.12 a	0.83 c	0.62 b	53.0 a	11.77 a	1.34	0.74	2.0
Whole-Canopy	6.28 b	1.35 a	0.73 a	0.0 b	8.87 b	1.50	0.79	0.0
P value	*0.0021*	*<0.0001*	*0.0344*	*<0.0001*	*0.0039*	*0.3683*	*0.1240*	*0.9889*
	Late application (T3)							
Control	8.31 b	1.47	0.70	0.0 b	5.77	1.02 b	0.69	1.5
Spot Spray	6.19 c	0.98	0.70	73.0 a	7.95	1.46 a	0.76	5.0
Whole-Canopy	11.91 a	1.03	0.71	0.0 b	6.03	1.19 b	0.70	0.0
P value	*0.0100*	*0.0793*	*0.4718*	*<0.0001*	*0.0555*	*0.0001*	*0.3092*	*0.8038*

[i]Means within a column without letters do not differ significantly.
[ii]Treatment means within a column and application period followed by different letters indicate statistical significance according to Fisher's LSD test (p = 0.05).
[iii]Data for the Spot Spray treatment was added to each application date to aid comparisons and therefore is the same for all application times within a year.
[iv]Statistical analysis of data was assessed according to randomized block design ANOVA (P < 0.05).

Table 5. Mean starch, sucrose and quebrachitol concentrations (expressed as percentage of wood dry weight (DW) in small branches), flushing rate, flowering rate and yield of 'Mauritius' litchi trees during rapid panicle development, at full bloom and at harvesting, in response to different ethephon treatments (Spot Spray and Whole-canopy) or no treatment (Control) across the four-year period of 2014-2017. Treatments were applied at the end of March (early, T1), mid-April (mid, T2) and end-April (late, T3). Values represent means (*n* = 10-20).

Treatment	During rapid panicle development					At full bloom				At harvesting			
	Starch (%DW)	Sucrose (%DW)	Quebrachitol (%DW)	Flushing rate (%)	Panicle rate (%)	Starch (%DW)	Sucrose (%DW)	Quebrachitol (%DW)	Flowering rate (%)	Starch (%DW)	Sucrose (%DW)	Quebrachitol (%DW)	Yield ($kg \cdot tree^{-1}$)
					Early application (T1)								
Control	6.32[i]	1.40	0.89	16.0 a[ii]	75.0	8.07	1.04	0.76	78.0 b	6.96	1.11	0.82	41.1 b
Spot Spray[iii]	7.32	1.57	0.76	6.0 b	86.0	8.20	1.11	0.75	89.0 a	5.88	1.09	0.77	44.9 b
Whole-Canopy	9.62	1.21	0.66	1.5 b	89.5	8.40	1.16	0.81	95.5 a	5.92	1.06	0.82	65.7 a
P value[iv]	*0.1482*	*0.1098*	*0.2244*	*0.0007*	*0.0764*	*0.8994*	*0.1359*	*0.3430*	*0.0039*	*0.1998*	*0.9853*	*0.7659*	*0.0046*
					Mid application (T2)								
Control	10.21	1.16	0.62	11.0	78.0 a	10.55	1.12	0.78	79.0	9.49	1.20	0.83	61.9
Spot Spray	10.57	1.32	0.70	5.0	81.0 a	11.73	1.06	0.71	89.0	6.96	1.23	0.74	57.6
Whole-Canopy	10.66	1.27	0.85	0.0	58.0 b	12.49	1.20	0.80	91.0	8.90	1.12	0.73	74.8
P value	*0.9905*	*0.8747*	*0.2913*	*0.0878*	*0.0282*	*0.3832*	*0.3140*	*0.2704*	*0.1860*	*0.3043*	*0.5103*	*0.0807*	*0.1875*
					Late application (T3)								
Control	7.59	1.48	0.68	22.5	61.0 b	8.00	1.14	0.78	60.0 b	8.37	1.07	0.78	40.8
Spot Spray	7.32	1.57	0.76	6.0	86.0 a	8.20	1.12	0.75	89.0 a	5.88	1.09	0.77	44.9
Whole-Canopy	6.26	1.72	0.75	2.5	18.5 c	8.41	1.29	0.79	52.5 b	7.68	1.10	0.89	44.5
P value	*0.6532*	*0.7371*	*0.3802*	*0.6086*	*< 0.0001*	*0.8991*	*0.2137*	*0.5528*	*<0.0001*	*0.0631*	*0.9444*	*0.1416*	*0.8211*

[i]Means within a column without letters do not differ significantly.
[ii]Treatment means within a column and application period followed by different letters indicate statistical significance according to Fisher's LSD test (p = 0.05).
[iii]Data for the Spot Spray treatment was added to each application date to aid comparisons and therefore is the same for all application times within a year.
[iv]Statistical analysis of data was assessed according to randomized block design ANOVA ($P < 0.05$).

Table 6. Pearson's correlation matrices (from Principal Component Analysis) performed between flushing rate, days to panicle emergence, chilling degree hour (CDH) accumulation, flowering rate, sucrose and starch concentrations (between May and July) in small branches of 'Mauritius' litchi trees. The original data was collected following different ethephon treatments (Spot Spray, Whole-Canopy) or no treatment (Control) applied at three different dates (late-March, mid-April and early-May) of 2014-2017. Values represent correlation coefficients between the respective variables. Values highlighted in bold denote significant differences at p = 0.05 according to Pearson's correlations.

	Flushing rate				Days to panicel emergence				CDH accumulation				Flowering rate			
Variables	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016	2017
Flushing rate	**1**	**1**	**1**	**1**	-0.262	**-0.768**	0.501	-0.448	-0.555	**-0.755**	-0.425	-0.602	-0.326	-0.715	-0.505	0.551
Days to panicle emergence	-0.262	**-0.768**	0.501	-0.448	**1**	**1**	**1**	**1**	0.620	0.360	-0.041	**0.923**	0.408	**0.878**	-0.714	-0.635
CDH accumulation	-0.555	**-0.755**	-0.425	-0.602	0.620	0.360	-0.041	**0.923**	**1**	**1**	**1**	**1**	-0.196	0.289	-0.483	**-0.878**
Flowering rate	-0.326	-0.715	-0.505	0.551	0.408	**0.878**	-0.714	-0.635	-0.196	0.289	-0.483	**-0.878**	**1**	**1**	**1**	**1**
Sucrose May	**-0.814**	-0.722	-0.273	0.653	0.700	**0.886**	0.018	-0.672	0.576	0.446	-0.682	**-0.905**	0.599	0.691	0.682	**0.991**
Sucrose June	-0.039	n/a	n/a	-0.292	-0.086	n/a	n/a	0.737	-0.184	n/a	n/a	**0.884**	0.199	n/a	n/a	**-0.929**
Sucrose July	n/a[i]	n/a	-0.444	-0.442	n/a	n/a	0.307	**0.998**	n/a	n/a	0.873	**0.903**	n/a	n/a	-0.513	-0.595
Starch May	-0.651	0.386	0.093	0.715	-0.116	**-0.757**	0.040	-0.656	-0.012	0.113	**-0.884**	**-0.890**	0.294	**-0.871**	0.628	**0.964**
Starch June	**-0.761**	n/a	n/a	0.459	0.163	n/a	n/a	-0.642	0.326	n/a	n/a	-0.863	0.364	n/a	n/a	**0.978**
Starch July	n/a	n/a	0.168	0.549	n/a	n/a	0.051	-0.795	n/a	n/a	**-0.936**	**-0.961**	n/a	n/a	0.605	**0.972**

[i]n/a means no data available.

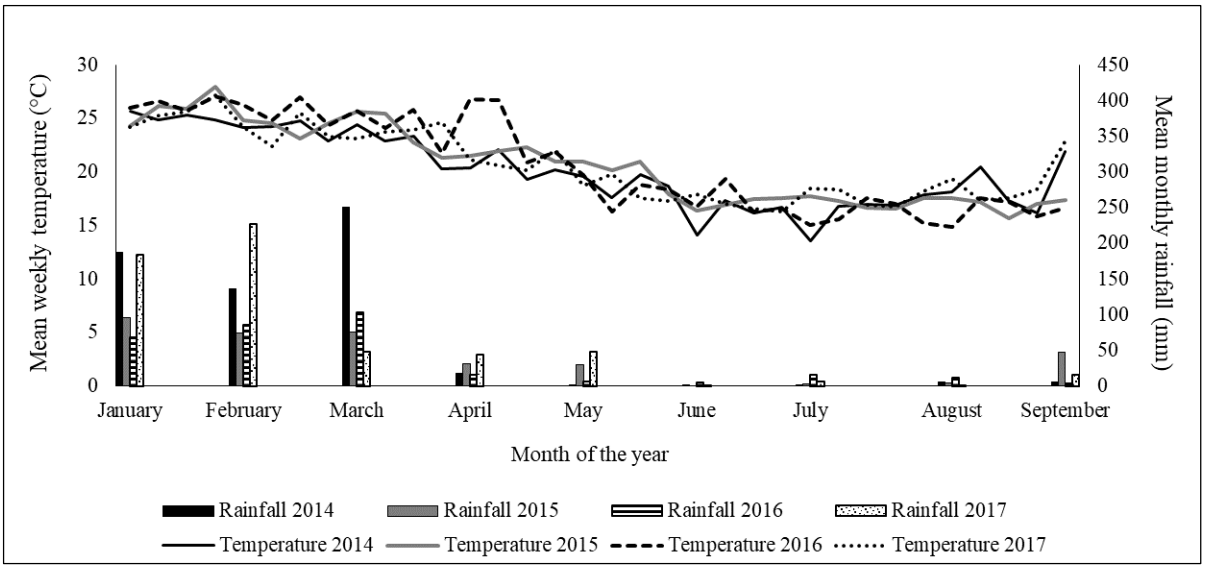

Fig. 1. Mean weekly temperature and mean monthly rainfall between the postharvest shoot growth and flowering period (January to September) at the experimental site near Mbombela, for the years 2014 to 2017.

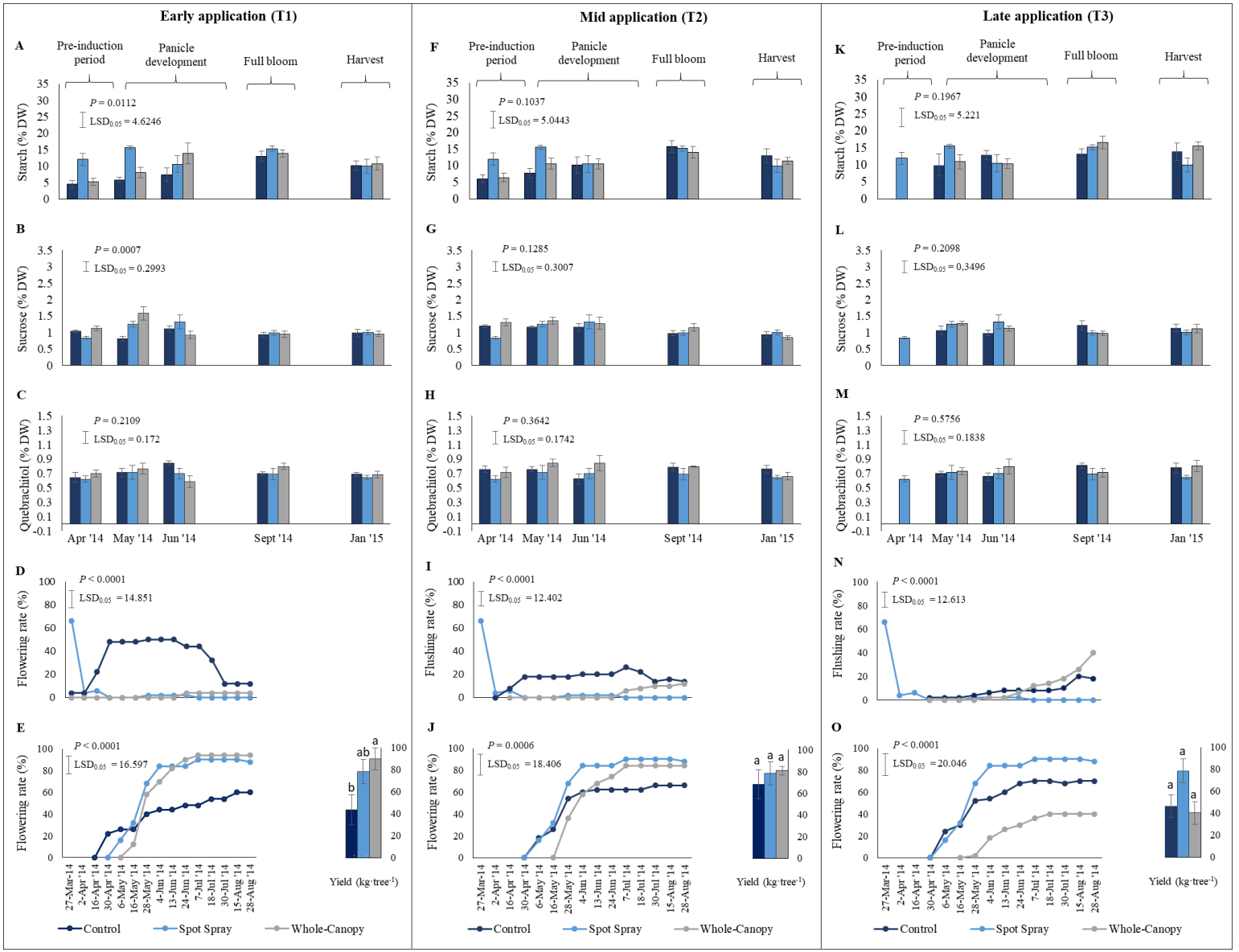

Fig. 2. Carbohydrate concentrations (starch (A, F, K), sucrose (B, G, L) and quebrachitol (C, H, M)), flushing rate (D, I, N), flowering rate and yield (E, J, O) of 'Mauritius' litchi trees in response to different ethephon treatments (Spot Spray, Whole-Canopy) or no treatment (Control), between treatment application and full bloom, and at harvest, respectively. Treatments were applied at the end of March (early, T1), mid-April (mid, T2) and end-April (late, T3) in 2014. Data points are value means ± *SE* (n = 5). Statistical analysis of data was assessed with repeated measures ANOVA (P < 0.05). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

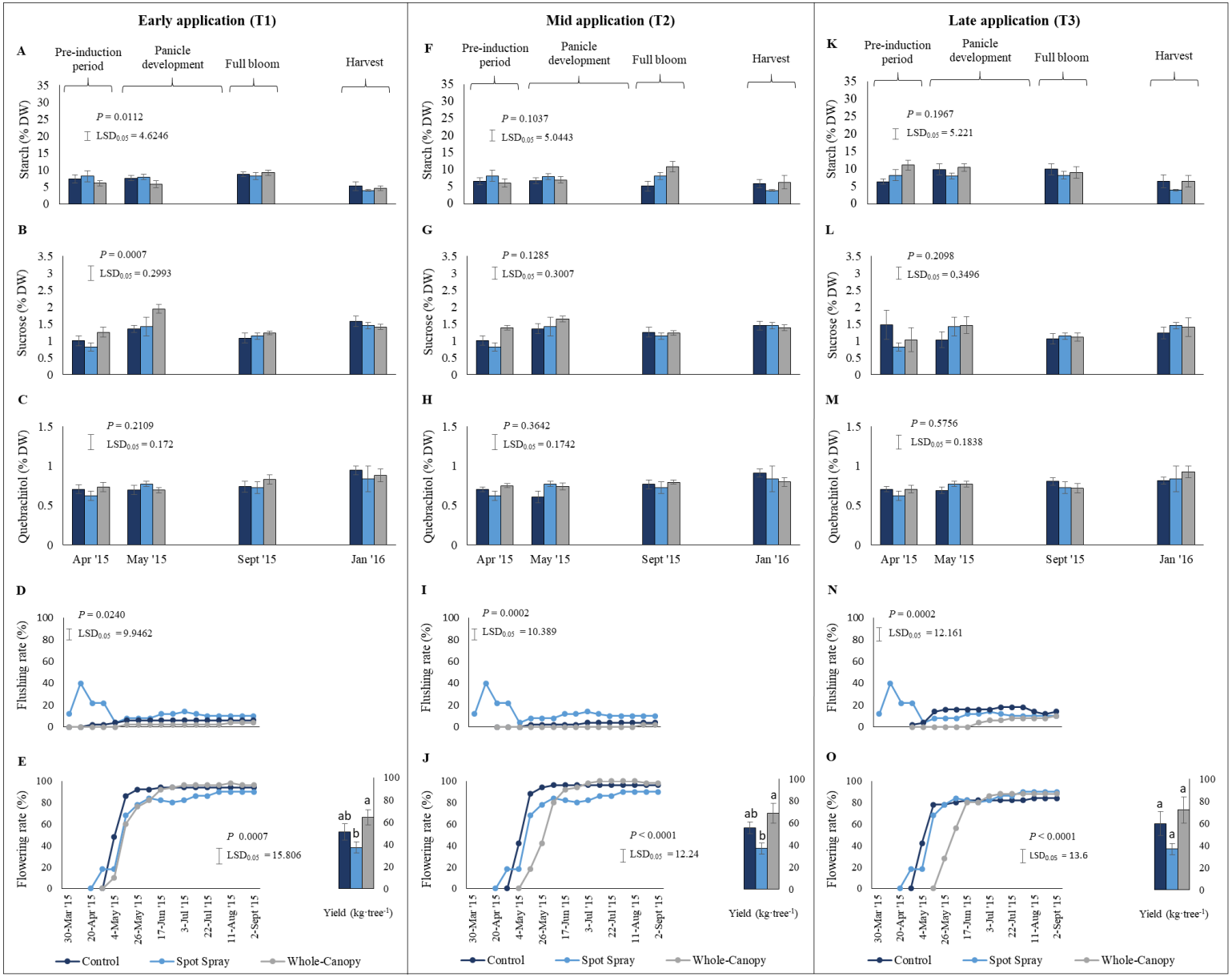

Fig. 3. Carbohydrate concentrations (starch (A, F, K), sucrose (B, G, L) and quebrachitol (C, H, M)), flushing rate (D, I, N), flowering rate and yield (E, J, O) of 'Mauritius' litchi trees in response to different ethephon treatments (Spot Spray, Whole-Canopy) or no treatment (Control), between treatment application and full bloom, and at harvest, respectively. Treatments were applied at the end of March (early, T1), mid-April (mid, T2) and end-April (late, T3) in 2015. Data points are value means ± *SE* (*n* = 5). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

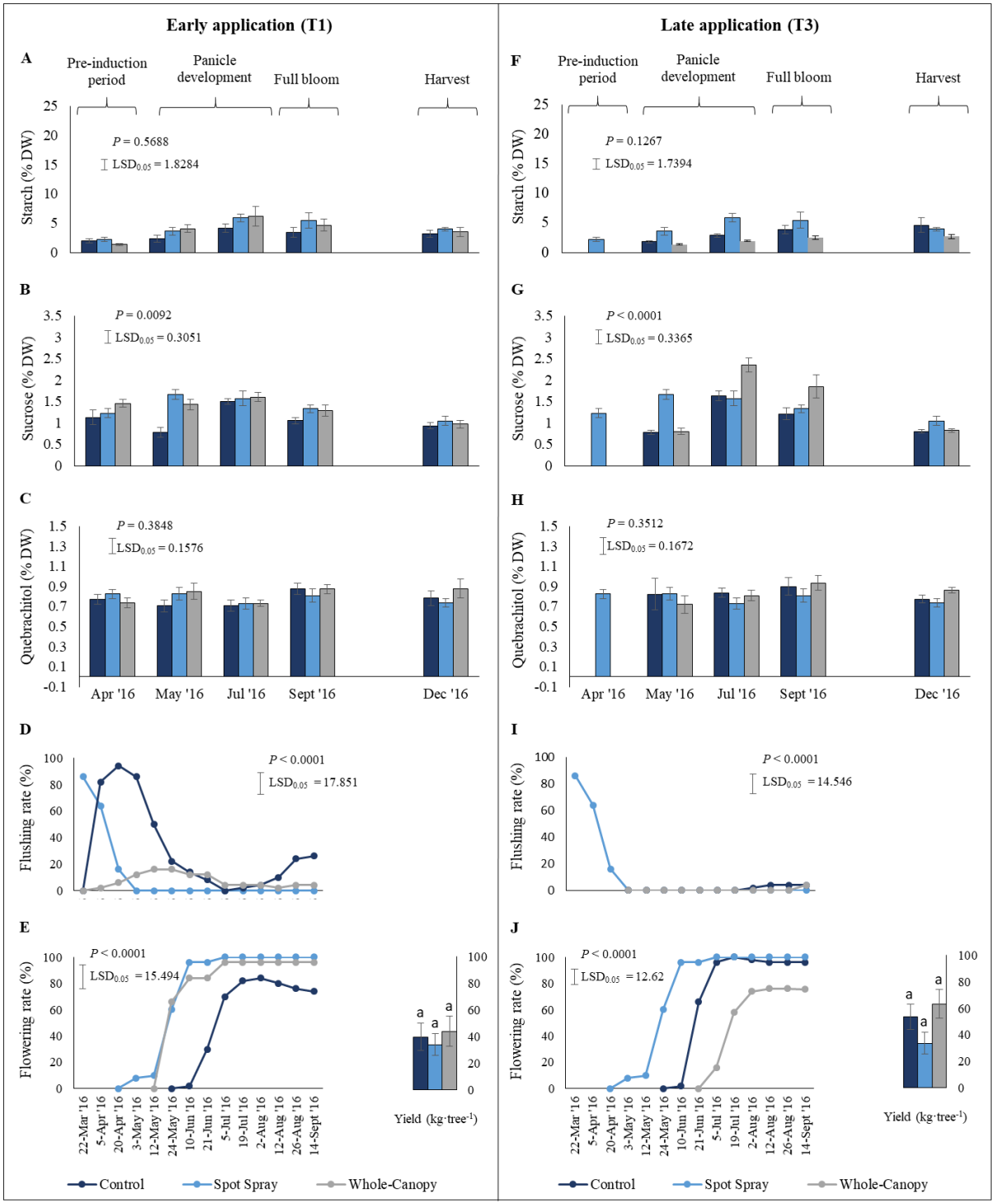

Fig. 4. Carbohydrate concentrations (starch (A, F), sucrose (B, G) and quebrachitol (C, H)), flushing rate (D, I), flowering rate and yield (E, J) of 'Mauritius' litchi trees in response to different ethephon treatments (Spot Spray, Whole-Canopy) or no treatment (Control), between treatment application and full bloom, and at harvest, respectively. Treatments were applied at the end of March (early, T1), and early-May (late, T3) in 2016. Data points are value means ± *SE* (n = 5). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

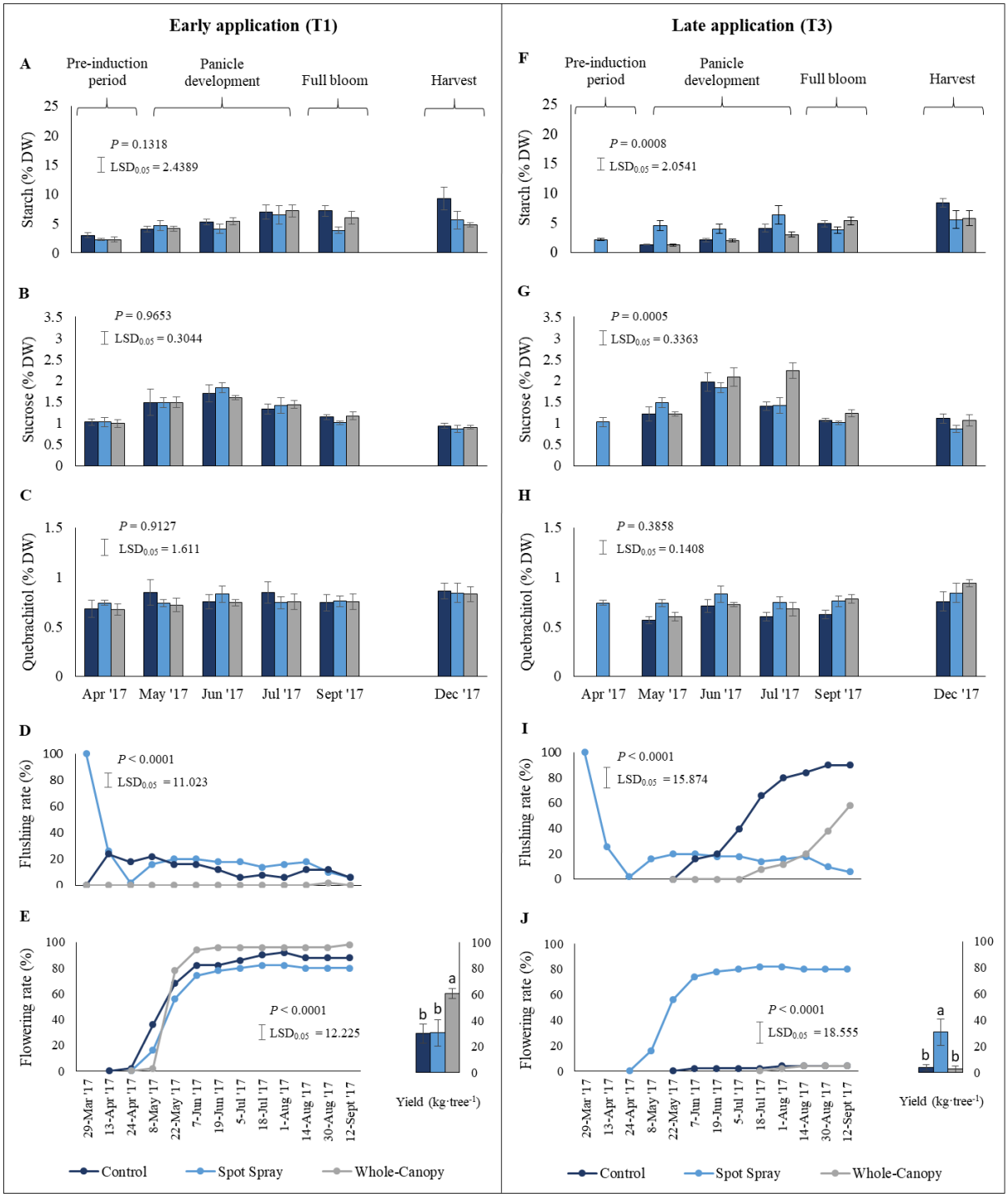

Fig. 5. Carbohydrate concentrations (starch (A, F), sucrose (B, G) and quebrachitol (C, H)), flushing rate (D, I), flowering rate and yield (E, J) of 'Mauritius' litchi trees in response to different ethephon treatments (Spot Spray, Whole-Canopy) or no treatment (Control), between treatment application and full bloom, and at harvest, respectively. Treatments were applied at the end of March (early, T1), and mid-May (late, T3) in 2017. Data points are value means ± *SE* (n = 5). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

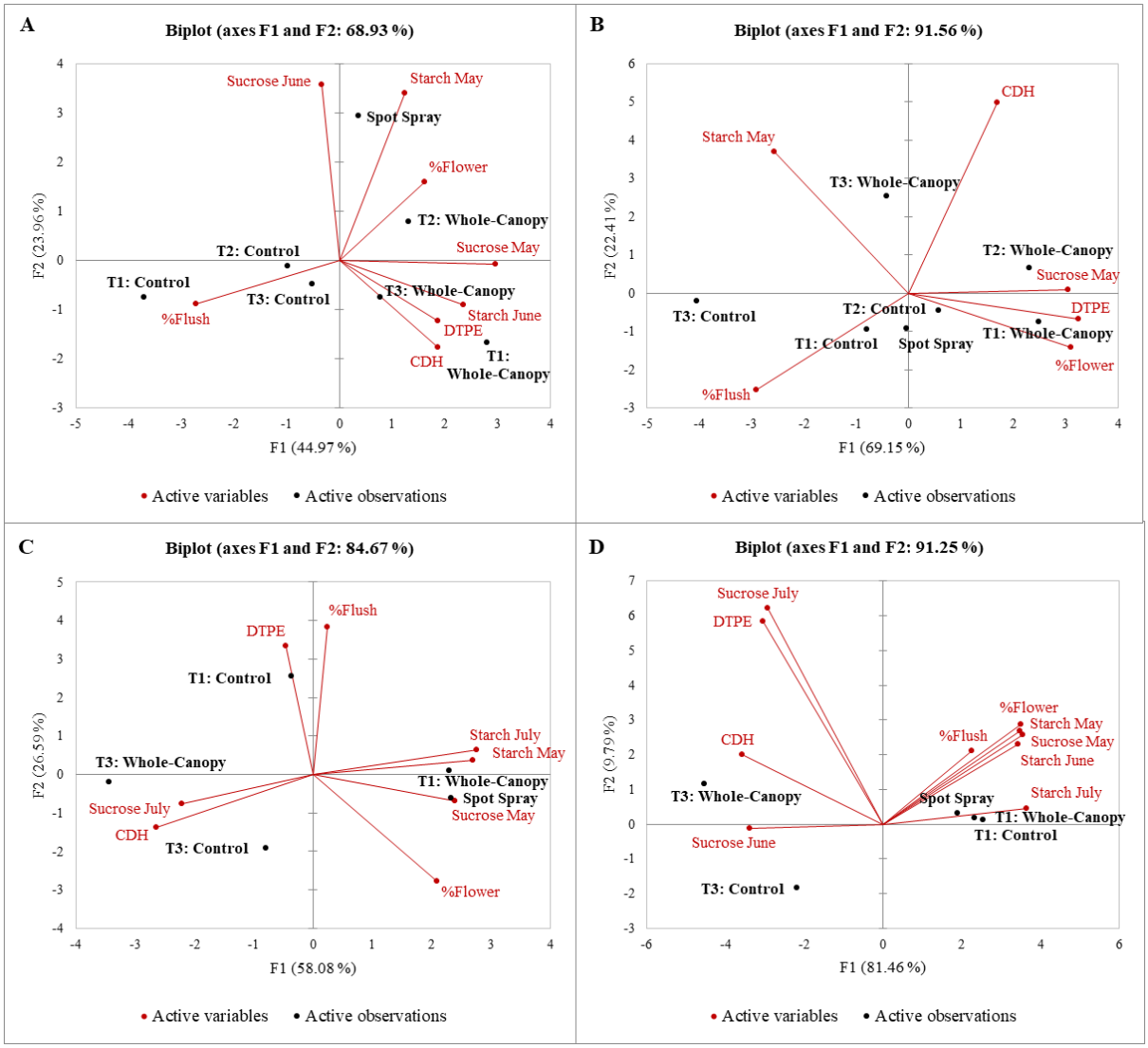

Fig. 6. Principal component analysis of flushing rate (%Flush), flowering rate (%Flower), chilling degree hours accumulation (CDH), days to panicle emergence (DTPE) and pre-flowering reserve carbohydrate levels (starch and sucrose between May and July) in response to different ethephon treatments (Spot Spray, Whole-Canopy) or no treatment (Control) at different application dates (T1: early, T2: mid, T3: late) in A) 2014, B) 2015, C) 2016 and D) 2017.

Paper 4: Foliar application of ethephon induces bud dormancy and affects gene expression of dormancy- and flowering-related genes in 'Mauritius' litchi (*Litchi chinensis* Sonn.)

Keywords. Carbohydrates, ethylene evolution, *LcEIN3*, *LcFLC*, *LcFT2*, floral formation

Abstract. **A previous study showed that foliar application of ethephon to litchi trees with mature shoots and dormant terminal buds during autumn successfully inhibited new vegetative shoot growth prior to floral induction thereby promoting carbohydrate accumulation and flowering. However, the functional mechanisms of ethylene, the breakdown product of ethephon, in the leaves and terminal buds of litchi and its involvement in the flowering process are largely unknown. Therefore, this study aimed to investigate the phenological, physiological and molecular changes underlying ethephon application and its associations with bud dormancy and flowering in litchi. Ethephon was applied as a single whole-canopy spray at a concentration of 1000 mg·L^{-1} to 'Mauritius' litchi trees with mature vegetative shoots and dormant terminal buds during late autumn of 2018 (mid-April; Southern Hemisphere). Untreated trees served as a control. Phenological characteristics, such as bud dormancy and panicle development, leaf chlorophyll content index (as an indicator of shoot maturity), ethylene evolution, gene expression levels of flowering- (*LcFT2*, *LcFLC* and *LcAP1*), dormancy- (*LcSVP1* and *LcSVP2*) and ethylene pathway-related (*LcEIN3*) genes, as well as non-structural carbohydrates were determined in terminal buds, leaves and/or shoots. Ethephon application induced bud dormancy, significantly delayed panicle emergence and promoted pure floral panicle development under more favorable inductive conditions. Ethylene evolution increased sharply two hours after application in both leaves and terminal buds, but decreased rapidly thereafter in the leaves, while remaining high in terminal buds for seven days before gradually declining. Ethephon application significantly increased relative expression of *LcEIN3* and *LcFLC* in terminal buds one day after application, while *LcFT2* expression in leaves and *LcAP1* expression in terminal buds were significantly increased at the bud break stage. Significant treatments differences were also observed for various carbohydrate metabolites in leaves and shoots at the bud break or floral initiation stage. The current study provided evidence that ethephon application plays an important role in the physiological and molecular regulation of bud dormancy of litchi. By influencing the time of bud break, ethephon application can be a useful tool to manage panicle emergence under less inductive conditions.**

Introduction

Ethephon (2-chloroethylphosphonic acid) has been widely used in agriculture for different applications, such as fruit ripening, flower stimulation and organ abscission (Ju and Chang, 2015). After absorption by the plant tissue, or in the presence of a base, ethephon rapidly degrades to ethylene, phosphate and chloride (Domir and Foy, 1978; Hartley and Kidd, 1983; Warner and Leopold, 1969). By binding to specific ethylene receptors in the membrane of the endoplasmic reticulum, ethylene then influences downstream gene expression of various transcription factors, such as *ETHYLENE INSENSITIVE3* (*EIN3*), which eventually leads to ethylene responses specific to its exogenous or endogenous triggers (Bleecker and Kende, 2000; Dubois et al., 2018; Ju and Chang, 2015; Wang et al., 2013). Ethylene is involved in the regulation of various aspects of plant growth and development, such as seed germination, inhibition of cell division in meristems, organ abscission, fruit ripening, as well as stress responses and stress tolerance under suboptimal conditions (Burg, 1973; Dubois et al., 2018; Iqbal et al., 2013; Olsen, 2010; Wang et al., 2013).

In South Africa, ethephon is commercially applied to litchi trees as repeated foliar spot sprays to burn off any young vegetative shoots that emerge during autumn and early winter, thereby preventing these new shoots from impeding floral development (Roets et al., 2010). A recent study suggested that a single whole-canopy application of ethephon to trees with fully mature shoots and dormant terminal buds can inhibit any new shoot growth occurring during periods of high temperature in autumn and winter, being more effective than repeated spot spray applications (Cronje and Ratlapane, 2017, 2018; Paper 2). In this study, ethephon not only prolonged bud dormancy and delayed panicle emergence to a period with more consistent low temperatures, but also promoted tree carbohydrate accumulation, thereby increasing flowering rate and yield. While ethylene, in the form of a commercial ethephon application, directly induced flowering in pineapple (Batholomew, 1977; Maruthasalam et al., 2010), the exact functional mechanisms of ethylene in the litchi flowering processes are unknown, despite its evident role in flowering alluded to in the previous study (Cronje and Ratlapane, 2017, 2018; Paper 2). Moreover, ethylene evolution and endogenous ethylene regulation have been closely linked to environmental cues, in particular temperature (Galvão and Schmid, 2014; Olien, 1976), which also plays an essential role in litchi floral formation (Batten and McConchie, 1995; Chen and Huang, 2005).

Successful flower induction in litchi is highly dependent on trees being exposed to sufficiently low temperatures (Batten and McConchie, 1995; Chen and Huang, 2005; Davenport and Stern, 2005). Furthermore, the maturity status of the vegetative shoots is critical for the chilling responsiveness of crops like litchi and mango (Wilke et al., 2008; Zhang et al., 2016). The signal for flowering, i.e., low temperature in the case of litchi, is perceived in the mature leaves and is transmitted in the vascular tissue to the shoot apical meristems through a series of signal

transduction mechanisms and complex regulatory networks. These regulate the expression of a set of flowering-related genes in the leaves and shoot apical meristems, such as *FLOWERING LOCUS T* (*FT*), *CONSTANS* (*CO*), S*UPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *APETALA1* (*AP1*), while suppressing flowering repressor genes, such as *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*) to prevent floral reversion (Galvão and Schmid, 2014; Hu et al., 2018; Lu et al., 2017; Yang et al., 2014; Zhang et al., 2014).

As the breaking buds are most responsive to low inductive temperatures in litchi, there is only a narrow window for floral induction and initiation (Batten and McConchie, 1995; Huang and Chen, 2005; Zhang et al., 2016). Thus, preventing untimely vegetative shoot growth prior to floral induction, as well as enabling a sufficient dormancy period, is crucial. In deciduous crops, such as peach, pear and apricot, dormancy was found to be regulated by *SVP* genes and the related *DORMANCY ASSOCIATED MADS-BOX* (*DAM*) genes (Zhang et al., 2016). Similarly, in litchi, *LcSVP1* and *LcSVP2* genes were correlated with growth cessation and dormancy maintenance, while *LcSVP3* was associated with bud break and growth of vegetative shoots (Zhang et al., 2016). Under floral inductive conditions, however, all three *LcSVP*'s declined to low levels during floral initiation and panicle development (Hu et al., 2018; Lu et al., 2017).

Apart from ensuring shoot maturity and bud growth at an opportune time, the dormancy period and its associated growth check is essential for the accumulation of carbohydrate reserves. Since flowering is a carbon costly process, carbohydrate accumulation during the winter dormancy period is important for successful flowering in litchi (Huang and Chen, 2014). Various studies confirmed that carbohydrate reserves increased in the main storage organs of flowering litchi trees during the autumn dormancy period (Cronje and Mostert, 2009; Cronje et al., 2020; Menzel et al., 1995). Carbohydrates, in particular soluble sugars, are known to play an important role in signal transduction, and control internal regulators and environmental cues that govern growth (Rolland et al., 2002). Furthermore, Yang et al. (2014) showed that the leaves of mature litchi shoots had higher carbohydrate levels as well as higher gene expression levels of the floral promoters *LcCO* and *LcFT2* under inductive temperatures, compared with leaves of immature litchi shoots. Research by Xiao et al. (2018) corroborated these findings and suggested that flower competence in litchi is associated with the accumulation of soluble sugars in the apical leaves of terminal shoots. Moreover, Huang and Chen (2014) postulated that sufficient carbohydrate accumulation prior to flower induction might increase the responsiveness of litchi to chill temperatures, which may reduce the chilling period required for flower induction, thus, promoting floral formation. Horticultural practices that control vegetative growth prior to floral induction and promote carbohydrate accumulation include the use of plant growth regulators, such as ethephon, water

stress and girdling to remove or suppress vegetative growth (Huang and Chen, 2014; Menzel et al., 1995; Roets et al., 2010; Wilke et al., 2008).

Results from previous reports on the growth-inhibiting and flowering-promoting effects of ethephon (Cronje and Ratlapane, 2017, 2018; Paper 2), as well as studies exploring the flowering mechanisms in litchi (Chen et al., 2016; Hu et al., 2018; Huang and Chen, 2005; Lu et al., 2017; Lu et al., 2020; Shen et al., 2016; Yang et al., 2014; Zhang et al., 2014), suggest that there may be a link between the ethylene and flowering pathways via dormancy regulation. To gain more insight into the regulatory mechanisms of bud dormancy and flowering in litchi as induced by ethephon application, the aim of this study was to investigate the effect of foliar-applied ethephon on physiological and molecular changes in the leaves and apical buds of terminal shoots of 'Mauritius' litchi.

Materials and Methods

Experimental site and plant material. The experimental site was located on a commercial farm near Mbombela in the Mpumalanga Province of South Africa (lat. -25.453, long. 30.945, elevation 680 m; subtropical climate with moderate autumns and cool winters). All experiments were conducted on 18-year old air-layered 'Mauritius' litchi trees. The trees were planted on sandy soil (84% sand, 13% clay, and 3% silt) at a planting distance of 6.0 × 6.5 m. All experimental trees were selected for uniformity of tree size and phenological growth stage and were subjected to standard cultural farming practices with regard to fertilization, irrigation (micro irrigation) and postharvest pruning. Hourly temperature recordings were obtained from a scientific-grade weather station of the Agricultural Research Council – Institute for Soil, Climate and Water located 2.6 km from the Mbombela experimental site (station name: ITSC, # 30420). Chilling degree hours (CDH) and CDH accumulation were calculated based on the formula provided by Chen et al. (2016), but using 20°C as the base temperature for 'Mauritius', as determined by Batten and McConchie (1995), and incorporating 12-h day and 12-h night mean temperatures instead of hourly temperatures. Chilling degree hour accumulation between ethephon application and bud break or panicle emergence was obtained by means of the equation:

$$CDH_d = \begin{cases} 0, & 12(T_{bi} - T_d) < 0 \\ 12(T_{bi} - T_d), & 12(T_{bi} - T_d) \geq 0 \end{cases}$$

$$CDH_n = \begin{cases} 0, & 12(T_{bi} - T_n) < 0 \\ 12(T_{bi} - T_n), & 12(T_{bi} - T_n) \geq 0 \end{cases}$$

$$CDH_i = CDH_d + CDH_n$$

$$CDH_t = \sum_{i=start\ of\ observation}^{t=bud\ break\ or\ panicle\ emergence} CDH_i$$

where CDH_d is the floral induction quantity for the 12-h day period, CDH_n is the floral induction quantity for the 12-h night period, T_d is the mean air temperature over the 12-h day period (°C), T_n is the mean air temperature over the 12-h night period (°C), T_{bi} is the base temperature for floral induction (20°C), CDH_i is the floral induction quantity for the 24-h cycle, CDH_t is the sum of chilling degree hours from predetermined start (i.e., ethephon application) to end of observation (i.e., bud break or panicle emergence).

Treatments and experimental design. Ethephon (Ethapon SL; Plaaskem (Pty) Ltd, Witfield, South Africa; 480 g·L^{-1} a.i.) was applied on 12 April 2018 at a concentration of 1000 mg·L^{-1} as a single whole-canopy spray to the point of run-off to trees with fully mature terminal shoots and dormant apical buds, using a motorized sprayer equipped with hand lances and a 2.5 mm nozzle, at a pressure of 15 bar. A wetting agent (Sanawett 90-940 SL; Dow AgroScience (Pty) Ltd, Bryanston, South Africa; 940 g·L^{-1} a.i.) was added at 10 mL·100 L^{-1} water. Untreated trees served as a control.

The experiment was laid out in a randomized complete block design with two treatments and five block replicates (i.e., five experimental units per treatment). Each experimental unit consisted of five trees and was separated from other experimental units by buffer trees.

Phenological observations. Six representative terminal shoots with fully mature leaves were selected per tree at a height of approximately 1.5–1.8 m above soil level and tagged. Tree responses between spray application and full bloom, i.e., female flower opening, were monitored at weekly or two-weekly intervals. Developmental stages for bud and flower development were identified as described by Wei et al. (2013) and the time of their appearance recorded accordingly. The most critical developmental stages considered for this study were the pre-induction dormancy period, the bud break stage, the floral initiation stage (i.e., panicle emergence), as well as anthesis, i.e., full female flower. Dormant buds (Fig. 1A) were characterized by brown colored rudimentary leaves tightly enclosing the growing point, while breaking buds (Fig. 1B) were greenish and slightly swollen (Zhang et al., 2016). The floral initiation stage (Fig. 1C), also known as the “white millet” stage, was defined as inflorescence primordia becoming visible in the leaf axils in the form of millet-like whitish hairy structures appearing (Huang and Chen, 2005). The interval between

application and bud break or floral initiation stage was calculated as the number of days between application and the visible appearance of at least 50% of shoots at the respective stage. The days to bud break (DTBB) and days to floral initiation stage (DTFI) served as demarcation points for the duration of the dormancy period and defined floral initiation, respectively. The percentage of vegetative shoots during floral induction (May), flowering shoots and leafy panicles was calculated from the total number of tagged shoots. At anthesis, the number of panicles per tagged shoot (including those arising from axillary buds) was counted and the length of the longest panicle per shoot was measured. The number of fruit per panicle was recorded at fruit set and at harvest.

Leaf chlorophyll measurements. One leaflet from the compound leaf closest to the terminal bud of each tagged terminal shoot (total of six leaves per tree and 30 leaves per treatment) was used to measure the relative chlorophyll content, expressed as chlorophyll content index (CCI), during dormancy (21 days after application), at the bud break and floral initiation stage, as well as at anthesis, using a portable CCM-200 chlorophyll content meter (Opti-Sciences, Husdon, NH, USA) (Fu et al., 2014).

Determination of ethylene evolution. The protocol for the quantification of ethylene evolution in tomato leaves developed by Kim et al. (2016) was adapted for litchi. For each sample, two mature leaflets (one leaflet each from two terminal shoots) from the compound leaf closest to the terminal bud and up to 20 terminal buds (4-8 mm in length, depending on bud stage and size) (Fig. 1), respectively, were randomly selected from all trees within a replicate. Samples were collected 24 hours prior to treatment, two hours after treatment, and 2, 4, 7, 14, 21, 35, 42, 56, 63 and 70 days after treatment. Two sub-samples per replicate were collected. The harvested leaflets and buds were inserted into 20 mL and 2 mL glass vials, respectively, and allowed to equilibrate for 10 min to release possible wound ethylene. Thereafter the vials were sealed airtight with aluminum crimp caps (including Si/PTFE septa) and incubated for 3 h at ambient temperature. Gas sampling from vials was achieved using disposable 3 mL syringes with luer-type valves, each connected to a 23 gauge needle of 51 mm length. Volumes aspirated were 1 mL and 0.5 mL from the 20 mL and 2 mL vials, respectively. All samples were analyzed using a Trace GC Ultra gas chromatograph (Thermo Scientific, Waltham, MA, USA) with flame ionization detection, and equipped with a TG-BOND Q+ column (Thermo Scientific, Waltham, MA, USA). The analyses were performed at 60°C with helium as carrier gas and a flow rate of 3.5 $mL \cdot min^{-1}$. Quantification was achieved based on a standard curve derived from an external standard gas calibration with certified ethylene gas (Afrox, Johannesburg, South Africa).

Leaf and shoot carbohydrate analysis. Sixteen leaflets (one leaflet from the compound leaf closest to the terminal bud per sampled shoot), and mid-stem sections of 12 terminal shoots (xylem and phloem; ~ 50 mm in length) were randomly selected from all trees within a replicate and split into two sub-samples per replicate. The samples were collected one day after application (representing the dormancy stage), at the bud break and white millet stage, as well as at anthesis. After collection, the leaves were washed with tap water. Both leaf and shoot samples were oven-dried at 60°C for 72 h and thereafter ground to a fine powder using a laboratory grinder (Fritsch, Idar-Oberstein, Germany) fitted with a 0.5 mm mesh.

Soluble sugars were extracted according to the method described by Chow and Landhäusser (2004), with slight modifications. Briefly, 100 mg sample was suspended in 3 mL of 80% (v/v) ethanol and total sugars extracted at 80°C for 30 min. The extraction process was repeated twice following the first extraction, and the respective supernatants pooled. Thereafter, the ethanol fraction in the pooled supernatants was evaporated at 80°C until dry. Prior to analysis, the sample extracts were re-suspended in 2 mL distilled water and treated with 150 mg of aluminium oxide (activated, acidic, Brockman 1) (Sigma-Aldrich, Johannesburg, South Africa) to remove organic acids and salts, which can co-elute with sugars and interfere with the analysis, based on recommendations by Waters (Waters Corporation, 2020). The samples were analyzed using a HP 1100 LC system (Agilent Technologies, Santa Clara, CA, USA) with refractive index detection and equipped with a Rezex RCM Monosaccharide Ca^{+2} column (Phenomenex, Torrance, CA, USA). The analyses were performed on 20 µl injection volume with deionized water as eluent, at a flow rate of 0.5 $mL \cdot min^{-1}$. The column temperature was kept at 80°C. Quantification was achieved based on external standard calibration derived from a standard curve with analytical-grade sucrose, fructose, glucose, raffinose, myo-inositol and quebrachitol (Sigma-Aldrich, Johannesburg, South Africa). The sugar concentrations were expressed as percentage of sample dry weight (DW).

Leaf and shoot starch were extracted and analyzed according to the iodine colorimetric method as described by Xu et al. (1998). Fifty milligram sample was ground in 5 mL of 1% PVP-40 (w/v). The homogenate was then centrifuged at 5000 rpm (~ 4700 × *g*) for 10 min at room temperature using an Allegra X-22 centrifuge (Beckman Coulter, Fullerton, CA, USA). After discarding the pigmented supernatant, starch was extracted by re-suspending the pellet in 10 mL of 80% calcium nitrate ($Ca(NO_3)_2$) at 100°C for 20 min and then centrifuged at 5000 rpm for 10 min at room temperature. The extraction process was repeated once following the first extraction, whereafter the respective supernatants were filtered through cellulose filter papers (Advantec No. 2, Advantec Toyo Kaisha, Tokyo, Japan) pooled and adjusted to 25 mL with 80% $Ca(NO_3)_2$ solution. For analyses, 0.1 mL of 0.01 N I_2-KI solution was added to 2.9 mL of the pooled sample supernatant, blanks (distilled water) and starch standard solutions to a final volume of 3.0 mL. The starch

concentration was determined from the changes in absorbance of the starch-iodine complex at 620 nm using a UV-1800 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of starch was calculated based on external standard calibration derived from a standard curve with analytical-grade soluble starch (Sigma-Aldrich, Johannesburg, South Africa). The starch concentration was expressed as percentage of sample dry weight (DW).

RNA sample collection and extraction. Leaf sections of eight mature leaflets from the compound leaf closest to the terminal bud (~10 mm^2, avoiding the mid rib), and 30 to 40 terminal buds (depending on bud size) were randomly collected from all trees within the three replicates, two days before application, one day after application, during the dormancy stage (21 days after application), as well as at the bud break and at the floral initiation stage (Fig. 1). On collection, all samples were frozen in liquid nitrogen and stored at -80°C until further processing. Samples collected prior to treatment application were pooled for each replicate. Immediately prior to analysis, the leaf and bud samples were finely ground in liquid nitrogen, whereafter total RNA was extracted using the Tiangen RNAprep Pure Kit (Tiangen, Beijing, China) according to manufacturer's instructions. The RNA samples were treated with RNase-free DNase I (Tiangen, Beijing, China) to remove genomic DNA. In addition, for bud samples, 2% PVP-40 (w/v) was added to the SL buffer of the RNA kit in the first clean-up step to remove contaminating polyphenols. The RNA integrity was assessed using 1% (w/v) agarose gel electrophoresis (80 V for 20 min). The quantity of the isolated RNA was measured using a NanoDrop UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). Only RNA samples with a 260/280 nm ratio of higher than 1.8 were used for subsequent analyses.

cDNA synthesis and generation of standard curves. First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SensiFast cDNA synthesis kit (Meridian Bioscience, London, UK) in a final volume of 20 µl, following the manufacturer's instructions. The undiluted cDNA samples were stored at -20°C until further use. Prior to analysis, the cDNA samples were diluted 50 times. For the generation of standard curves of all reference and target genes, 1 µL of each cDNA sample was pooled, and a five-fold dilution series was prepared from the cDNA sample mix.

Target and reference gene selection. The genes of interest included the dormancy genes *LcSVP1* and *LcSVP2*, the ethylene-signaling pathway gene *LcEIN3*, and the floral genes *LcFLC*, *LcFT2* and *LcAP1*. Primer sequences for the studied reference and target genes are displayed in Table 1. The primers were synthesized by Integrated DNA Technologies (Whitehead Scientific, Johannesburg, South Africa). The *LcActin* and *LcGAPDH* genes were selected as reference genes

due to their stability in qRT-PCR analyses across different tissue types, developmental stages, and treatments in litchi (Zhong et al., 2011). The stability of reference gene expression in leaf and bud samples was assessed using the Excel-based application BestKeeper (Pfaffl et al., 2004). The descriptive statistics for the reference genes and the BestKeeper index are presented in Table 2.

Gene expression analysis with qRT-PCR. Quantitative RT-PCR analysis was performed on a Rotor Gene Q instrument (Qiagen, Hilden, Germany) with three technical replicates for each biological replicate, using the SensiFast SYBR No-Rox kit (Meridian Bioscience, London, UK) in a final volume of 15 µL, following the manufacturer's instructions. The analyses were run with the following program: hot start at 95°C for 2 min, followed by 45 cycles of 10 s at 95°C, 10 s at 54°C to 58°C (depending on primer annealing temperature; Table 1), and 15 s at 72°C. Melt-curve analyses were performed at temperatures ranging between 60 and 95°C, with a 1°C increase every 5 s to identify primer-dimers and non-specific amplification.

The Rotor-gene Q software 2.3.1 (Qiagen, Hilden, Germany) was utilized to calculate PCR efficiency, quantification cycle (Cq)-, and quantification values for all samples. These calculations were performed using the slope of the standard curve derived from the five-fold dilution series for each primer. The third dilution point (125x) of the five-fold dilution series for each primer was included in all runs to compensate for inter-assay variability. The relative expression value for each sample was calculated with the geometric mean of the triplicate reactions and normalized using a reference gene index, which was calculated from the geometric mean of the two reference genes.

Statistical analysis. All data was subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4, SAS Institute Inc, Cary, USA). Data collected in a repeated measures design was subjected to repeated measures analysis of SAS software with treatment, time and treatment × time as fixed factors. The Shapiro-Wilk and Levene's tests confirmed normality of the data and homogeneity of variances, respectively. Bartlett's test for homogeneity of variances was used where outliers had to be removed, to ensure consistent reliability of ANOVA data. Where ANOVA indicated significant treatment effects at $P < 0.05$, Fisher's Least Significant Difference (LSD) test was performed at a 5% significance level to compare treatment means for main effects and interactions. Pearson's product-moment correlations were performed separately for each studied plant organ (leaves, buds, shoots) to identify significant relationships ($r > 0.5$) between dormancy- and carbohydrate-related parameters, respectively, and the relative expression levels of each gene for a given developmental stage, using the correlation procedure (PROC CORR) of SAS software (Version 9.4, SAS Institute Inc, Cary, NC, USA) at a 5% significance level, unless indicated otherwise. Principal component analysis

(PCA) was performed to investigate the relationship between treatments and the different variables using XLSTAT (Version 2015.1.03.15485, Paris, France).

Results

Ethylene evolution in leaves and terminal buds following ethephon application. Minimal ethylene was detected from leaves and terminal buds prior to the application of ethephon (Fig. 2). However, 2 h after the ethephon treatment, ethylene evolution rapidly increased to 111 $\mu L \cdot kg^{-1} \cdot h^{-1}$ in treated leaves, whereafter it dropped sharply to almost one-tenth of the initial peak within 7 days after application. Ethylene evolution from treated terminal buds peaked at 64 $\mu L \cdot kg^{-1} \cdot h^{-1}$ 2 h after treatment and remained elevated for a period of 7 days after application, whereafter it decreased gradually. Ethylene evolution from treated leaves dropped to below 5.0 $\mu L \cdot kg^{-1} \cdot h^{-1}$ from day 35 onwards, whereas in the treated buds, ethylene evolution only decreased to below the 5.0 $\mu L \cdot kg^{-1} \cdot h^{-1}$ threshold level after 63 days. Ethylene levels for untreated leaves and terminal buds stayed below 1.4 and 3.0 $\mu L \cdot kg^{-1} \cdot h^{-1}$, respectively, throughout the observation period (Fig. 2).

Temperature appeared to have an effect on ethylene evolution in treated buds, particularly between day 2 and day 7 and again around day 42, showing slight increases in ethylene release as temperatures increased and vice versa (Fig. 2). Pearson's correlations performed on the data between day 2 and 42 revealed a significant positive correlation between temperature and ethylene release from treated buds (r = 0.82448, P = 0.0225), whereas no significant correlation could be found for treated leaves (r = 0.63404, P = 0.1262) during the same period.

Treatment effect on tree phenology. Ethephon treatment increased the number of days to bud break and delayed panicle emergence (floral initiation stage) by 22 days compared with the control (Table 3). However, the time between bud break and the floral initiation stage was 5 days shorter for treated trees compared with control trees. Furthermore, the accumulated chilling degree hours (CDH) were higher for treated trees than for control trees due to the delayed bud break. This resulted in an increase of 1374 and 2057 accumulated CDH for treated trees at the bud break and floral initiation stage, respectively, compared with untreated trees (Table 3). The application of ethephon influenced various phenological parameters compared with the control (Table 4). No vegetative shoot growth occurred in ethephon-treated trees during floral induction compared with the 13.3% flushing rate in the control trees. The flowering rate was 75% and 80% for the ethephon and control treatment, respectively, with no statistical difference between them. The percentage leafy panicles was significantly decreased, while the number of flower panicles per shoot was significantly increased by the ethephon treatment. Ethephon application reduced panicle length and increased the initial and final number of fruit set per panicle, albeit not significantly (Table 4).

Furthermore, the ethephon treatment was associated with a higher ($P < 0.1$) leaf chlorophyll content index at the bud break stage compared with the untreated control (Fig. 3), while the leaf chlorophyll content indices at dormancy, floral initiation and anthesis were similar for both treatments.

Treatment effect on leaf and shoot carbohydrate concentrations. Changes in starch and soluble sugar concentrations were observed in response to ethephon application as well as over time (Fig. 4). Both leaf and shoot sucrose concentrations were significantly increased in ethephon-treated trees at bud break, but were significantly lower at the floral initiation stage compared with the control trees (Fig. 4A and 4E). Glucose concentrations in leaves of treated trees were significantly higher at bud break and significantly lower at the floral initiation stage compared with the control, while the treatment effect was inverse in the shoots (Fig. 4B and 4F). Fructose concentrations in both leaves and shoots were below the level of detection and are therefore not presented. Quebrachitol was found in similarly high concentrations as sucrose in both leaves and shoots (Fig. 4C and 4G). Quebrachitol concentrations in leaves did not differ between treatments, but were significantly lower in shoots of ethephon-treated trees at bud break and the floral initiation stage compared with the control treatment. Starch concentrations in the leaves of both treatments fluctuated throughout the trial period and were considerably lower than in the shoots (Fig. 4D and 4H). In the leaves, starch concentrations of ethephon-treated trees were significantly increased at the floral initiation stage and significantly decreased at anthesis compared with the control trees (Fig. 4D). In the shoots, starch concentrations increased over time in both treatments, but no significant treatment differences were found (Fig. 4H).

Treatment effect on expression levels of ethylene pathway and dormancy-related genes. Gene expression levels of *LcEIN3* were generally lower in leaves compared with terminal buds (Fig. 5A and 5D). Ethephon application did not influence *LcEIN3* expression levels in the leaves, however, it significantly increased *LcEIN3* expression in terminal buds one day after application and at dormancy, whereafter it declined to the same low level recorded for the control.

Expression levels of *LcSVP1* in the leaves were increased at dormancy and again at the floral initiation stage, but no significant differences between treatments or interactions were observed (Fig. 5B). In terminal buds, *LcSVP1* showed similar expression levels for both treatments throughout the observation period, with a significant reduction in expression levels one day after application and at dormancy and a subsequent significant increase during floral formation (Fig. 5E). At bud break, expression levels of *LcSVP1* were significantly increased, while at the floral initiation stage they were significantly reduced in ethephon-treated trees compared with the control trees (Fig. 5E). Relative gene expression of *LcSVP2* in leaves remained stable throughout the observation period, except at bud break where ethephon-treated trees had lower ($P > 0.05$) *LcSVP2*

expression levels compared with the control trees and when compared with the other developmental stages (Fig. 5C). In the terminal buds, *LcSVP2* expression levels decreased after application, similar to *LcSVP1*, and remained stable thereafter in both treatments, except for bud break, where *LcSVP2* expression was increased in ethephon-treated trees (Fig. 5F). However, no significant treatment × stage interactions were found.

Treatment effect on expression levels of flowering-related genes. Relative expression of the flowering antagonist *LcFLC* remained stable in leaves for both treatments throughout the observation period with a slight, but non-significant, increase in treated trees at bud break (Fig. 6A). In terminal buds, however, *LcFLC* expression levels increased significantly in treated trees one day after application. Thereafter, *LcFLC* expression levels declined to the same level as the control, and no further treatment differences were observed (Fig. 6D).

Expression levels of *LcFT2* in leaves of both treated and untreated trees remained low between pre-application and dormancy (Fig. 6B). At bud break, *LcFT2* expression significantly increased in treated trees compared with the control, while at the floral initiation stage, *LcFT2* expression of both treatments reached the same level. In terminal buds, *LcFT2* expression levels of both treatments remained low until dormancy, but significantly increased at bud break and again at the floral initiation stage. However, no significant differences between treatments were observed (Fig. 6E).

Relative gene expression of *LcAP1* in leaves was stable throughout the observation period for both treatments with no significant treatment differences (Fig. 6C). In terminal buds, *LcAP1* expression levels before and one day after ethephon application were low, but gradually increased thereafter in both treatments and exhibited the similar *LcAP1* expression levels at the floral initiation stage (Fig. 6F). However, while the control trees displayed significantly higher *LcAP1* expression levels at dormancy, *LcAP1* expression levels in ethephon-treated trees were significantly higher at bud break.

Associations between phenology, carbohydrates and gene expression. Pearson's correlations confirmed the growth-inhibiting effects of ethephon one day after application by significant positive correlations between *LcEIN3* and *LcFLC* expression in buds (r = 0.85219, P = 0.0312), and between *LcEIN3* expression and the number of days to bud break (DTBB) (r = 0.90229, P = 0.0139). Expression levels of *LcEIN3* were also negatively correlated with flushing rate at the dormancy stage (r = -0.87720; P = 0.0507). The influence of ethephon on bud dormancy was further highlighted by a positive correlation between *LcFLC* and *LcSVP2* expression in buds one day after application (r = 0.79067, P = 0.0611), albeit only significant at 10%. In addition, at

dormancy, *LcSVP1* expression in buds was significantly negatively correlated with *LcFT2* and *LcAP1* expression in buds (r = -0.79691, P = 0.0019 and r = -0.75153, P = 0.0048, respectively). The role of dormancy in carbohydrate accumulation was established by significant positive correlations between *LcSVP1* expression in buds and leaf sucrose concentration at the dormancy stage (r = 0.89314, P = 0.0165), as well as between DTBB and sucrose concentrations in leaves and shoots at bud break (r = 0.87545, P = 0.0009 and r = 0.70281, P = 0.0234, respectively). The importance of shoot maturity in carbohydrate accumulation was emphasized at the dormancy stage by a significant positive correlation between shoot sucrose concentrations and leaf chlorophyll content index (as a shoot maturity indicator) (r = 0.69163, P = 0.0267). Furthermore, at bud break, leaf sucrose concentration was significantly positively correlated with leaf chlorophyll content index (r = 0.66274, P = 0.0368). An association between carbohydrates and flowering was established at bud break by leaf and shoot sucrose concentrations being significantly positively correlated with *LcFT2* expression in leaves (r = 0.69283, P = 0.0263) and *LcAP1* expression in buds (r = 0.78387, P = 0.0026).

Processes governing flowering were identified at the floral initiation stage by a strong significant negative correlation between *LcAP1* and *LcSVP2* expression in buds (r = -0.90010, P = 0.0145), as well as by a significant positive correlation between *LcAP1* expression and flowering rate (r = 0.66581, P = 0.0181). Significantly positive correlations between *LcFT2* expression in leaves and *LcAP1* expression in buds (r = 0.80049, P = 0.0054) were only observed at bud break, but not at the floral initiation stage.

The principal component analysis (PCA) was limited to key developmental stages and variables for flower initiation. These included the leaf chlorophyll content index as an indicator of shoot maturity, shoot starch concentration as an indicator of reserve carbohydrates, leaf sucrose concentration as a signaling molecule, accumulated CDH, DTBB and DTFI as factors relating to temperature influences and duration of dormancy, as well as the expression of certain genes involved in dormancy and flowering regulation. The principal components F1 and F2 explained 92.77% of the variation in the data (Fig. 7). The variables contributing the most to F1 were leaf chlorophyll content index, leaf sucrose concentration, shoot starch concentration, *LcFT2* and *LcFLC*, while CDH and DTBB contributed the most to F2. The variables in F1 and F2 can thus be considered as indicators for flowering and dormancy, respectively. Highly positive correlations between leaf chlorophyll content index, leaf sucrose concentrations, shoot starch concentrations, *LcFT2* and *LcAP1* were found. These variables were also highly associated with the ethephon treatment at the bud break and floral initiation stage, whilst *LcEIN3* and *LcFLC* were associated with the ethephon treatment at the dormancy stage. Furthermore, DTBB, DTFI, and their corresponding accumulated CDH were closely associated with the ethephon treatment. Flowering

was associated with the control treatment at bud break, however, no association between the ethephon treatment and flowering was detected.

Discussion

Post-ethephon treatment ethylene release patterns and associated temperature effects. Ethylene release showed distinct patterns in leaves and terminal buds (Fig. 2). The high peak and subsequent rapid decline in ethylene evolution observed in the leaves immediately after ethephon application suggests that ethephon absorption by the leaves was limited, most likely due to the thick wax layer of the leaf cuticle. Therefore, ethylene evolution detected in leaves may have primarily been the result of a chemical breakdown reaction of ethephon on the leaf surface rather than ethylene release after absorption by the plant tissue itself (Domir and Foy, 1978; Hartley and Kidd, 1983). However, ethephon appeared to have been absorbed by the bud tissue, where it subsequently degraded to ethylene as the active component over an extended period and could then exert its influence on a physiological and molecular level. In contrast, ethephon uptake in peach required the presence of leaves to stimulate endogenous ethylene production in the buds (Liu et al., 2021), possibly because peach buds are protected by scales while litchi buds are naked (Zhang et al., 2016).

It was also observed that ethylene evolution followed similar variations as the mean daily temperatures recorded on the day of observation (Fig. 2). Olien (1976) reported that ethylene evolution from ethephon in cherry occurred at an exponential rate for temperatures between 10°C and 34°C and was higher when temperatures changed from lower to higher temperatures compared with a change from higher to lower temperature. Similar results were observed for other crops (Domir and Foy, 1978; Klein et al., 1978; Walters and Lopez, 2018; Warner and Leopold, 1969). Although not performed under controlled temperature conditions, our results confirmed similar trends. The effect of environmental conditions, such as ambient temperature in reference to ethephon applications, is an important aspect to consider when evaluating the effectiveness of a spray application. For the current study, this suggests that intermittent temperature increases may have contributed to an extended ethylene release in the terminal buds.

Role of ethylene in bud dormancy and subsequent growth events. In litchi, floral induction and initiation can only take place when the terminal buds are exposed to floral-inducing chilling temperatures (Batten and McConchie, 1995; Huang and Chen, 2005). For buds to be released from dormancy, the leaves of the terminal shoot need to be fully mature (Fu et al., 2014; Zhang et al., 2016). A previous study has shown that ethephon applications to trees with fully mature shoots delayed bud break to a time when temperatures were more conducive to floral induction by

inhibiting new shoot growth during warmer periods prior to floral induction (Cronje and Ratlapane, 2017, 2018; Paper 2). However, the exact functional mechanisms remained unclear. It is known from other crops that ethylene binds to the relevant receptors within a plant cell and exerts its influence on physiological and molecular processes within the cell, such as the inactivation of cell growth in the bud apical meristem (Burg, 1973; Ju and Chang, 2015; Dubois, et al., 2018). Ethylene and ethephon treatment were also associated with a delay in bud growth and flower development (Abeles et al. 1992; Hansen and Grossmann 2000). Quantification of ethylene evolution in our study revealed an extended presence of ethylene in the terminal buds of litchi, which induced bud dormancy and subsequently inhibited new flush growth whilst promoting shoot maturity, i.e., leaf chlorophyll accumulation. These observations were also confirmed by increased *LcEIN3* expression levels in terminal buds one day after application and during dormancy, as well as by *LcEIN3* expression being positively correlated with DTBB and being negatively correlated with flushing incidence. In addition, ethephon application and the subsequent ethylene release maintained bud dormancy irrespective of temperature increases during the pre-induction and induction period (at around day 42), thus, preventing shoot growth during a warmer period.

The subsequent delay in panicle emergence under more favorable inductive conditions significantly reduced the percentage of leafy panicles and increased the number of panicles per shoot in ethephon-treated trees (Table 4; Fig. 2). Despite delayed bud break and panicle emergence, panicle development in ethephon-treated trees was accelerated, as was indicated by a shorter period between the bud break and floral initiation stage (Table 3), leading to anthesis at the same time as in the control trees (data not shown). This may be related to the prevailing temperatures at the time of bud break and panicle emergence. In this regard, Menzel and Simpson (1988) found that panicles emerged later at a maximum/minimum temperature regime of 15°/10°C than at 20°/15°C, but required a shorter time to reach anthesis. Most of the changes in tree phenology in response to ethephon application were consistent with previous reports on ethephon used for shoot control and delay of bud break in litchi (Cronje and Ratlapane, 2017, 2018; Paper 2). Our study did not find significant differences in the flowering percentage (Table 4), possibly because temperatures at the time of flower panicle emergence in untreated trees were sufficiently low to achieve high floral induction and initiation success. Nevertheless, and despite lack of significant treatment differences in flowering rate, the higher number of fruit set per panicle (Table 4) indicated that the ethephon treatment in our study had the potential to improve the crop, similar to what was reported by Cronje and Ratlapane (2017, 2018) and in Paper 2.

Role of ethylene in carbohydrate accumulation. Carbohydrates are considered to play an important role in the regulation of flowering in litchi (Cronje and Mostert, 2009; Menzel et al.,

1995; Yang et al., 2014) and were found to be correlated with floral formation in litchi (Zhang et al., 2017). In contrast, initial shoot growth is known to consume carbohydrate reserves and may deplete necessary reserves for floral development, if it occurs prior to flowering (Cronje and Mostert, 2009; Cronje et al., 2020; Huang and Chen, 2014; Menzel et al., 1995; Paper 3). By inhibiting shoot growth and extending dormancy (Table 3), ethephon application in our study provided a prolonged opportunity to accumulate carbohydrates, which led to increased concentrations of sucrose, glucose, quebrachitol and starch in leaves and shoots, mainly at bud break and the floral initiation stage, compared with untreated trees (Fig. 4). These results also corroborate the findings on carbohydrate accumulation in small branches following ethephon application (Paper 3).

Furthermore, shoot maturity, as promoted by the ethephon treatment, played an important role in carbohydrate assimilation. This was highlighted by leaf chlorophyll content index being significantly positively correlated with shoot and leaf sucrose concentration at dormancy and bud break, respectively. Fu et al. (2014) showed that CO_2 assimilation and photochemical efficiency were highest when leaves of the litchi cultivars 'Baili' and 'Heiye' reached full maturity, which corresponded to 90% of the maximum chlorophyll concentration of fully mature leaves. Moreover, Yang et al. (2014) found that leaf chlorophyll and carbohydrate concentration was higher for dark-green mature leaves compared with yellowish-green or yellowish-red immature leaves of three different litchi cultivars during the floral induction period. In our study, the role of dormancy in carbohydrate accumulation was particularly evident in shoot starch concentrations, which significantly increased over time in both treatments (Fig. 4H). Significant positive correlations between DTBB and sucrose concentrations in leaves and shoots, as well as between *LcSVP1* expression and leaf sucrose concentration during the dormancy stage further accentuated the link between dormancy and carbohydrate accumulation.

While both starch and sucrose are important non-structural carbohydrates, sucrose is a primary product of photosynthesis and generally the principle form of translocated carbon in vascular plants (ap Rees, 1984). Sucrose also plays an important role in the transition from the vegetative to the reproductive phase (Cho et al., 2018) and was found to act as a primary messenger in signal transduction during floral induction in *Arabidopsis thaliana* (Rolland et al., 2002). Cho et al. (2018) discussed various studies where an increase or mobilization of sugar and/or starch concentration in leaves, phloem sap or shoot apex in response to different treatments, such as changes in photoperiod, irradiation, exogenous sucrose application or by use of transgenic plants like *A. thaliana*, promoted floral induction and the expression of regulatory genes involved in flowering and carbohydrate metabolism. Similarly in our study, significantly increased concentrations of soluble sugars (sucrose, glucose and quebrachitol) in the leaves of ethephon-treated trees at bud

break corresponded with significantly higher expression levels of *LcFT2* in leaves and *LcAP1* in buds at bud break, as well as with increased CDH accumulation (Table 3; Figs. 4 and 6). These results suggest increased chill responsiveness in ethephon-treated buds due to ethephon-mediated physiological changes, such as increased carbohydrate accumulation.

Principal component analysis highlighted strong associations between ethephon treatment and shoot maturity, carbohydrates and flowering-related genes at bud break and the floral initiation stage, and confirmed that shoot maturity and leaf sucrose are key factors for the expression of flowering-related genes (Fig. 7). The decrease of leaf sucrose and glucose concentrations of ethephon-treated trees at the floral initiation stage suggests the possibility of higher sugar demand for respiratory processes at bud break (Zhang et al., 2016) to facilitate the accelerated panicle development (ap Rees, 1984) (Table 3). Similarly, increased quebrachitol concentrations in the leaves and decreased concentrations in the shoots of ethephon-treated trees at bud break and the floral initiation stage (Fig. 4C and 4G) suggest that quebrachitol was possibly mobilized from the shoots to meet the high sugar demand for sucrose and glucose for floral development in the ethephon-treated trees. Similar, yet less pronounced trends were observed in small branches of ethephon-treated trees with high flowering rates (Paper 3). Quebrachitol represents about 50% of soluble sugars in mature litchi leaves and occurs in similar high concentrations as sucrose (Liu et al., 2022; Wu et al., 2018). It was previously reported to have a unique function in litchi as transportable photosynthate under reduced sucrose conditions (Wu et al., 2018). In addition to these reports and the findings in Paper 3, our results suggest that quebrachitol may also play a role as easily transportable carbohydrate that supports flowering processes in litchi.

Role of ethylene in modulating gene expression. ETHYLENE INSENSITIVE 3 (*EIN3*) is a transcription factor in the ethylene-signaling pathway (Wang et al., 2013) and regulates the expression of ethylene-responsive genes (*ERF*s), which in turn are directly responsible for a wide range of ethylene-induced (stress) responses (Bleecker and Kende, 2000; Wang et al., 2013). In our study, ethephon application and the associated ethylene release had a direct effect on *LcEIN3* expression levels in terminal buds immediately after application and during the dormancy period (Fig. 5). This led to specific responses, such as shoot inhibition due to extended bud dormancy, as mentioned earlier, and corresponded directly with ethylene evolution (Fig. 2). Furthermore, these results also explain the responses observed and described in Paper 2. In support of our results, research on the expression of ethylene-related genes in litchi fruitlets following ethephon application revealed that several key regulatory genes in the ethylene-signaling pathway, such as *ERF*s and *EIN3/EIL*, were highly expressed one day after application (Li et al., 2015). Likewise, gene expression profiling during natural bud development in litchi showed that major components

of the ethylene-signaling pathway, such as *LcEIN3*, were upregulated during growth cessation and dormancy of terminal buds in litchi, thus, suggesting that ethylene is a key player in inducing growth cessation and dormancy entrance in litchi (Zhang et al., 2016). The overall low *LcEIN3* expression levels in treated leaves throughout the observation period compared with treated buds (Fig. 5) further provide evidence in support of the view that ethephon may not have been absorbed by the leaves, but that the observed peak in ethylene evolution (Fig. 2) can be attributed to a chemical breakdown reaction of the applied ethephon on the leaf surface.

SHORT VEGETATIVE PHASE genes (*SVP*s) are transcription factors that act as repressors of flowering during the vegetative phase and play an important role during floral meristem specification in *A. thaliana* (Gregis et al., 2013; Hu et al., 2018). The *SVP* genes also control flowering time under ambient temperature changes in *A. thaliana* by repressing the expression of the flowering gene *FLOWERING LOCUS T* (*FT*) (Lee et al., 2007). In litchi, *LcSVP1* regulates growth cessation and dormancy maintenance, while *LcSVP2* regulates the entrance into dormancy and flowering (Zhang et al., 2016). In our study, pre-treatment expression levels of both *SVP*s were high in terminal buds, possibly indicating that the buds might have been in a late stage of growth cessation and dormancy entrance. The dormancy maintenance function of *LcSVP1* can be supported by *LcSVP1* expression being significantly negatively correlated with *LcFT2* and *LcAP1* expression at the dormancy stage. However, relative gene expression of *LcSVP1* (Fig. 5B and 5E) did not reveal significant treatment differences one day after application or at dormancy. The increased, albeit not significantly, expression levels of *LcSVP2* in buds of treated trees one day after application and at bud break (Fig. 5F), could indicate a possible interaction between *LcSVP2* and the ethylene and/or flowering pathways in litchi. It has been proposed that some genes in the *SVP* family may be regulated by ethylene, abscisic and gibberellic acid (Gregis et al., 2013; Liu et al., 2018).

The *FLOWERING LOCUS C (FLC)* gene is a flowering repressor that integrates the autonomous flowering and vernalization pathways (Zhang et al., 2014). Overexpression of *FLC* was associated with the downregulation of the flowering genes *FT* and *AP1*, as well as with a delay in flowering in *A. thaliana* and litchi (Lu et al., 2017; Zhang et al., 2014). Similar interactions between *LcFLC* and the flowering genes, *LcFT2* and *LcAP1*, in terminal buds were found in our study for the period between application and bud break (Fig. 6D-6F). Generally, *FLC* is expressed under conditions that are not conducive for flowering, such as high temperatures (Liu et al., 2018). However, in our study, overexpression of *LcFLC* one day after ethephon application may have been in direct response to the ethephon treatment or regulated by *LcEIN3*, and may have contributed to the extended dormancy period and delayed bud break (Table 3). A possible interaction between *LcFLC* and *LcSVP2* expression, as indicated by the positive correlation between these two genes

one day after ethephon application, may also have played a role in bud dormancy of ethephon-treated trees, since some genes in the *SVP* family in *A. thaliana* were found to interact with the flowering repressor *FLC* to downregulate flower promoters, such as *FT* (Gregis et al., 2013).

Due to the delay in flower panicle emergence, ethephon-treated trees initiated floral development at lower temperatures (~ 4°C difference to control) (Fig. 2) and had accumulated more CDH at the bud break and floral initiation stage compared with untreated trees at the same phenological stages (Table 3; Fig. 2). Similar results were found by Liu et al. (2021) and Islam et al. (2021) after applying ethephon to delay bloom in peach. The stronger chill effect on ethephon-treated trees was associated with significantly increased expression levels of *LcFT2* in leaves as well as with significantly increased expression levels of *LcAP1* in buds at the bud break stage compared with control trees (Fig. 6B and 6F). However, similarly high *LcAP1* expression levels in both treatments at the floral initiation stage indicate that temperatures were still sufficiently low for successful flower differentiation in the control trees, hence, leading to equally high flowering rates in both treatments (Table 4). The interdependence of *FT* and *AP1* genes in response to cold was also described in other studies on *A. thaliana* and litchi (Gregis et al., 2013; Lu et al., 2017). The stronger chill effect on ethephon-treated trees can also explain their significantly lower percentage leafy panicles compared with control trees (Table 4), because under low temperature conditions, the mixed floral buds of litchi, which consist of leaf and panicle primordia as well as rudimentary leaves, abort their rudimentary leaves and develop into pure leafless flower panicles (Huang and Chen, 2005; Zhou et al., 2008).

Reactive oxygen species (ROS) may also possibly be involved in ethephon responses that cause abscission of young leaves in litchi and inhibit bud growth in other crops (Abeles et al., 1992; Cronje and Ratlapane, 2017, 2018; Roets et al., 2010; Sexton, 1997; Paper 2). In our study, the prolonged presence of ethylene in the bud tissue, as a result of slow ethephon degradation in the shoot apex, most likely generated ROS (Lu et al., 2014; Zhou et al., 2013). Indeed, Zhou et al. (2013) detected ROS in apical buds as well as in petioles and stems of abscising rudimentary leaves after applying ethephon to leafy panicles for the purpose of promoting pure panicle development in litchi. Likewise, ethephon treatment induced increased levels of ROS and ROS-generating enzymes in floral buds of peach (Islam et al., 2021). Lu et al. (2014) found that treating litchi shoots with the ROS-generating chemical methyl viologen dichloride hydrate (MV) increased expression levels of several genes in the ethylene-signaling pathway, among them *LcEIN3*, similar to ethephon application in our study. Furthermore, the most recent study on genome-wide transcriptomic analysis of oxidative stress-induced flowering genes in litchi leaves revealed that ROS, such as those induced by MV, significantly upregulated *LcFT1* and *LcFT2* and even promoted litchi flowering under less inductive temperatures (Lu et al., 2020). In an earlier study by the same

research group, Lu et al. (2017) also discovered an overexpression of *LcAP1* in low temperature-treated as well as in medium temperature plus MV-treated trees. The MV application was associated with an increase of hydrogen peroxide as well as an increase in the expression of the floral gene *LcLFY*, which interacts with *LcAP1* to establish floral meristem identity. Although applied at a different time and phenological stage, ethephon application and the subsequent breakdown to ethylene in our study may also have generated ROS, which, together with the ethylene effect, could have triggered a cascade of physiological and molecular processes. This may explain the observed changes in the expression of related genes, such as *LcFLC*. Apart from promoting a higher percentage of pure floral panicles, the application of ethephon may hold potential to induce litchi flowering under less inductive conditions similar to MV in the above-mentioned study.

Based on our results, we developed a conceptual model that illustrates the effect of ethephon-derived ethylene on key regulatory genes primarily involved in the regulation of bud dormancy, and the ensuing effects on, and interactions between shoot maturity, carbohydrate accumulation, chilling degree hour accumulation and floral gene expression, which eventually lead to successful floral formation in litchi (Fig. 8).

Conclusion

This study aimed to investigate the effect of ethephon application on physiological and molecular mechanisms governing shoot maturity, bud dormancy, carbohydrate accumulation and expression of key regulatory genes involved in dormancy and flowering of 'Mauritius' litchi. Our study has provided evidence that ethephon application to trees with mature shoots and dormant terminal buds during autumn resulted in an upregulation of ethylene- and dormancy-related genes, particularly in terminal buds. Consequently, this led to an extended dormancy with an increased accumulation of carbohydrates as well as a subsequent delay in bud break and panicle emergence, which promoted pure floral panicle development under more favorable inductive conditions, thereby indirectly influencing phenological, physiological and molecular changes associated with flowering in litchi. Our study confirmed that ethylene plays an important role in the physiological and molecular regulation of bud dormancy in litchi, and that ethephon application can be a useful tool to prevent panicle emergence under less inductive conditions. The role of ROS in this regard, however, warrants further research.

With the current knowledge, future studies should focus on finding strategies that increase the responsiveness of litchi trees to induce flowers under less inductive temperatures or that may reduce their chill requirements for flowering as an approach to mitigate unfavorable seasonal climatic changes, which otherwise would lead to partial or complete flower and crop failure.

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Table 1. Forward and reverse primer sequences for reference and target genes used in the quantitative RT-PCR assays.

Gene abbreviation	Annotation	Forward primer sequence (5'→ 3') Reverse primer sequence (5'→ 3')	Annealing temperature (°C)	Primer efficiency (%)	Reference
LcActin	Actin	AGTTTGGTTGATGTGGGAGAC TGGCTGAACCCGAGATGAT	54	101	Yang et al., 2014
LcGAPDH	Glyceraldehyde 3-phosphate dehydrogenase	GATACAGTTCCCGTGTTGTTGAC CATAAAGACACATAACACCACACTC	56	99	Zhong et al., 2011
LcSVP1	Short Vegetative Phase 1	CTTTCTGTTCTTTGCGATGC TTCTCAAGGTTCTTGGAGTGC	56	96	Zhang et al., 2016
LcSVP2	Short Vegetative Phase 2	AGGAAATGCTAACCCCAACT AGACAAGGGAACAACAACTGAT	56	98	Zhang et al., 2016
LcEIN3	Ethylene Insensitive 3	TTTCCTGTCTGCTTTTCCTGG GCGTTGCTTTGCATTGTCA	55	95	South China Agricultural University (unpublished)
LcFT2	Flowering Locus T	CCAAGTGAACCAAGCCTGAG GGAACAACACGAATACGAACC	58	93	Yang et al., 2014
LcFLC	Flowering Locus C	AAAATAGCACATCCAACAGGC AACAAACTTACATAGGTAGTGAGGC	56	95	Yang et al., 2014
LcAP1	Apetala 1	TTAGGGCAGCCACCTCAGTC GTGCAGCAACCTCTTTACATCA	58	96	Zhang et al., 2014

Table 2. Descriptive statistics of two candidate reference genes (*LcActin* and *LcGAPDH*) based on their quantification (Cq) values according to the software program BestKeeper (Pfaffl et al., 2004). In the last column, the BestKeeper index is computed together for both reference genes with the same descriptive parameters.

	Candidate reference genes		
	LcActin	*LcGAPDH*	BestKeeper (n=2)
Number of samples	50	50	50
Geometric mean (Cq)	18.45	19.53	18.98
Arithmetic mean (Cq)	18.50	19.58	19.02
Minimum (Cq)	16.38	17.67	17.27
Maximum (Cq)	21.09	22.92	21.98
Standard deviation (±Cq)	1.19	1.07	0.91
Coefficient of variation (%Cq)	6.41	5.46	4.76
BestKeeper index vs. correlation coefficient (*r*)	0.856	0.844	
P value	*0.001*	*0.001*	

Table 3. Growth response time and accumulated chilling degree hours (CDH) between treatment application and the bud break (BB) or floral initiation (FI) stage in response to ethephon treatment at 1000 mg·mL^{-1} (Ethephon) or no treatment (Control) of 'Mauritius' litchi trees with mature shoots and dormant terminal buds. Data are mean values (n = 5).

Treatment	Days to bud break (BB)	Days to floral initiation (FI)	Days between BB and FI	Accumulated CDH to BB stage	Accumulated CDH to FI stage	CDH difference between stages/treatments
Control	31	53	22	2072	3446	1374
Ethephon	53	70	17	3446	5503	2057

Table 4. Phenological parameters for vegetative and reproductive growth in response to ethephon treatment at 1000 mg·mL^{-1} (Ethephon) or no treatment (Control) of 'Mauritius' litchi trees with mature shoots and dormant terminal buds. Data are mean values (n = 5).

Treatment	Flushing rate during floral induction (%)	Flowering rate (%)	Leafy panicle rate (%)	Panicles/shoot (incl. axillary) (no.)	Panicle length (cm)	Fruit/panicle at fruit set (no.)	Fruit/panicle at harvest (no.)
Control	13.3 a[i]	80.0 a	38.7 a	1.7 b	23.2 a	8.6 a	5.3 a
Ethephon	0.0 a	74.7 a	4.7 b	2.7 a	21.5 a	11.0 a	6.1 a
P value[ii]	*0.1324*	*0.5803*	*0.0017*	*0.0035*	*0.1388*	*0.0614*	*0.1524*

[i]Different letters within columns indicate significant differences between treatments according to Fisher's LSD test (p = 0.05).
[ii]Statistical analysis of data was assessed with randomized block design ANOVA (P < 0.05).

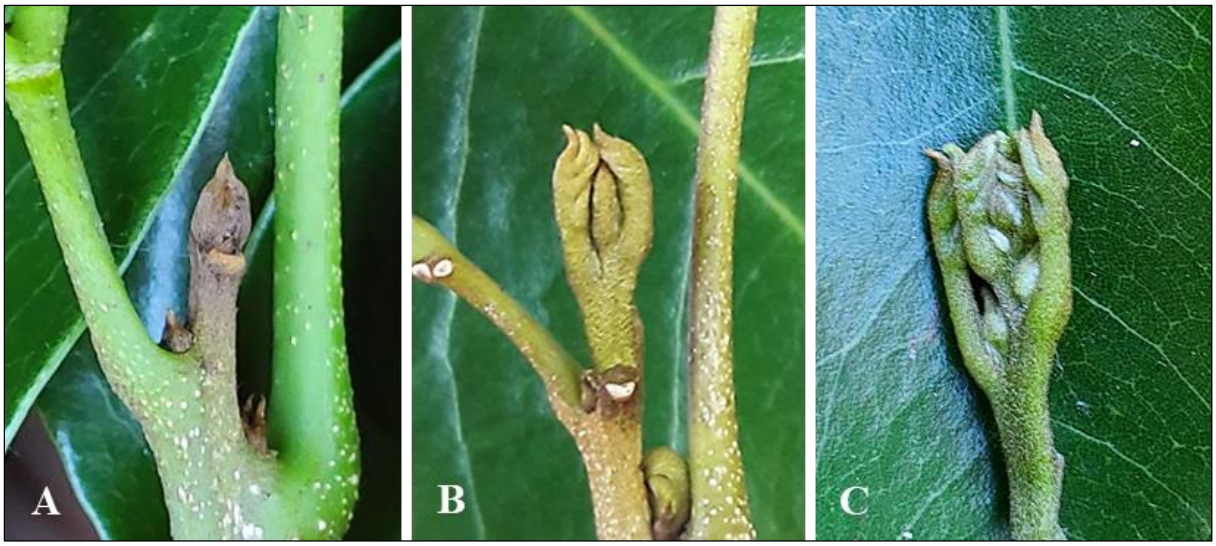

Fig. 1. Morphology of terminal buds at different developmental stages between dormancy and floral initiation in ‘Mauritius’ litchi. Sub-figures specify the developmental stages used for sample collection for ethylene and gene expression analysis: (A) dormant bud stage, (B) bud break stage, and (C) floral initiation stage.

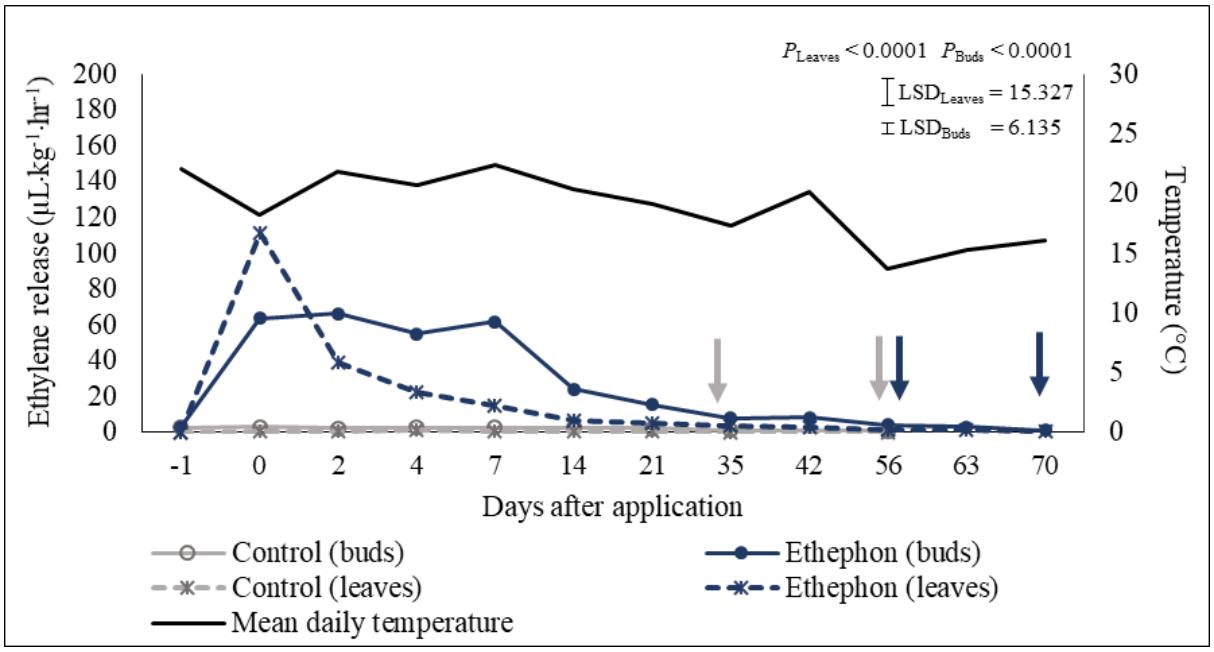

Fig. 2. Ethephon application increased ethylene evolution in leaves and terminal buds of 'Mauritius' litchi. Samples were collected one day prior to application, on the day of application, and over a 70-day period following ethephon treatment at 1000 mg·L^{-1} (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Detached leaves and terminal buds were incubated for 3 h prior to analysis with gas chromatography. Grey arrows indicate the bud break (day 33) and floral initiation stage (day 56) of the control trees. Black arrows indicate the bud break (day 56) and floral initiation stage (day 70) of the ethephon-treated trees. Data points represent mean values (n = 10). Statistical analysis of data was assessed with repeated measures ANOVA (P < 0.05), for leaf and bud data individually. Statistical significance of the treatment × time interaction effect is indicated as LSD bar according to Fisher's LSD test (p = 0.05).

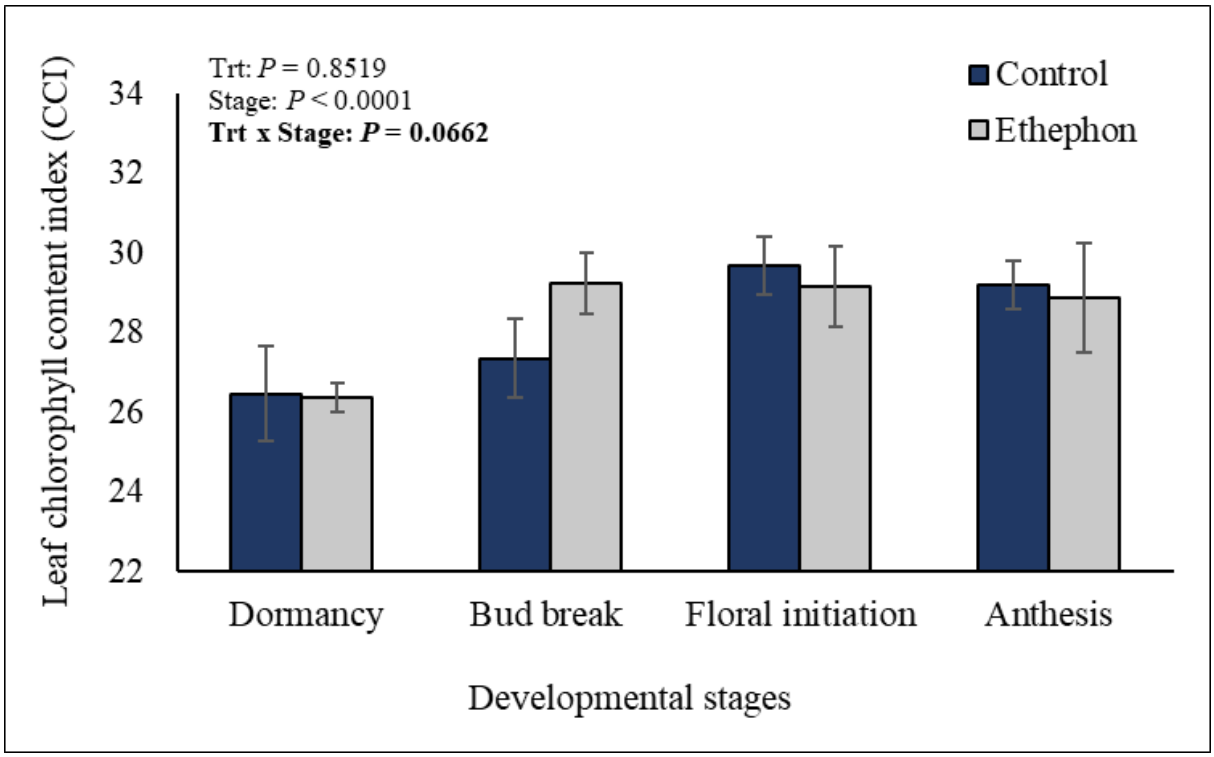

Fig. 3. Leaf chlorophyll content index of 'Mauritius' litchi leaves at different developmental stages. Leaf chlorophyll content index from a leaflet of the compound leaf closest to the terminal bud was determined at the indicated developmental stages following ethephon treatment at 1000 mg·L^{-1} (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Columns represent mean values ± *SE* (n = 5). Statistical analysis of data was assessed with repeated measures ANOVA (P < 0.05).

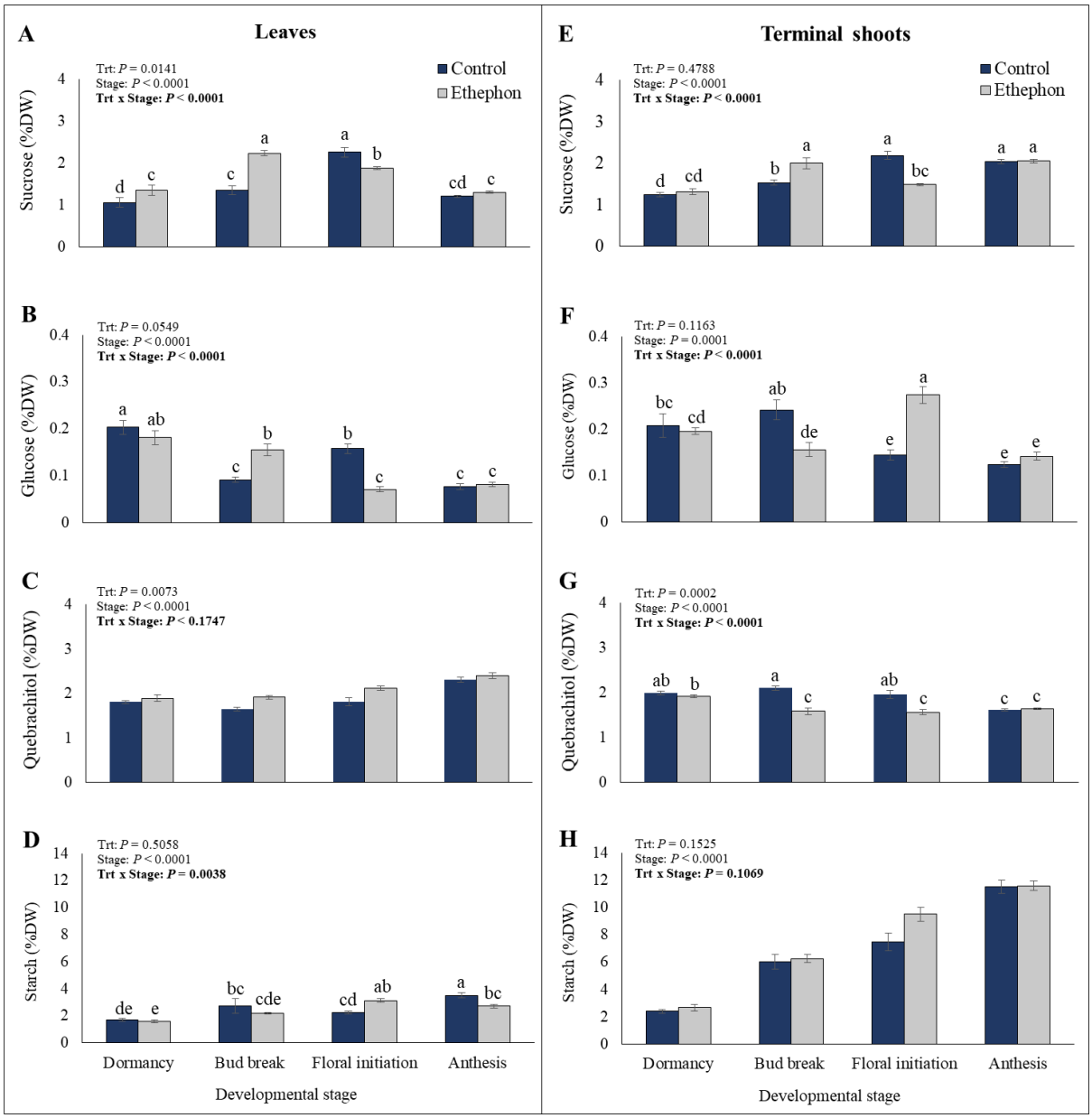

Fig. 4. Ethephon application increased carbohydrate concentrations in leaves and terminal shoots of 'Mauritius' litchi at specific developmental stages. Figures A to D display changes in carbohydrate concentrations in leaves for A) sucrose, B) glucose, C) quebrachitol and D) starch. Figures E to H display changes in carbohydrate concentrations in terminal shoots for E) sucrose, F) glucose, G) quebrachitol and H) starch. Data was collected from a leaflet of the compound leaf closest to the terminal bud, as well as from terminal shoots at the indicated developmental stages following ethephon treatment at 1000 mg·L^{-1} (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Columns represent mean values ± *SE* (n = 5). DW denotes dry weight. Statistical analysis of data was assessed with repeated measures ANOVA (P < 0.05). Different letters above columns indicate significant differences for treatment × stage interactions according to Fisher's LSD test (p = 0.05).

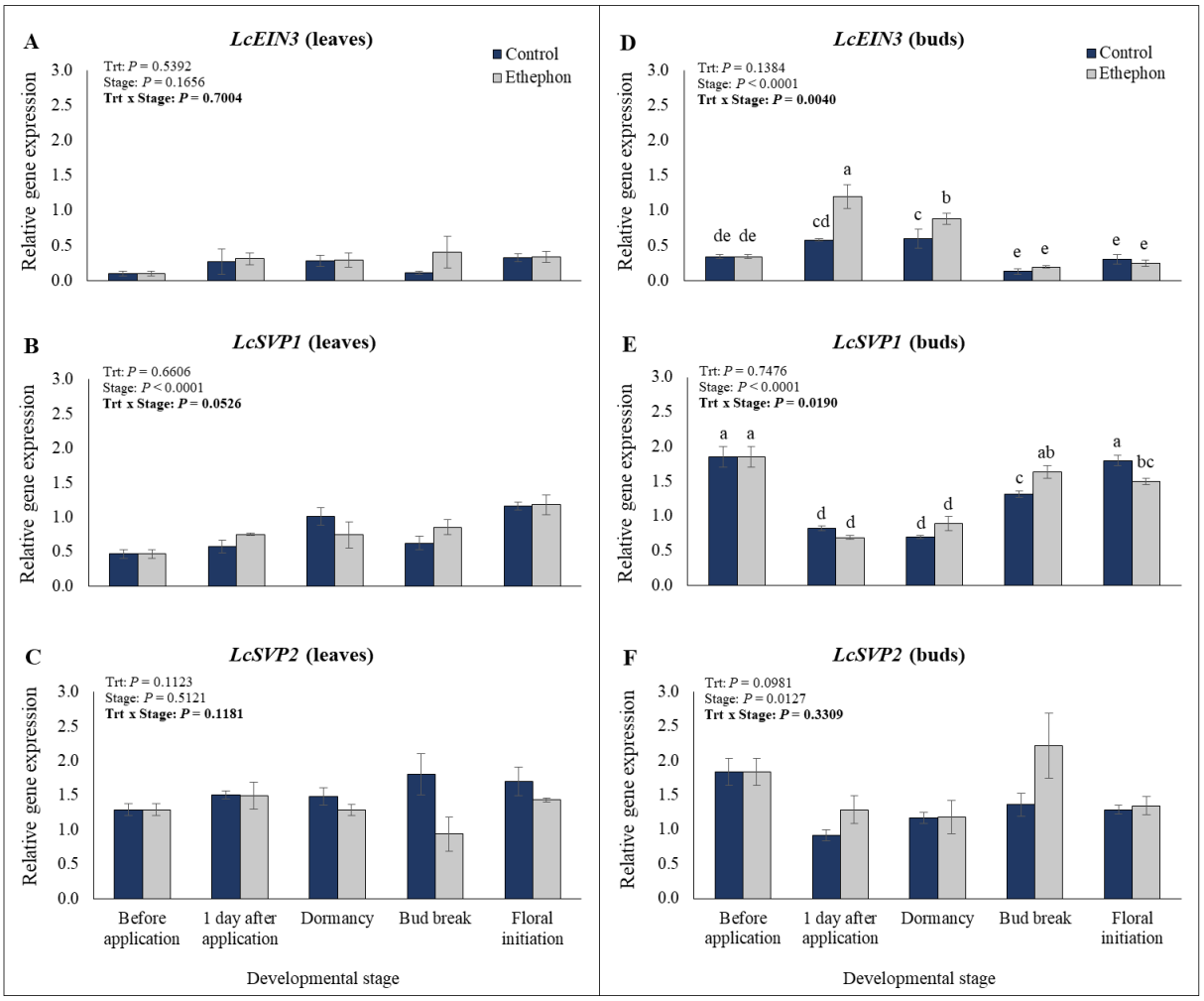

Fig. 5. Relative expression levels of ethylene- and dormancy-related genes in leaves and terminal buds of 'Mauritius' litchi in response to ethephon treatment (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Figures A to C display changes in relative gene expression in leaves for A) *LcEIN3*, B) *LcSVP1* and C) *LcSVP2*. Figures D to F display changes in relative gene expression in terminal buds for D) *LcEIN3*, E) *LcSVP1* and F) *LcSVP2*. Data was collected from a leaflet of the compound leaf closest to the terminal bud, as well as from terminal buds at the indicated developmental stages. Relative gene expression was determined with qRT-PCR. Columns represent mean values ± *SE* (n = 3). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Different letters above columns indicate significant differences for treatment × stage interactions according to Fisher's LSD test (p = 0.05).

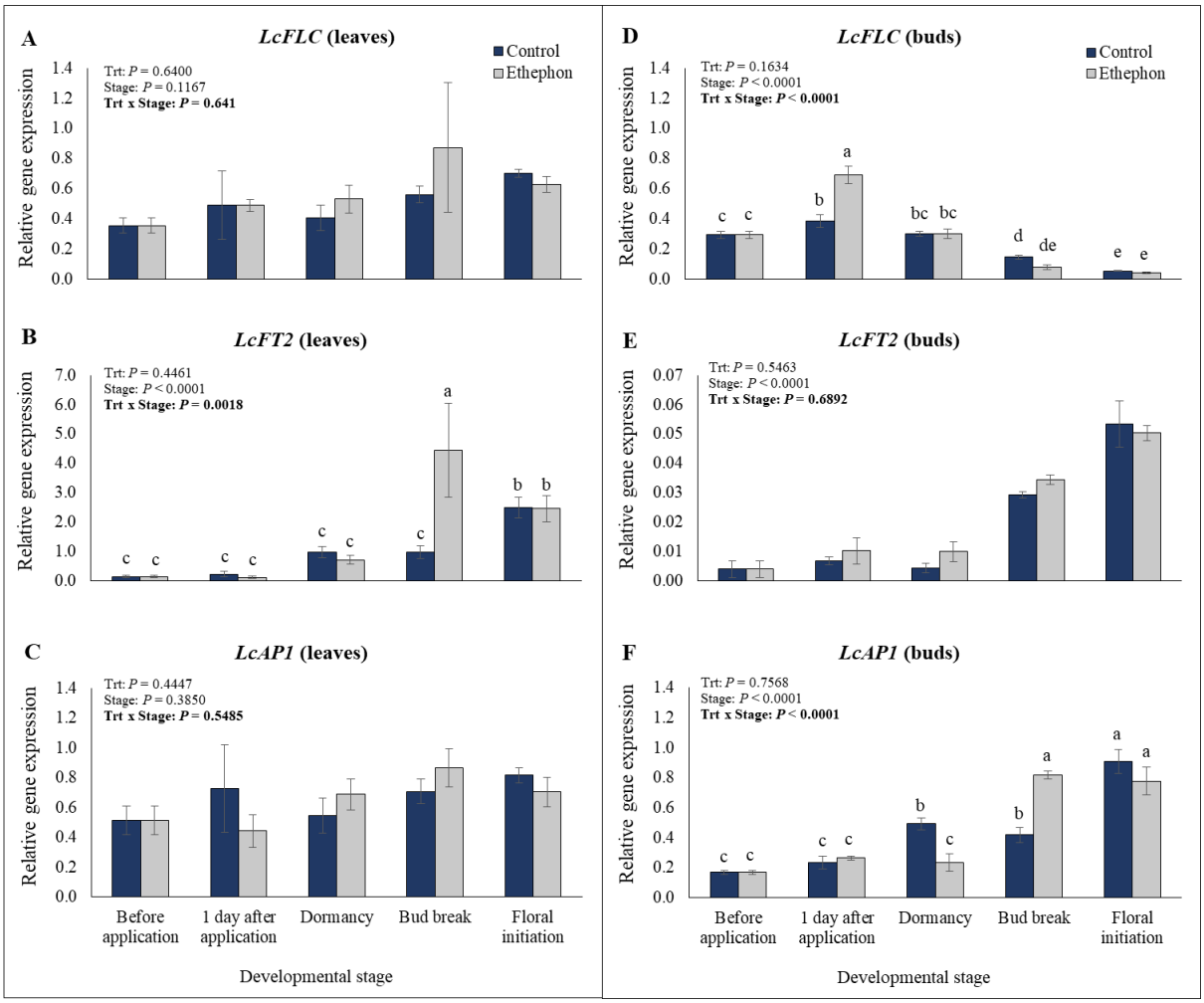

Fig. 6. Relative expression levels of flowering-related genes in leaves and terminal buds of 'Mauritius' litchi in response to ethephon treatment (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Figures A to C display changes in relative gene expression in leaves for A) *LcFLC*, B) *LcFT2* and C) *LcAP1*. Figures D to F display changes in relative gene expression in terminal buds for D) *LcFLC*, E) *LcFT2* and F) *LcAP1*. Data was collected from a leaflet of the compound leaf closest to the terminal bud, as well as from terminal buds at the indicated developmental stages. Relative gene expression was determined with qRT-PCR. Columns represent mean values ± *SE* (n = 3). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Different letters above columns indicate significant differences for treatment × stage interactions according to Fisher's LSD test (p = 0.05).

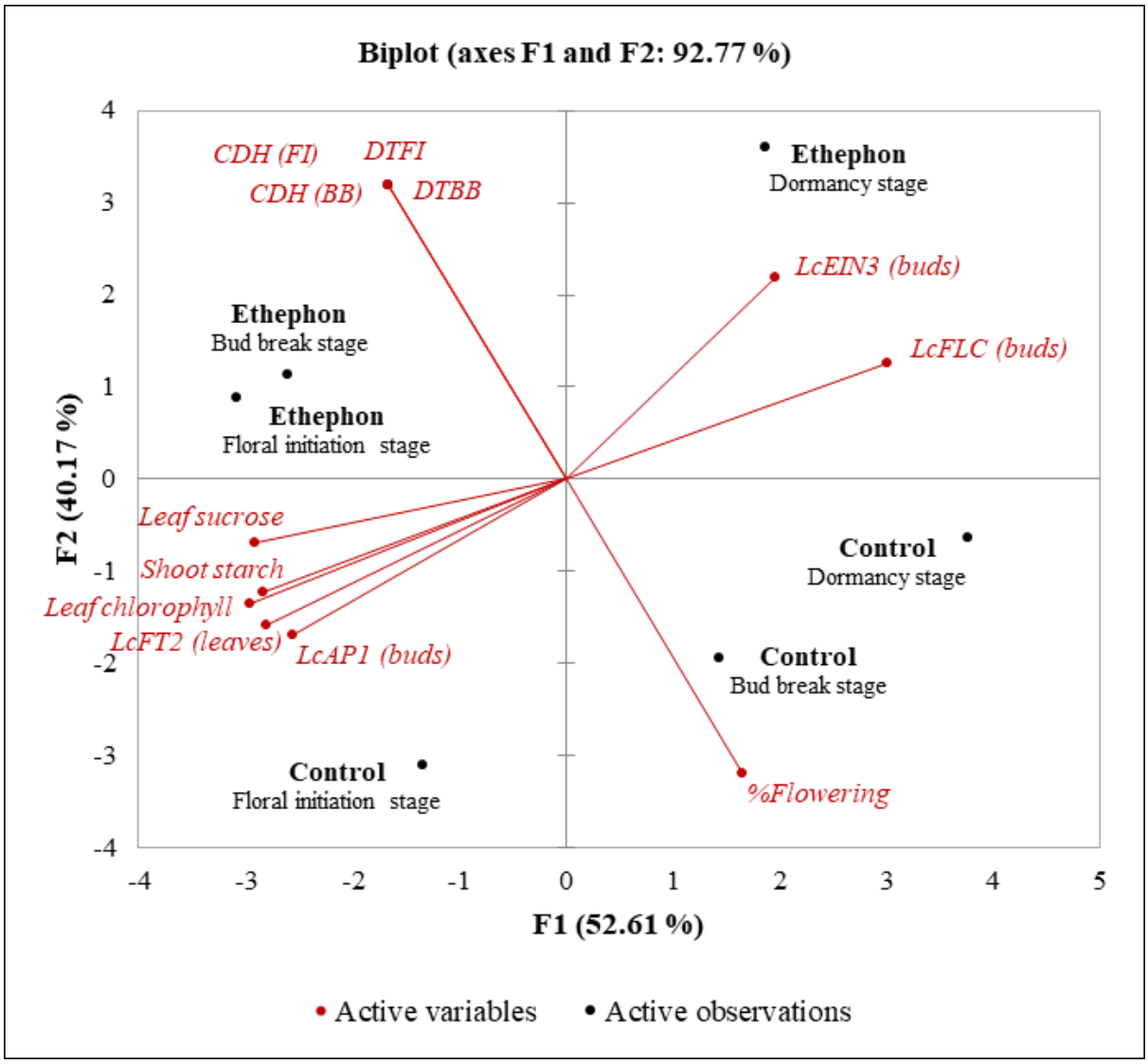

Fig. 7. Principal Component Analysis (PCA) revealed close associations between ethephon treatment and key regulatory genes involved in dormancy (*LcEIN3*, *LcFLC*) and flowering (*LcFLC*, *LcFT2*, *LcAP1*), shoot maturity (leaf chlorophyll), as well as carbohydrates (leaf sucrose, shoot starch) in 'Mauritius' litchi. PCA was calculated from data collected at the indicated developmental stages (dormancy, bud break and floral initiation stage) in response to ethephon treatment (Ethephon) or no treatment (Control). *LcEIN3*, *LcFLC*, *LcAP1* represent relative gene expression in terminal buds. *LcFT2* represents relative gene expression in leaves. CDH indicates chilling degree hour accumulation at the bud break (BB) or floral initiation (FI) stage. DTBB indicates days to bud break. DTFI indicates days to floral initiation.

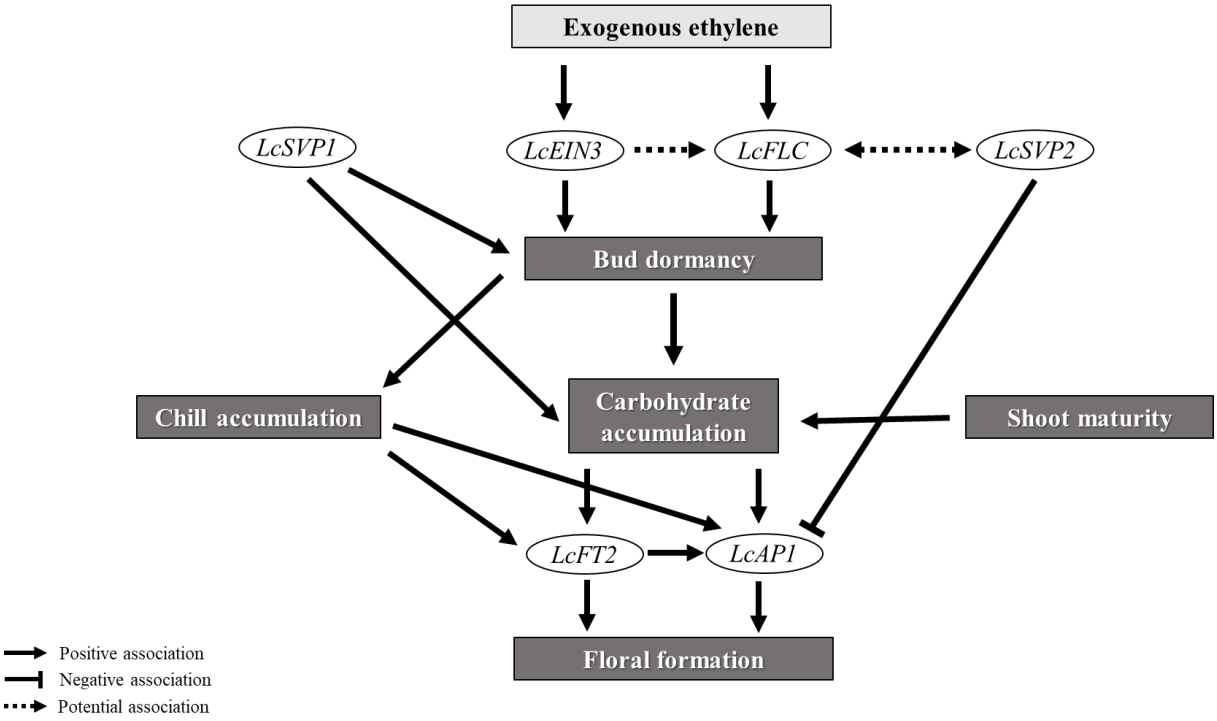

Fig. 8. Conceptual model illustrating the interactions between morphological, physiological and molecular processes affecting floral formation in litchi in response to exogenous ethylene. Exogenous ethylene (via ethephon application) stimulates expression of *LcEIN3* and *LcFLC* in terminal buds, which, in possible association with *LcSVP*s, enforces and prolongs bud dormancy. The extended dormancy period promotes shoot maturity and carbohydrate accumulation. The subsequent delay in bud break and panicle emergence stimulates upregulation of flowering-related genes (*LcFT2*, *LcAP1*) under more favorable inductive conditions with higher chill accumulation leading to successful floral panicle development. Arrows with solid lines indicate positive associations; solid lines ending with a bar indicate negative association; arrows with dotted lines indicate potential, but unconfirmed associations.

General Discussion and Conclusion

Erratic flowering in litchi (*Litchi chinensis* Sonn.) is a great challenge in most litchi producing countries and has worsened in the past decades due to changes in seasonal weather pattern, which often interfere with normal floral development processes (Chen and Huang, 2005; Menzel and Simpson, 1988, 1994; Nghi et al., 2020; Wang et al., 2017). Low climatic adaptability, as well as being a terminal bearer, makes litchi particularly prone to irregular flowering under unfavorable weather conditions, as shoot maturity and inductive temperatures need to coincide for successful floral induction and initiation to take place (Batten and Lahav, 1994; Menzel and Simpson, 1994).

Similar to other countries, litchi production regions in South Africa have also been affected by extended periods of rainfall during autumn, as well as above-average temperatures and a late onset of chilling temperatures in winter, which lead to higher incidences of vegetative shoot growth during late autumn and early winter. This poses a great challenge for growers to maintain sufficient shoot maturity until the onset of low inductive temperatures (Crane, 2004; Cronje et al., 2017; Huang and Chen, 2014).

Shoot control practices for litchi in South Africa have been limited to only a few options, such as girdling, water stress and ethephon applications. As vegetative shoot control by means of girdling and water stress do not consistently suppress flushing prior to floral induction and require additional chemical intervention during warm and wet autumns (Crane, 2004; Huang and Chen, 2014), most South African growers rely on ethephon spot spray applications to remove untimely young vegetative shoots, owing to its ease of use. Such applications also provide growers with a more reliable control for the management of vegetative shoot growth compared with above-mentioned practices. However, conventional ethephon spot spray applications have become less economical over the years due to unfavorable weather conditions resulting in continuous flushing and therefore, requiring numerous ethephon applications to achieve the desired shoot control effect. In addition, inconsistent low temperatures during winter often caused vegetative shoot growth concurrent with panicle emergence, which has made growers hesitant to continue with ethephon applications out of concern to damage the developing panicles. To ensure adequate flowering under these conditions and enable sustainable litchi production in the future, a new approach to pre-flowering shoot control was required.

Since the litchi industry in South Africa is relatively small and the testing and registration of new chemicals is costly, the aim of the current study was to adjust the conventional use of ethephon by applying it at a different phenological stage to enhance its efficiency. It was hypothesized that an application to mature terminal shoots with dormant buds, instead of already developing young vegetative shoots, may induce bud dormancy and inhibit new vegetative growth prior to floral

induction, which subsequently would prolong the dormancy period, promote carbohydrate accumulation and promote flowering and yield accordingly. To determine the most effective concentration, a range of concentrations and applications times were evaluated over a four-year period. The effects of ethephon on phenological, physiological and molecular changes in terminal shoots and buds prior to and after floral initiation were then investigated by studying ethylene evolution, gene expression and reserve carbohydrate dynamics.

Phenological changes associated with ethephon application. The results presented in the current study confirmed that ethephon is effective in modulating litchi tree phenology. The four-year field experiment found that ethephon applied to mature terminal shoots induced bud dormancy and extend the dormancy period for up to six weeks, resulting in vegetative shoot growth inhibition prior to floral induction and improved flowering. Applications were most effective when applied as whole-canopy treatment to dormant buds or buds that had not progressed beyond the bud swelling stage. An integrated dose-, time- and temperature response was observed at both experimental sites, with higher concentrations exerting a stronger inhibitory effect than lower concentrations within each application period.

In general, early applications and high prevailing temperatures required higher ethephon concentrations for effective shoot inhibition and panicle emergence delay compared with later applications, particularly in the warmer production area. However, late ethephon applications at high concentrations under already prevailing lower temperatures caused an accumulative inhibitory effect and delayed bud break past the inductive period, thus, affecting flowering negatively.
The double ethephon application to mature shoots did not provide an additional effect in terms of shoot inhibition when compared with the highest concentration used in the single application, but delayed bud break and panicle emergence by another five days. An extended delay in panicle emergence could mean the difference between a mediocre and a high flowering rate, particularly in warm production areas, since buds emerging at an earlier stage are more likely to be affected by intermittent warm temperatures.

The current study also aimed to provide insight into the efficacy of ethephon when applied as whole-canopy spray to trees that had passed the mature dormant stage and already showed initial signs of vegetative shoot growth, but had not progressed to the young soft leaf flush stage yet, which would normally be controlled by spot spray applications. Such a situation can easily occur in non-uniform orchards. Although some follow-up sprays were necessary to remove the already developing shoots once big enough for spot spray treatment, a whole-canopy application at first signs of shoot growth maintained bud dormancy in the rest of the tree and therefore also offered better shoot inhibition, dormancy extension and flowering response compared with spot spray applications alone.

In general, the conventionally used spot spray applications controlled immature vegetative shoots adequately, but since this treatment only targeted already emerged shoots, the untreated shoots within the same tree could start their growth unrestricted at any time and permitted earlier panicle emergence under less inductive conditions, resulting in the development of leafy flower panicles. Furthermore, spot spray applications could not prevent new shoots from emerging during periods of intermittent high temperatures in winter after spot spraying had discontinued. To ensure sufficient and continuous shoot control, several spot sprays applications are required, rendering this treatment less economical, compared with a single whole-canopy application to trees with mature shoots, which generally provided a longer-lasting and more reliable inhibition response, even during periods of intermittent high temperatures in winter.

While the level of shoot inhibition determined the flowering rate, the duration of dormancy or timing of panicle emergence influenced the quality characteristics of panicles. Ethephon applied to mature shoots, particularly at the higher concentrations, successfully delayed panicle emergence to a period with more consistently low temperatures, allowing for further chill accumulation and leading to better flowering with an increased number of panicles per shoot, i.e., axillary panicle development, less leafy and shorter panicles, and higher yields in most years when compared with untreated trees. Despite delayed bud break and panicle emergence, floral development in trees treated with whole-canopy ethephon applications was accelerated compared with spot spray-treated and untreated trees, leading to comparable full bloom dates and with a tendency to advance fruit maturity. Furthermore, reduced leafiness and/or panicle length has great relevance for litchi production, as both shorter and leafless flower panicles, both of which are produced under cooler temperatures, were found to produce more female flowers and fruit compared with leafy panicles (Huang and Chen, 2014; Lee and Chang, 2019; Menzel and Simpson, 1992). Together with the higher number of panicles per shoot, shorter and less leafy flower panicles could also have been the main contributing factor to the overall higher yields in the whole-canopy ethephon treatments in the current study.

The importance of accumulated carbohydrate reserves for litchi flowering and fruiting has been discussed in previous studies (Chen et al., 2004; Cronje and Mostert, 2009; Jiang et al., 2012; Menzel et al., 1995; Yang et al., 2014; Yuan et al., 2009). The current study provided evidence that pre-induction vegetative shoot growth reduced or arrested carbohydrate accumulation prior to flowering, while prevention of such shoot growth promoted accumulation. Moreover, the ethephon-mediated delay in panicle emergence provided additional time for carbohydrate accumulation. In this regard, complete prevention of shoot growth by applying ethephon to mature shoots proved to be more beneficial compared with elimination of existing immature shoots by spot spray applications, particularly for the accumulation of sucrose reserves. Furthermore, the current study

showed that vegetative growth and rapid panicle growth appeared to be more reliant on sucrose reserves, while early fruit development was supported by starch reserves. In addition, quebrachitol concentration tended to decrease in trees with high flushing or flowering rates. Previously, quebrachitol had only been reported in xylem and phloem tissue of terminal shoots, leaves, roots and the respective parts of the fruit (Wu et al., 2018). The current study is therefore the first report of quebrachitol in small branches. Principal component analysis revealed strong associations between the whole-canopy ethephon application to mature shoots and the time interval to panicle emergence, chilling degree hour (CDH) accumulation and sucrose/starch concentrations prior to flowering, emphasizing the role of ethephon in promoting pre-flowering carbohydrate accumulation and flowering in litchi. Furthermore, both the whole-canopy and spot spray ethephon treatments were positively associated with sucrose and starch concentrations during the pre-induction period, as well as with flowering rate. In contrast, no associations between untreated trees, carbohydrate reserves and flowering rate were evident. Although CDH accumulation was positively correlated with sucrose levels in the month during which panicle emergence occurred in the respective years, no correlations with flowering rate was found. Therefore, the four-year study could not establish whether high pre-flowering carbohydrate levels in small branches can contribute to the chilling responsiveness of trees, as proposed by Huang and Chen (2014).

*Physiological and molecular changes associated with ethephon application. S*tudies on ethylene evolution, reserve carbohydrate dynamics and gene expression in leaves and buds of terminal shoots contributed substantially to a better understanding of the physiological and molecular mechanisms underlying the ethephon effects that were observed in the four-year experiment. For example, ethylene evolution revealed that the inhibitory effect of ethephon was limited to the terminal buds. The prolonged presence of ethylene in the bud tissue caused a significant upregulation of the ethylene-signaling pathway gene *LcEIN3* and of the floral antagonist *LcFLC*, and confirmed the involvement of ethylene, the active form of ethephon, in bud dormancy in the current study. This was further supported by the increase in gene expression of *LcSVP2*, a DAM-gene that regulates entrance into dormancy, and the positive correlation between *LcSVP2* and *LcFLC* in buds one day after ethephon application. It has been proposed that some genes in the *SVP* family may be regulated by ethylene amongst other hormones (Gregis et al., 2013; Liu et al., 2018). Gene expression profiling of terminal buds during natural bud development found that major components of the ethylene-signaling pathway, such as *LcEIN3*, were upregulated during growth cessation and dormancy of terminal buds in litchi (Zhang et al., 2016). The current study added to this understanding by showing that also exogenous ethylene, via application of ethephon, can trigger some of the same downstream processes in litchi buds, thus, suggesting that ethylene is a key player in inducing growth cessation and dormancy in litchi. It is also possible that ethephon application

triggered endogenous ethylene production, amplifying the downstream ethylene response. However, direct causation could not be established in the current study and would require the analysis of 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase (ACO) enzyme activity for confirmation. Furthermore, ethephon treatment was found to trigger a stress response with an increase in reactive oxygen species (ROS) levels in peach (Islam et al., 2021; Liu and Sherif, 2019) and litchi (Zhou et al., 2013). Although these studies did not investigate specific genes associated with the ethylene-signaling pathway, Lu et al. (2014) found that treating litchi shoots with another ROS-generating chemical, namely methyl viologen dichloride hydrate (MV), increased expression levels of several genes in the ethylene-signaling pathway, among them *LcEIN3*, similar to ethephon application in the current study. For the current study, analysing ROS and ABA production levels could have provided more insight into the level of stress response exerted on the buds by the ethephon treatment.

The ethephon-mediated bud dormancy found in the current study also suggested that the terminal buds were less responsive to high temperatures during the pre-floral induction period, which was evident from the significantly increased delay in bud break and panicle emergence compared with the control treatments at both experimental sites. Selected studies on the effects of ethephon on bloom delay in stone fruit also proposed that ethephon reduced bud sensitivity to elevated temperatures and the associated de-acclimation conditions after mid-winter thaws (Gianfagna et al., 1992; Liu et al., 2021). Furthermore, results from the current study reported higher CDH accumulation in ethephon-treated litchi trees in both the four-year and gene expression experiment, which was attributed to the extended dormancy period prior to panicle emergence. Subsequently, panicle emergence took place under lower inductive temperatures compared with untreated trees, and resulted in significantly increased expression levels of the *LcFT2* gene in the leaves and of the *LcAP1* gene in buds of ethephon-treated trees at the bud break stage. In addition, the shorter time interval between bud break and panicle emergence observed in ethephon-treated trees across all experiments indicated that ethylene possibly promoted the transition from vegetative bud to floral bud. Thus, the combination of a stronger inductive stimulus, and possibly ethephon-induced increased responsiveness of shoots to these inductive temperatures, resulted in a higher percentage of pure flower panicles and reduced panicle length in ethephon-treated trees compared with untreated trees in both the four-year orchard-based and the gene expression experiment of the current study. The use of ethephon as mitigating tool for adverse climatic conditions is not new. In a range of temperate crops, such as stone and pome fruit (Crisosto et al., 1990; Ebel et al., 1999; Islam et al., 2021; Liu and Sherif, 2019; Liu et al., 2021; Mertoğlu et al., 2019), blueberries (Krewer et al., 2005) and pistachio (Askari et al., 2011), ethephon has been used to delay bloom as a mitigating strategy against spring frosts. However, the use of ethephon for the same purpose has

not been explored much in subtropical crops. The only report found in this regard was the use of ethephon to delay bloom in mangos to avoid the negative effect of low temperatures on pollination and fruit set (Mullins, 1984).

The importance of shoot maturity for chill responsiveness and successful floral induction in litchi has long been recognized (Batten and McConchie, 1995; Chen and Huang, 2005; Fu et al., 2014; Huang and Chen, 2005; Zhang et al., 2016). More recent studies showed that shoot maturity was also correlated with the carbohydrate status and the level of *LcFT* expression in the leaves (Fu et al., 2022; Xiao et al., 2018; Yang et al., 2014; Zhang et al., 2016). The current study found that ethephon-mediated bud dormancy and delay in bud break enhanced shoot maturity and increased concentrations of starch and soluble sugars (sucrose, glucose and quebrachitol) in the leaves and/or stems of terminal shoots prior to bud break, confirming the trends observed in small branches. However, the significant decrease of leaf sucrose and glucose concentrations as well as shoot sucrose and quebrachitol concentrations in ethephon-treated trees at the floral initiation stage indicated a high carbohydrate demand to support respiratory processes at bud break, as suggested by Zhang et al. (2016), as well as the accelerated panicle development compared with the untreated trees. The decline of shoot glucose concentrations at bud break and of shoot quebrachitol concentrations at the bud break and floral initiation stage in ethephon-treated trees further suggested that both soluble sugars, but particularly quebrachitol, were possibly mobilized from the shoots to meet the high sugar demand required for the floral initiation process. Quebrachitol was previously reported to have a unique function in litchi as a transportable photosynthate under reduced sucrose conditions (Wu et al., 2018). The current study supports this notion and it is proposed that quebrachitol may play a role as easily transportable carbohydrate under conditions of high energy demand during the flowering process in litchi.

Highly positive correlations between shoot maturity, *LcSVP1* gene expression in buds as well as leaf and shoot sucrose were found at the dormancy stage. Whereas shoot maturity, bud break date, leaf sucrose concentration, *LcFT2* expression in leaves and *LcAP1* expression in buds were significantly correlated at the bud break stage. These correlations were strongly associated with the ethephon treatment, and do not only confirm that shoot maturity and carbohydrate levels, particularly of leaf sucrose, are key factors for the expression of flowering-related genes, but also highlight the important role of ethephon in modulating physiological and molecular processes that promote floral induction and initiation in litchi. Although low inductive temperatures concurred with high carbohydrate and high floral promoter expression levels at bud break in ethephon-treated trees, the results of the gene expression study were not conclusive on whether the higher carbohydrate status in leaves and stems of terminal shoots of ethephon-treated trees contributed to the chilling responsiveness of the trees, since untreated trees also presented high flowering rates. In

order to determine the absolute contribution of carbohydrates to the chilling responsiveness of leaves and buds, experiments under different temperature regimes and different carbohydrate levels in the stems, leaves and buds of terminal shoots need to be conducted.

Limitations of the current study and future research. The current study provided valuable information on how adjustments to a current horticultural practice can improve crop production under changing climates. However, the unbalanced trial design in the four-year study due to the unavailability of sufficient number of trees at the designated phenological stage for each planned treatment and application date posed a limitation to the trial, and the missing data restricted interpretation to a certain extent. Particularly at the warmer Malalane experimental site, a more complete data set including all three application dates for at least two of the four years could possibly have created clearer differences between the whole-canopy and spot spray treatments for all measured parameters, and thus could have provided additional information for a more comprehensive grower guideline for this production region. In addition, temperature fluctuations during and after ethephon applications in both experiments may have influenced the rate of ethephon degradation, as ethephon evolution is highly temperature dependent (Olien, 1976). Complex interactions between environment, i.e., temperature fluctuation in the current study, plant growth regulator effect and tree response challenged sound interpretations of the true ethephon effect, particularly when attempting to compare the same treatment concentration between different application dates and years. The temperature variations together with the intricate and temperature-specific floral developmental processes in litchi add to the complexity when endeavoring to develop a prediction model that intends to incorporate ethephon concentration and chilling degree hours to estimate the panicle emergence date. Therefore, from a scientific point of view, experiments under controlled conditions would have been preferred and would have enabled investigating the effect of ethephon under inductive and less inductive temperature regimes. This may also have allowed insight into possible changes in chilling responsiveness of litchi trees when subjected to ethephon treatment, and their subsequent ability to induce flowers under less inductive temperatures. Controlled conditions could probably also have yielded stronger correlations between treatment effects and associated phenological responses. However, from a grower's point of view, the current study provided ample information on the performance of whole-canopy sprays applied to mature shoots, as well as the spot spray applied to developing immature vegetative shoots, under different climatic scenarios.

Considering the higher frequency with which above-average temperatures in winter were experienced in most subtropical litchi production regions in recent decades, future studies addressing erratic flowering in litchi should focus on finding strategies that increase the responsiveness of litchi trees to induce floral development under less inductive temperatures.

Recommendations. Based on the results of the current study, whole-canopy ethephon applications after maturation of the last desired postharvest shoot growth can be recommended for 'Mauritius' litchi in South Africa and will deliver superior results compared with conventional shoot control measures. The best results are achieved in uniform orchards with all trees having fully mature terminal shoots. As this is not always achievable, application can commence when at least 95% of the trees have completed their final postharvest flush growth. Terminal buds should be dormant, or not beyond the bud swelling stage. As the first signs of new flush growth are easily overlooked, whole-canopy applications can also be applied when the trees show less than 10% newly developing vegetative shoots, which is generally first observed at the top of the canopy. At this stage, some buds would have commenced growth, but not have fully unfolded their leaves, rendering them insensitive to ethephon. Therefore, these shoots need to be removed by spot spray application once their leaves have completely unfolded. Early whole-canopy applications to mature shoots between end-March and mid-April, under prevailing warm and wet conditions, require high ethephon concentrations, such as 1000 $mg \cdot L^{-1}$. For warm production regions, two whole-canopy applications can be used with the first application at 1000 $mg \cdot L^{-1}$ followed by a second application of 750 $mg \cdot L^{-1}$ two weeks later. Where trees only complete shoot maturation between mid-April and early-May under prevailing warm conditions or forecasts of warm weather conditions, ethephon concentrations between 750 and 1000 $mg \cdot L^{-1}$ are advisable, while under conditions of declining temperatures, a reduction of ethephon concentrations to 500 or 750 $mg \cdot L^{-1}$ is recommended. If temperatures are already close to the 20°C threshold for floral induction (Batten and McConchie, 1995) by the end of April or early May with forecasts of cold winter condition, no ethephon applications are necessary, since the trees will already be at the optimal phenological stage to perceive and respond to the low temperature stimulus for floral induction. In fact, the current study showed that ethephon application under cold conditions will extend bud dormancy beyond the floral induction period, risking a reduction in flowering. However, should sporadic leaf flush still appear, spot spray applications may be used instead of whole-canopy applications. The dates recorded for bud break and panicle emergence during 2014 to 2017 can serve as a future reference for spray date estimations during cold and warm seasons. Other practical considerations for whole-canopy applications include a good spray coverage to the point of run-off to ensure that the terminal buds are wetted optimally. The addition of a suitable wetting agent to the spray solution is recommended for better application and uptake efficiency. Moreover, general observations by growers found that care should be taken not to subject trees to additional stress factors during autumn and early winter, e.g., by using high concentrations of ethephon together with severe water stress, as this may force trees into a prolonged dormancy and reduce flowering. Likewise, other litchi cultivars will likely react differently to the same ethephon concentrations, similar to what has already been known for

spot sprays (Roets et al., 2010), and separate experiments would be needed to determine the most effective treatment conditions.

Implications for the South African litchi industry. Modifying existing horticultural practices can be an important short-term strategy to reduce the effect of unfavorable climatic conditions. This is particularly important for perennial fruit tree crops, where changing to a more climate-resilient cultivar or alternative crop generally requires high investment cost and is a long-term endeavour. In the current study, the efficacy and downstream effects of ethephon were amplified by simply applying ethephon at a different phenological stage, thus, providing a practical and effective tool to mitigate unfavorable weather conditions during the pre-flowering period. Compared with multiple spot spray applications, a single whole-canopy application also enables better ease of application and higher cost-effectiveness, which is important particularly when considering the ever-increasing input costs in agriculture. With a deeper understanding of the flowering mechanisms of litchi when subjected to plant growth regulator manipulation with ethephon and the recommendations emanating from it, growers will have a powerful tool for managing the effects of adverse climatic conditions that influence litchi flowering. Implementation of revised strategies can minimize erratic flowering and achieve sustainable production for the cultivar 'Mauritius'. For a small industry, such as the litchi industry in South Africa, with limited resource for research and development, this modification has great implications for sustainable litchi production, farming productivity, profitability, and competitiveness on international markets.

Conclusion

The current study showed that a single whole-canopy ethephon application to trees with fully mature shoots inhibited pre-induction vegetative growth and delayed panicle emergence to a period with consistently low inductive conditions, thus, improving flowering and yield of 'Mauritius' litchi under South African conditions. Whole-canopy ethephon application is also an effective alternative to the frequent conventional spot spray applications. The study further revealed that ethylene plays an important role in the physiological and molecular regulation of bud dormancy and subsequent floral formation in litchi. Due to its potential to induce and maintain bud dormancy, ethephon treatment of mature shoots can therefore be regarded as a useful tool to mitigate adverse climatic conditions prior to floral induction and to promote flowering in litchi.

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APPENDIX I

Effect of full-cover ethephon applications on flowering and yield of 'Mauritius' litchi in South Africa

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Abstract

Currently, ethephon is used by South African growers as spot spray to control autumn/winter flush, but such applications generally have to be repeated several times due to re-occurring flush. In order to reduce the amount of applications, trials were conducted to determine whether a once-off full-cover ethephon application after the last post-harvest flush (at dormant stage) could inhibit new leaf flush long enough until temperatures become conducive for flower induction in order to increase flowering and yield. A trial was conducted in Nelspruit, South Africa, on 'Mauritius' ('Tai So') litchi trees during the 2014 and 2015 litchi seasons. Ethephon (a.i. 48%) was applied as a once-off full-cover spray after hardening of the last desired post-harvest flush at the concentrations of 0, 500 and 1000 ppm at the end of March, mid and end of April. The control trees were treated with spot sprays as per current farm practice (at 1000 ppm whenever young shoots appeared). Shoot control on the control trees started at the end of March and needed to be repeated twice. Tree response was monitored at regular intervals after spray applications. Shoot growth, days to flower panicle emergence, flowering and yield was recorded. In both years, concentration and timing of full-cover ethephon applications affected the growth of leaf flush and time of flower panicle emergence. As a result there was no or little vegetative growth during the flower induction period (April/May) as well as later flower panicle emergence. The higher the applied concentration was, the later was flower panicle emergence. Overall, applications up to mid-April increased flowering and yield. Application after mid-April delayed flower panicle emergence too long, leading to reduced flowering and yield, and increased winter/spring flush. It could be shown that one timely full-cover ethephon spray can substitute the frequent spot sprays that are currently used in the South African litchi industry.

Keywords: shoot control, ethephon, dormancy, flower induction, flower panicle emergence

INTRODUCTION

Mature shoots and mean temperatures below 20°C are the prerequisite for successful flowering in litchi (Batten and McConchie, 1995; O'Hare, 2002; Chen and Huang, 2005; Davenport and Stern, 2005). Shoot growth just before and during the flower induction period can lead to crop failure as these shoots will not mature in time to produce flowers (O'Hare, 2002; Davenport and Stern, 2005). It is therefore important to manipulate the flushing cycle in such a way that the latest flushes are fully mature when inductive temperatures occur. Water stress or drought can suppress leaf flush during autumn/winter and thus indirectly enhance flowering, but can only be successfully used in areas with little or no rain during this period (Menzel and Simpson, 1991; Chaikiattiyos et al., 1994; Stern et al., 1998; Carr and Menzel, 2013). Plant growth regulators, such as paclobutrazol and ethephon, have been used to regulate vegetative growth prior to flower induction in various countries with varying degrees of success to improve flowering (Chaitrakulsub et al., 1992; Kift and Roets, 2001; Huang and Chen, 2014; Mandal et al., 2014).

In South Africa leaf flush in the cultivar 'Mauritius' is a common phenomenon during autumn (March/April) and early winter (May) due to the relatively high temperatures and

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autumnal rainfall. Flower induction and initiation in the mid-season production areas generally takes place during April and May. A drop in temperature below a mean of 20°C for a few days is sufficient enough for flower induction in 'Mauritius' if the buds are in the correct stage of development, although a longer cold period would be more preferable (Kift and Roets, 2001; Huang and Chen, 2005). However, during this period temperatures are not always consistently low enough so that leaf flush and early flower panicle emergence can occur at the same time. Early flower panicles are also often leafy and thus less fruitful than pure flower panicles (Huang and Chen, 2014). In order to control leaf flush, South African growers have been using ethephon since the early 2000's (Roets et al., 2010). It is applied as spot spray to young soft flush whenever it appears during autumn and early winter. Ethephon, an ethylene releasing compound, kills the young flush including the terminal bud. Four to six weeks later, when temperatures have become consistently low enough, flower panicles start to emerge from the axillary buds (Kift and Roets, 2001).

As flush control by spot spraying requires several applications and every appearing flush consumes tree reserves, the aims of this study were to inhibit any shoot growth during April and May and at the same time prevent early flower panicle emergence, and to investigate the effects of the different ethephon concentrations and application times on the length of the inhibition period.

MATERIALS AND METHODS

Experimental site and design

The trial was conducted at Nelspruit, a mid-season litchi production area of the Mpumalanga Province of South Africa, which is characterized by moderate to cool autumns and winters, respectively. The treatments were applied to 14-year old 'Mauritius' litchi trees during 2014 and 2015. Application dates in 2014 were on 25 March (T1), 8 April (T2) and 25 April (T3) and in 2015 on 30 March (T1), 13 April (T2) and 28 April (T3). The applications were made with a motorised sprayer with hand lances at 15 bar pressure and a 2.5 mm nozzle. A wetter was added at 10 mL 100 L^{-1} water. Table 1 displays the application rates and times for the trial site. All treatments were applied with ethephon (a.i. 48%) as a single full-cover spray after hardening of the last desired post-harvest flush, i.e., to dormant trees. Control trees were sprayed with ethephon as spot sprays whenever new leaf flush appeared prior or during the induction period.

Table 1. Treatments applied at Nelspruit during 2014 and 2015.

Treatment	Description	Tree growth stage at time of application
Commercial sprays (Control)	1000 ppm as spot sprays (farm practice) (repeated 2 times in both years)	Young soft flush
0 ppm	No ethephon application	Hardened flush
500 ppm	500 ppm ethephon single full-cover application	Hardened flush
1000 ppm	1000 ppm ethephon single full-cover application	Hardened flush

Treatments: 500 ppm = 104 mL 100 L^{-1} water; 1000 ppm = 208 mL 100 L^{-1} water.

Application dates in 2014: 25 March (T1), 8 April (T2) and 25 April (T3).

Application dates in 2015: 30 March (T1), 13 April (T2) and 28 April (T3).

Data collection and statistical analysis

Tree response after the application of ethephon was monitored by observing 10 branches $tree^{-1}$ at weekly to bi-weekly intervals. Temperature and humidity was measured using data loggers. Rainfall data was obtained from a nearby weather station. Percentage leaf flush, days to flower panicle emergence and flowering was calculated from the observed branches. At harvest, yield was determined as kg $tree^{-1}$.

The trial was laid out in a randomized complete block design with 5 replicates

treatment^{-1} and 3 trees replicate^{-1}.

The continuous data of yield and percentage flowering were subjected to a combined analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc., Cary, USA). Shapiro-Wilk test showed normal distributions for both flowering and yield. The residuals of both flowering and yield were normally distributed. Levene's test (Levene, 1960) for comparable seasonal variances (homogeneous variances) indicated that the seasonal variability in percentage flowering observations were not of comparable magnitude. Therefore, weighted ANOVA was performed for percentage flowering data. Treatment means were separated using the Student's *t*-test with Least Significant Difference (LSD) at the 5% level of significance.

RESULTS AND DISCUSSION

Climatic effects during 2014 and 2015

The climatic conditions in 2014 and 2015 differed from each other with regard to temperatures and rainfall (Figure 1). The year 2014 was cooler and wetter compared to 2015. In 2014, mean temperatures started to drop to 20°C during mid-April and below 20°C from beginning of May. During autumn, rainfall was high with 280 mm, dropping to almost no rain until August. Climatic conditions in 2015 were characterized by high mean temperatures until the beginning of June and very little rain between March and August. These conditions influenced tree phenology in relation to treatments to varying extent.

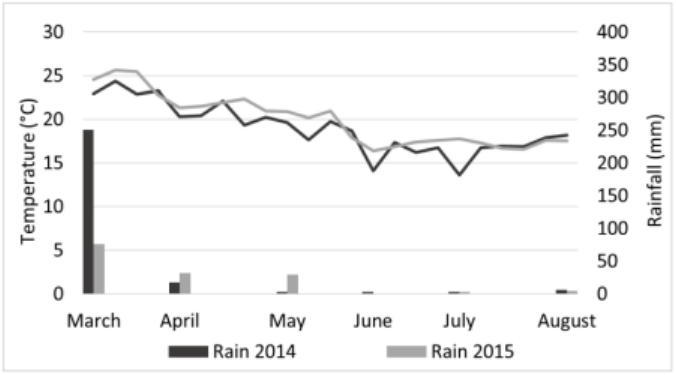

Figure 1. Mean temperature and rainfall between ethephon application and flowering of litchi in 2014 and 2015.

Experimental year 2014

Figure 2 (left) shows leaf flushing for the all treatments and ethephon application times in 2014. Standard flush control as per industry practice (control treatment) successfully reduced shoot growth in 2014 whenever it appeared. The 0 ppm treatment, where no flush control was applied, allowed 50, 18 and 4% leaf flush during April/May for the T1, T2 and T3 application times, respectively. As the temperatures and rainfall decreased towards end of April the untreated trees were naturally less prone to produce leaf flush even without ethephon application. The 500 ppm treatment only allowed 2% leaf flush during April/May, when applied at the end of March (Figure 2a (left)), and completely inhibited leaf flushing during the same period, when applied in mid or end of April (Figure 2b,c (left)). The 1000 ppm treatment inhibited all leaf flush during April/May for all application times. When applied at the end of April, the 0, 500 and 1000 ppm treatments all showed increased leaf flush again from June onwards (Figure 2c (left)). This might be due to prolonged dormancy, either naturally by low temperature (Figure 1), as in the case of the 0 ppm treatment, or as a treatment-temperature effect in the case of the 500 and 1000 ppm treatments. However, Figure 3 clearly illustrates that full-cover ethephon applications significantly reduced leaf flush during April/May.

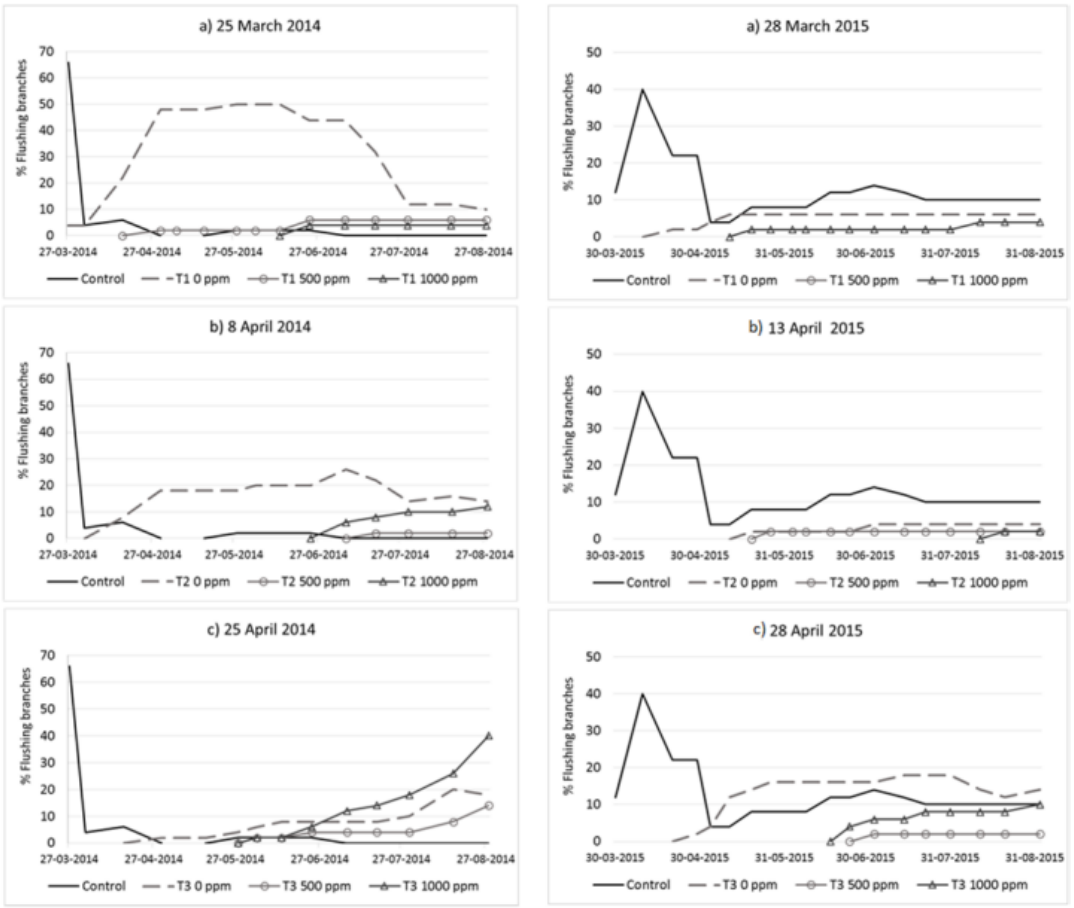

Figure 2. Percentage shoot growth of the treatments 0, 500 and 1000 ppm in comparison with the control for the application time at a) 25 March, b) 8 April and c) 25 April 2014 (left) and for the applications time at a) 28 March, b) 13 April and c) 28 April 2015 (right).

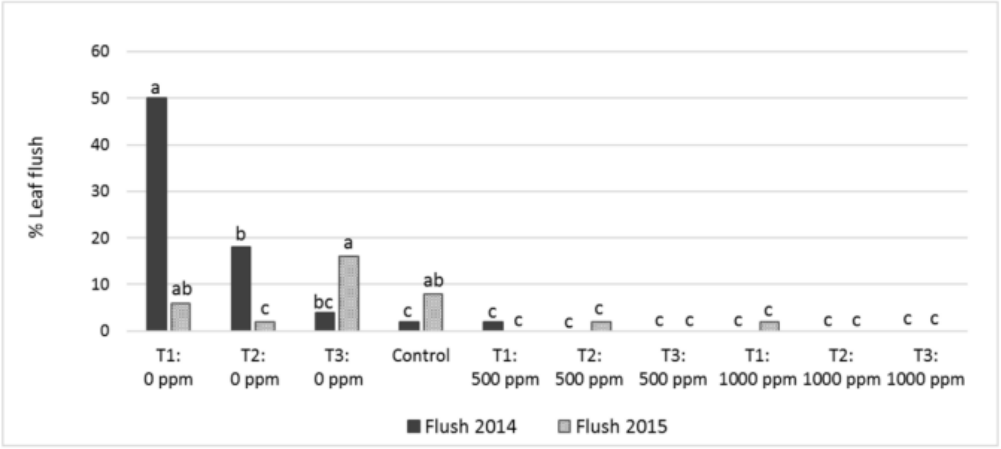

Figure 3. Percentage leaf flush of various treatments and application times during April and May 2014 and April and May 2015. Different letters denote significant differences for the separate years according to Student's *t*-test ($p<0.05$).

The presence or absence of leaf flush during April/May influenced percentage flowering in litchi, as immature flush during the induction period was not able to produce flowers. Figure 4 displays percentage flowering and days to flower panicle emergence after ethephon application for the year 2014. Due to good flush control, 88% of all marked branches in the control treatment flowered, having one of the highest percentages of flowering for that year. The 0 ppm treatment had one of the lowest percentages of flowering (56% for T1, 62% for T2, 54% for T3) due to high leaf flush before and during the induction period. The 500 and 1000 ppm treatments had the highest percentage flowering for T1 and T2. However, for T3, flowering was drastically reduced probably due to the combined effect of ethephon and lower temperature which mainly caused the trees to stay dormant too long, missing the inductive temperatures. For all three application times, days to panicle emergence showed a similar pattern, i.e., the higher the concentration, the longer the dormant period to panicle emergence. This also reduced the number of leafy flower panicles (data not shown). However, there was a tendency that the later the application time was, the shorter was the period to panicle emergence. This could indicate that buds were either naturally getting more active towards the cooler period or that the lower temperatures restricted the ethylene response (Beyer, 1973). Nevertheless, less flower panicles and more flush developed, especially in T3. High ethephon concentration applied later had a strong inhibition effect on the buds, which, together with the lower temperatures, may have withheld bud break during the induction period. When the buds eventually were active, the inductive temperatures had passed and leaf flush instead of flower emerged. Overall ethephon applications done at T1 and T2 were more beneficial than when done at T3.

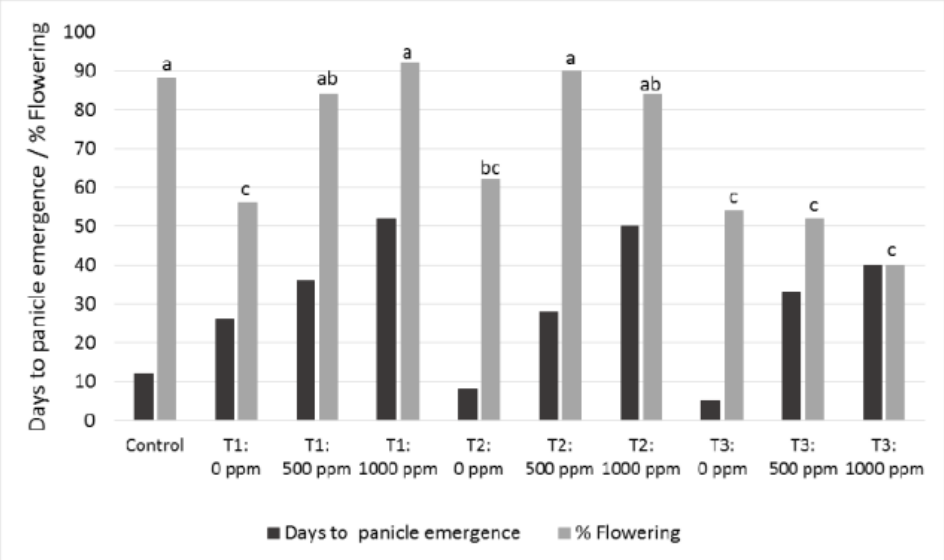

Figure 4. Days from application to first flower panicle emergence and percentage flowering branches at anthesis for various ethephon applications during 2014 at Nelspruit. (T1: 25 March, T2: 8 April and T3: 25 April 2014). Different letters denote significant differences according to Student's *t*-test ($p<0.05$).

Yield data revealed similar trends to flowering (Figure 5). Where leaf flush was low during April/May and flowering was high, yield was also high. The control, 500 and 1000 ppm treatments at T1 and T2 gave the best yields (78, 90 and 102 kg tree^{-1}, respectively). The 0 ppm treatment had the lowest yields for T1 and T2. At T3, all treatments had low yields due to reasons mentioned above. However, delay in panicle emergence did not cause delay in fruit maturity.

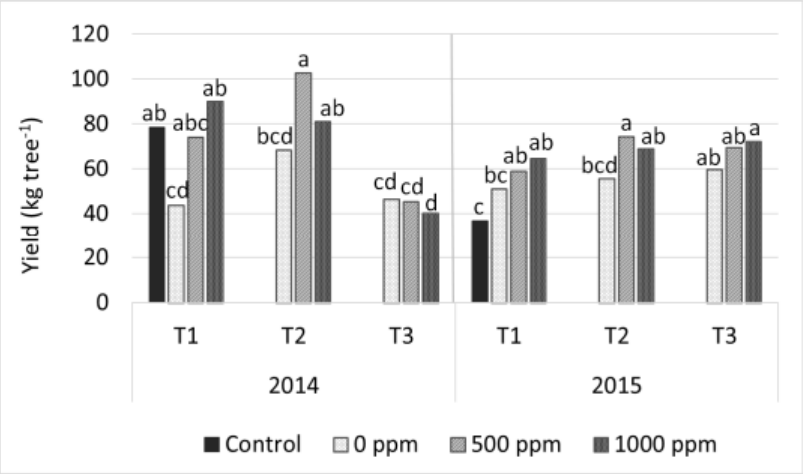

Figure 5. Yield (kg tree^{-1}) of litchi for different ethephon treatments in December 2014 and December 2015 at Nelspruit. Different letters denote significant differences for the separate years according to Student's *t*-test (p<0.05).

Experimental year 2015

The control treatment also controlled leaf flush successfully during April/May in 2015 (Figure 2 (right)). During June, however, there was a slight increase in leaf flush (up to 14%) due to the higher than usual temperatures during May and early June (Figure 1). During April, leaf flush was very low for the 0 ppm treatment for the T1 application time (Figure 2a (right)) and non-existent for T2 and T3 (Figure 2b,c (right)). However, from May onwards leaf flush increased between 2% (for T2) and 16% (for T3). The reasons for these different flushing patterns in this treatment compared to 2014 may be due to different climatic condition in 2015 (Figure 1). Although temperatures were higher in 2015, drought conditions inhibited leaf flush even without ethephon. Temperature also had an effect on the 500 and 1000 ppm treatments, which inhibited flush growth almost entirely up to end of June for all application times, with only 2-8% leaf flush appearance after that. Although leaf flush was low in all treatments during April/May 2015, again full-cover ethephon applications significantly reduced leaf flush compared to the control and 0 ppm treatment (Figure 3).

Higher temperatures along with drought condition suppressed leaf flush during April/May, as well as later than usual inductive temperatures (beginning of June, compared to beginning of May in 2014) resulted in a high percentage of flowering in all treatments and application times in 2015 (above 90%), except for the 0 and 1000 ppm treatments in T3 (Figure 6). On the other hand, trends in days to panicle emergence were similar to 2014, although overall slightly shorter. This is probably due to the higher temperatures during April/May (Figure 6).

Yield was lower compared to 2014, although overall percentage flowering was higher than in 2014 (Figure 5). The control treatment showed the lowest yield (37 kg tree^{-1}), followed by the 0 ppm treatments. Due to the long period of high temperatures, new leaf flush emerged in the control during the end of May, which impacted negatively on yield. The 500 and 1000 ppm treatments showed the highest yields for all three application times (Figure 5). While late application (end of April) of high concentrations of ethephon impacted negatively on panicle emergence, flowering and yield during a year with normal temperature patterns like in 2014, it was beneficial for an unusually warm year, like 2015, where a longer inhibition time was required to prevent leaf flush until the temperature eventually dropped at the beginning of June (Figure 1). Like in 2014, a delay in panicle emergence did not cause a delay in harvesting time.

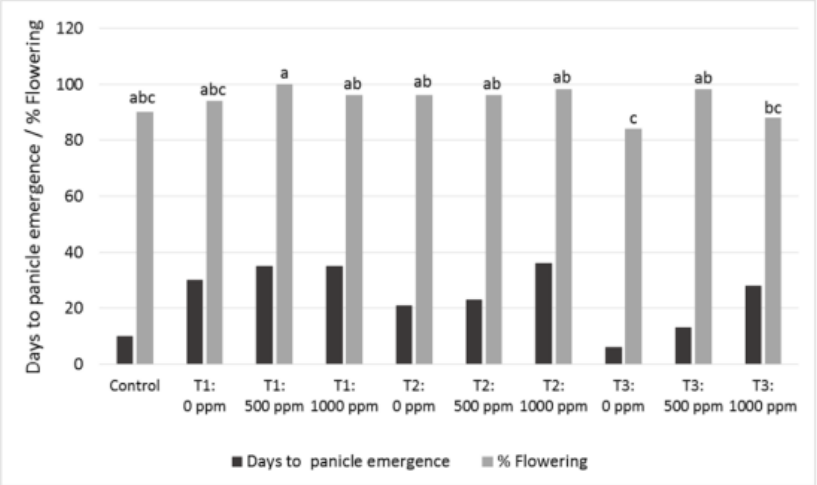

Figure 6. Days from application to first panicle emergence and percentage flowering branches at anthesis for various ethephon applications during 2015 at Nelspruit (T1: 30 March, T2: 13 April and T3: 28 April 2015). Different letters denote significant differences according to Student's *t*-test ($p<0.05$).

Similar to water stress, ethephon appears to exert stress on the trees, which in response prolongs bud dormancy or inhibits meristematic activity in the apical and axillary buds. The duration of inhibition was closely related to ethephon concentration and to a certain extend to climatic factors. This could be clearly seen when comparing full-cover ethephon applications with no or partial ethephon applications. The higher the ethephon concentration was, the later the new growth appeared, almost irrespective of climatic factors and vice versa. Ethylene is known for its stimulatory and inhibitory effects on vegetative and reproductive growth depending on concentration, growth conditions and developmental stage, and is species-dependent (Vandenbussche and Van Der Straeten, 2012). Ethylene influences shoot apical meristem activity in certain species, which can affect floral transition and flowering time (Ogawara et al., 2003; Liu et al., 2011; Vandenbussche and Van Der Straeten, 2012). From above results, ethylene appears to have similar effects in litchi. The exact mode of action in the apical meristem in dependence of temperature and other abiotic factors, its interaction with other hormones and effect on possible floral gene expression is yet to be determined in litchi.

CONCLUSION

Two years of experiments with single full-cover ethephon application to dormant trees have shown that in a mid-season litchi production area of South Africa, leaf flush during autumn and early winter can be inhibited until temperatures become conducive for flower induction. Flower panicle emergence can be delayed to avoid leafy flower panicles and flowering and yield can be increased. Time of application as well as concentrations of ethephon play an important role in the success of flush inhibition and delay in flower panicle emergence, as could be seen in the trial results. Other important factors to determine the inhibitory role of ethephon are climatic conditions after ethephon application until temperatures are low enough for flower induction. Despite climatic differences between the two experimental years one single full-cover ethephon application to dormant trees (up to mid-April) proved to be equal or better than the spot sprays currently used by the litchi industry. In addition, one single application is more cost-effective than multiple spot sprays.

Future research is aimed at an in-depth study on the effect of ethephon/ethylene on bud dormancy and meristematic activity in litchi. An investigation into the changes of plant hormones levels, their interactions, and gene expression patterns, as a result of ethephon application alone or in combination with abiotic stresses (temperature, water stress), could

shed more light on the mechanisms of floral formation in litchi.

ACKNOWLEDGEMENTS

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APPENDIX II

Carbohydrate reserve dynamics as influenced by shoot control strategies and climatic conditions prior to flowering in 'Mauritius' litchi

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Abstract

Changes in seasonal weather patterns due to climate change show an increasing impact on the phenological growth cycles of subtropical crops, including litchi. In the last decade, the onset of chilling temperatures in winter was reported to be later, with milder winter temperatures in general across the subtropical regions of South Africa. This change in environmental conditions results in continuous vegetative shoot growth during times of expected dormancy that is required for the accumulation of carbohydrate reserves to support flowering and fruiting of the next season. Non-structural carbohydrates, such as soluble sugars and starch, are important indicators of carbon source capacity of a plant and enable adaptation to environmental changes. In order to study carbohydrate levels and their effect on flowering in dormant versus vegetative growing trees during the pre-flowering period, experiments were conducted on 16-year-old 'Mauritius' trees in the north-east of South Africa using ethephon to inhibit shoot growth during autumn/early winter. Ethapon® (a.i. 48% ethephon) was applied as a single full cover spray to dormant, hardened off trees at concentrations of 500 and 1000 mg L^{-1} to inhibit any further shoot growth. Control treatments either consisted of untreated trees or tree where ethephon was applied at 1000 mg L^{-1} as a spot spray to the young developing vegetative flushes, as is the current management strategy for flush control in South Africa. Wood samples of small branches were collected at various phenological stages and analysed for starch and soluble sugar content. Results indicated that starch and soluble sugars fluctuated in response to growth events. Shoot inhibition by ethephon preserved carbohydrates during the pre-flowering period and improved flowering. The relationship between growth phase, ethephon concentration and carbohydrate levels is discussed.

Keywords: *Litchi chinensis* Sonn., plant growth regulator, shoot control, dormancy, starch, soluble sugars

INTRODUCTION

Irregular bearing due to unreliable flowering is a major challenge in litchi (*Litchi chinensis* Sonn.) production. Successful flower induction is dependent on low temperatures and maturity of terminal shoots (Batten and McConchie, 1995; O'Hare, 2002; Chen and Huang, 2005; Davenport and Stern, 2005). Changes in seasonal weather patterns with delayed onset of chilling temperatures in winter and milder winter temperatures results in continuous vegetative shoot growth just prior to and/re during the flower induction period. These shoots will not mature in time to produce flowers, therefore leading to crop failure (O'Hare, 2002; Davenport and Stern, 2005). As flowering is a carbon costly process, a period of growth check prior to flowering is important for the accumulation of sufficient carbohydrate reserves to support the flowering and fruiting processes of the next season (Huang and Chen, 2014). Various studies confirmed that carbohydrate reserves in the main storage organs of flowering trees increase in autumn, thus confirming their important role in litchi flowering (Menzel et

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al., 1995; Cronje and Mostert, 2009; Yang et al., 2014). A study by Yang et al. (2014) showed that mature shoots had higher carbohydrate and expression levels of the floral promoters *LcCO* and *LcFT2* in the leaves as well as higher flowering rates compared with immature shoots. Huang and Chen (2014) hypothesized that sufficient carbohydrate reserve accumulation prior to flower induction might increase the competence or responsiveness of litchi to flower-inducing chill temperatures and shorten the chilling period required for flower induction. The main carbohydrate reserves in litchi are starch and sucrose (Menzel et al., 1995; Yang et al., 2014). While starch is merely stored reserve energy for later metabolic activities, soluble sugars, such as sucrose, glucose and fructose, have a multitude of functions in the plant, such as regulation of growth, photosynthesis, carbohydrate partitioning, osmotic homeostasis, signalling molecules during abiotic stresses and regulation of source-sink activities to name but a few (Sami et al., 2016). Sugar accumulation may also serve as a signal for entrance and maintenance of bud dormancy (Zhang et al., 2016). In a more recent study, the methylated cyclitol, quebrachitol, has been found to be ubiquitous in litchi in considerable quantities and more so in autumn leaves compared with summer leaves. It is considered as an inert source of reserve energy and carbon metabolic strategy to maintain osmolality under low sucrose conditions (Wu et al., 2018). In order to promote accumulation of carbohydrate reserves, vegetative growth of shoots should be inhibited prior to flowering, either through autumn water stress, girdling or use of plant growth regulators, such as paclobutrazol and ethephon (Menzel and Simpson, 1991; Chaitrakulsub et al., 1992; Roets et al., 2010; Carr and Menzel, 2013; Huang and Chen, 2014; Mandal et al., 2014). In South Africa, the main shoot control strategy used for litchi is the application of ethephon to kill young developing flush, as water stress is not always reliable in seasons with late autumn rainfall. Following uptake by the plant, ethephon rapidly degrades to phosphate, ethylene, and chloride (Anonymous, 1983). Ethylene subsequently causes senescence and abscission of the young leaves and shoots (Olsen, 2010; Ju and Chang, 2015). After 4-6 weeks, bud break occurs in axillary buds and leads to floral development provided that temperatures are low enough for flower induction (Roets et al., 2010).

As the developing litchi shoots consume carbohydrate reserves, the aim of the current study was to compare the carbohydrate dynamics of small branches, the main carbon storage organ in litchi, in relation to flowering, as affected by various shoot control strategies, i.e., spot sprays to developing young shoots versus application to mature shoots with dormant buds, and its interaction with prevalent climatic conditions.

MATERIALS AND METHODS

Experimental site and design

The trial was conducted on a commercial farm near Nelspruit (Mpumalanga Province, South Africa) with moderate to cool autumns and winters. The treatments were applied to 16-year old 'Mauritius' litchi trees during the pre-flowering season on March 25 and April 25, 2014, and on March 30 and April 28, 2015, respectively, to evaluate the effect of temperature differences on the treatments. Treatments were applied with ethephon (a.i. 48%), either at 500 or 1000 mg L^{-1} as a single full-cover spray after hardening of the last desired postharvest flush, i.e., to dormant trees, or as a spot spray (control) at 1000 mg L^{-1} whenever any new leaf flush appeared prior or during the induction period (Table 1). A wetter was added at 10 mL 100 L^{-1} water to all ethephon applications.

The trial was laid out in a randomized complete block design with five replicates per treatment and three trees per replicate, of which the middle tree was used for data collection. All experimental trees were selected for uniformity of tree size and vigor, and subjected to the same cultural practices, i.e., adequate fertilizer and irrigation. In both years, water stress (<50% soil moisture content until flower panicles appeared) was induced during the pre-flowering period.

Table 1. Treatments applied to 'Mauritius' litchi trees in Nelspruit during 2014 and 2015 as shoot control strategies to improve flowering.

Treatment	Description	Tree growth stage at time of application
Control	1000 mg L^{-1} as spot sprays (repeated 2 times, in both years)	Young soft flush
0 ppm	No ethephon application	Mature dark green shoots
500 ppm	500 mg L^{-1} ethephon single full-cover application	Mature dark green shoots
1000 ppm	1000 mg L^{-1} ethephon single full-cover application	Mature dark green shoots

Data collection and statistical analysis

Tree response after the ethephon application was monitored by observing 10 branches per tree at weekly to bi-weekly intervals to determine percentage leaf flush and flowering. Climatic information included temperature recordings (HOBO data loggers, Onset Computer Corporation, USA) and weather station-based rainfall data (Figure 1). For determination of soluble sugars and starch, wood of four small branches per tree were pooled. Soluble sugars and sugar alcohols were extracted with 80% ethanol from milled, oven-dried (60°C) samples according to the method described by Chow and Landhäusser (2004), with slight modifications. Prior to analysis, the samples were cleaned with activated acidic alumina to reduce the load of colour, organic acids and salts. Analyses were performed using an HP 1100 LC system (Agilent Technologies, California) with refractive index detection, equipped with a Rezex RCM Monosaccharide Ca^{2+} column (Phenomenex, California). Quantification was performed according to external standard solution calibration. Extraction and determination of starch was done using the iodine colourimetric method of Xu et al. (1998).

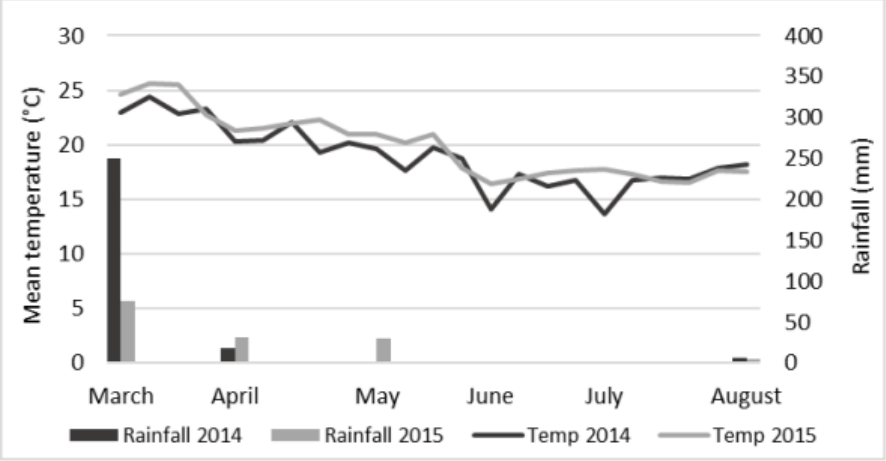

Figure 1. Mean temperature and rainfall between the pre-flowering and flowering period of 'Mauritius' litchi in Nelspruit during 2014 and 2015.

The data were subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc., Cary, USA). Comparison of treatment means was done by Fisher's least significant difference at the 5% level. Pearson's product-moment correlation was performed using the correlation procedure (PROC CORR) of SAS software (Version 9.4; SAS Institute Inc., Cary, USA). Correlations with r^2-values higher than 0.5 were regarded as physiologically significant. Principal component analysis (PCA) with a Pearson correlation matrix was performed to investigate the relationship between treatments and the different variables using XLSTAT (Version

2015.1.03.15485, Paris).

RESULTS AND DISCUSSION

Effect of shoot control strategies on vegetative and reproductive growth

The climatic conditions for the two experimental year differed in terms of temperature and rainfall (Figure 1). In 2014, high temperatures and rainfall during March influenced treatments that were applied at the end of March. Flushing incidence in the untreated trees (0 ppm treatment) increased to 50% and persisted until July, thus reducing flowering considerably (Figure 2a). For the control trees, initial flush growth prior to their treatment with ethephon was recorded at 66%, where after it was drastically reduced by the spot spray application. During April, some flush growth (<10%) occurred for the control treatment but was successfully removed by another two spot spray applications, which consequently allowed for almost 90% flowering. Both full cover ethephon applications to dormant trees successfully inhibited new flush growth almost entirely throughout the pre-flowering and flowering period and promoted flowering of up to 94% (Figure 2a). Treatments applied at the end of April, were affected by the decreasing temperatures. Untreated trees induced flowers instead of flushes due to the conducive temperatures and achieved a flowering rate of 70% (Figure 2b). Full cover ethephon applications not only inhibited flush growth during the induction period, but also prevented flower induction possibly due to an ethephon-temperature effect that inhibited bud break when temperatures were conducive for induction (Yang and Hoffman, 1984). As a result, delayed bud break caused vegetative flushing from July onwards, thereby reducing flowering to below 50% (Figure 2b). Ethylene is known for its stimulatory and inhibitory effects on vegetative and reproductive growth depending on concentration, growth conditions and developmental stage, and is species-dependant (Vandenbussche and Van Der Straeten, 2012). From the above results, ethylene appears to have similar effects in litchi.

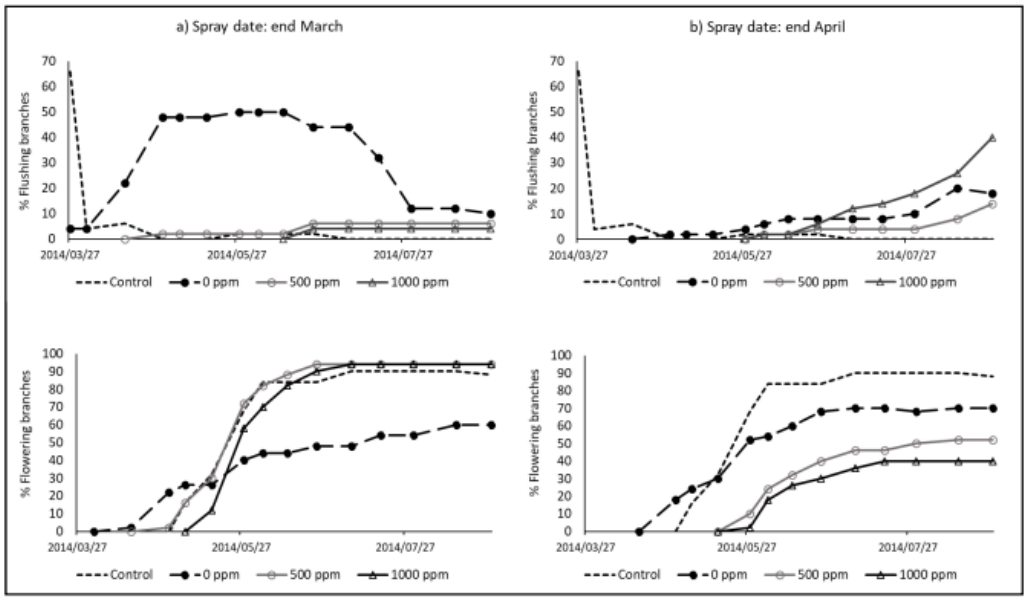

Figure 2. Percentage vegetative and reproductive growth in 'Mauritius' litchi trees when subjected to the treatments 0 ppm, 500 ppm and 1000 ppm in comparison with the control (spot sprays) for the application times at a) March 25 (left) and b) April 25, 2014 (right).

In 2015, lower rainfall in March reduced flush growth in the control treatment during the pre-flowering period compared with 2014, but higher temperatures caused more

continuous flushing during April as indicated by the wider peak in Figure 3a. Consistent high temperatures until the beginning of June, as well as above average rainfall during April and May (Figure 1) caused new flush growth for the control during June, thus reducing flowering compared with the other treatments. Shoot growth in the 0 ppm treatment remained low for the early application time probably due to a combination of low rainfall during March the applied water stress, but increased for the late application time due to climatic reasons as mentioned for the control. Both full cover ethephon applications successfully inhibited shoot growth almost entirely like in 2014, promoting almost 100% flowering (Figure 3a). For the late application time, full cover ethephon treatments could only inhibit new vegetative growth until end of May, but allowed some new flush in June (<10%) due to the high temperatures that persisted until the beginning of June (Figure 3b). Nevertheless, flowering in both treatments still reached up to 98%. Flush growth in the untreated trees reached almost 20% due to above-mentioned climatic conditions.

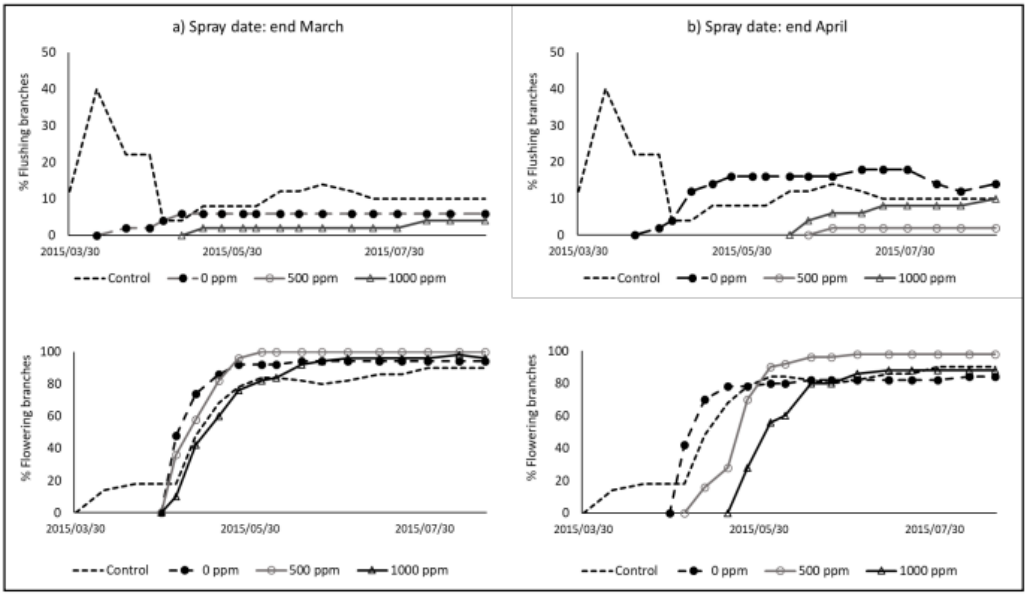

Figure 3. Percentage vegetative and reproductive growth in 'Mauritius' litchi trees when subjected to the treatments 0, 500 and 1000 ppm in comparison with the control (spot sprays) for the application times at: a) March 30 (left) and b) April 28, 2015 (right).

It should be noted that in both years flower panicle emergence in the control occurred earlier and under less inductive temperatures compared with the other ethephon treatments thus causing more leafy panicles (data not shown).

Effect of various shoot control strategies on carbohydrate reserves

When samples were analysed for all major soluble sugars and sugar alcohols, the metabolites glucose, fructose, inositol and bornesitol were found in very small quantities with no distinct differences between treatments (data not shown). Therefore, the focus was placed on sucrose, quebrachitol, starch, and raffinose (the latter for its role as osmoprotectant). Overall, the most abundant carbohydrate present in the small branches was starch. Sucrose and quebrachitol accounted for more than 50 and 35% of the total sugars, respectively.

In 2014 for the early application time, shoot growth in May in the untreated trees caused a decrease in sucrose levels. In contrast, inhibition of shoot growth by the ethephon applications, in particular the 500 and 1000 ppm treatments, caused a significant accumulation of sucrose during the flower induction period (May) (Figures 2a and 3a). The

untreated trees only showed a delayed increase in sucrose levels in June, however, this spike was unable to promote flowering in this treatment (Figure 2a). Flower development reduced sucrose levels, especially for the full cover treatments, which also exhibited the highest flowering rates. For the late application time, the full cover treatments also had higher sucrose levels during the flower induction period compared with the untreated trees. However, none of the treatments exhibited the same fluctuations as observed for the early application time, due to reduced shoot response because of lower temperatures at the time of application and soon afterwards (Figure 4b). Besides the inhibition effect of ethephon on bud growth, low temperatures and photo inhibition also most likely inhibited photosynthesis and thus the export of sucrose to storage organs (Rosa et al., 2009). Quebrachitol levels remained fairly stable for all treatments and application times except for the June levels in the early application time, where the 0 and 1000 ppm treatment differed significantly. The decrease in quebrachitol levels in the 1000 ppm treatment occurred at the time of early flower panicle emergence and coincided with a steep decline in sucrose levels. This may confirm suggestions by Wu et al. (2018) that quebrachitol might be part of a unique carbon metabolic strategy that maintains osmolality under reduced sucrose conditions in actively growing tissue. Raffinose was found in very low concentrations and all treatments followed the same trends. There was a marked peak for both applications times during the June sampling interval (Figure 3a). Raffinose is known to accumulate during stress conditions such as chilling in order to provide protection from oxidative damage (Sami et al., 2016). The observed accumulation of raffinose during winter suggests a putative protective mechanism in trees exposed to lower winter temperatures during 2014. The significant difference in raffinose levels between the untreated and ethephon treated trees in the late application time, particularly in June (Figure 4b), may be because the untreated trees were already more actively growing at the time (as indicated by earlier flower panicle emergence (Figure 2b)), thus rendering them more sensitive to low temperatures. Starch levels for the early application time for the control were significantly higher for April and May compared with the other treatments most likely because of their advanced phenological growth cycle having been able to accumulate carbohydrates before the new flush appeared. However, thereafter shoot growth prior to flowering in the control treatment affected the starch level, indicating the high demand for carbohydrates by developing vegetative shoots (Figure 4a). Nevertheless, starch accumulation occurred again after successful shoot control. Starch accumulation in the full cover ethephon treatments occurred continuously until flowering and was higher for the 1000 ppm than for the 500 ppm treatment. Starch accumulation in the untreated trees was the lowest until the June sampling time due to strong flush growth, but thereafter increased to the same levels as for the other treatments in September. For the late application time, no and only slight starch accumulation took place until June in the full cover treatments and untreated trees, respectively, most likely due to the effect of low temperatures and photo inhibition on photosynthesis (Figures 1 and 4b).

In 2015, less differences in shoot and flower development between treatments caused less variation in sucrose, quebrachitol, raffinose and starch levels when compared with 2014 (Figures 3 and 5). For the early application time, trends in sucrose fluctuations were similar for all treatments. However, the 1000 ppm treatment accumulated considerably more sucrose in April and May compared with the other treatments (Figure 5a). For the late application time, above average rainfall and temperatures in April and May caused flush growth during May and June, which caused a decline in sucrose levels in the untreated trees. The limited flush growth in the control did not have any effect on its sucrose levels (Figure 4b). Full cover ethephon applications inhibited any shoot growth during the flower induction period thereby promoting sucrose accumulation (Figure 5b). No significant differences for quebrachitol and raffinose levels between treatments and application times in 2015 were observed. There was no starch accumulation in any of the treatments applied at the early application time probably due to the suppressive effect of high temperatures and late summer drought conditions on photosynthesis (Figure 5a). In contrast to the early application time of 2015 and the late application time of 2014, starch accumulated in all treatments applied at the late application time in 2015 due to more favourable climatic conditions for photosynthesis (rainfall in April

and May and high temperatures; Figure 1).

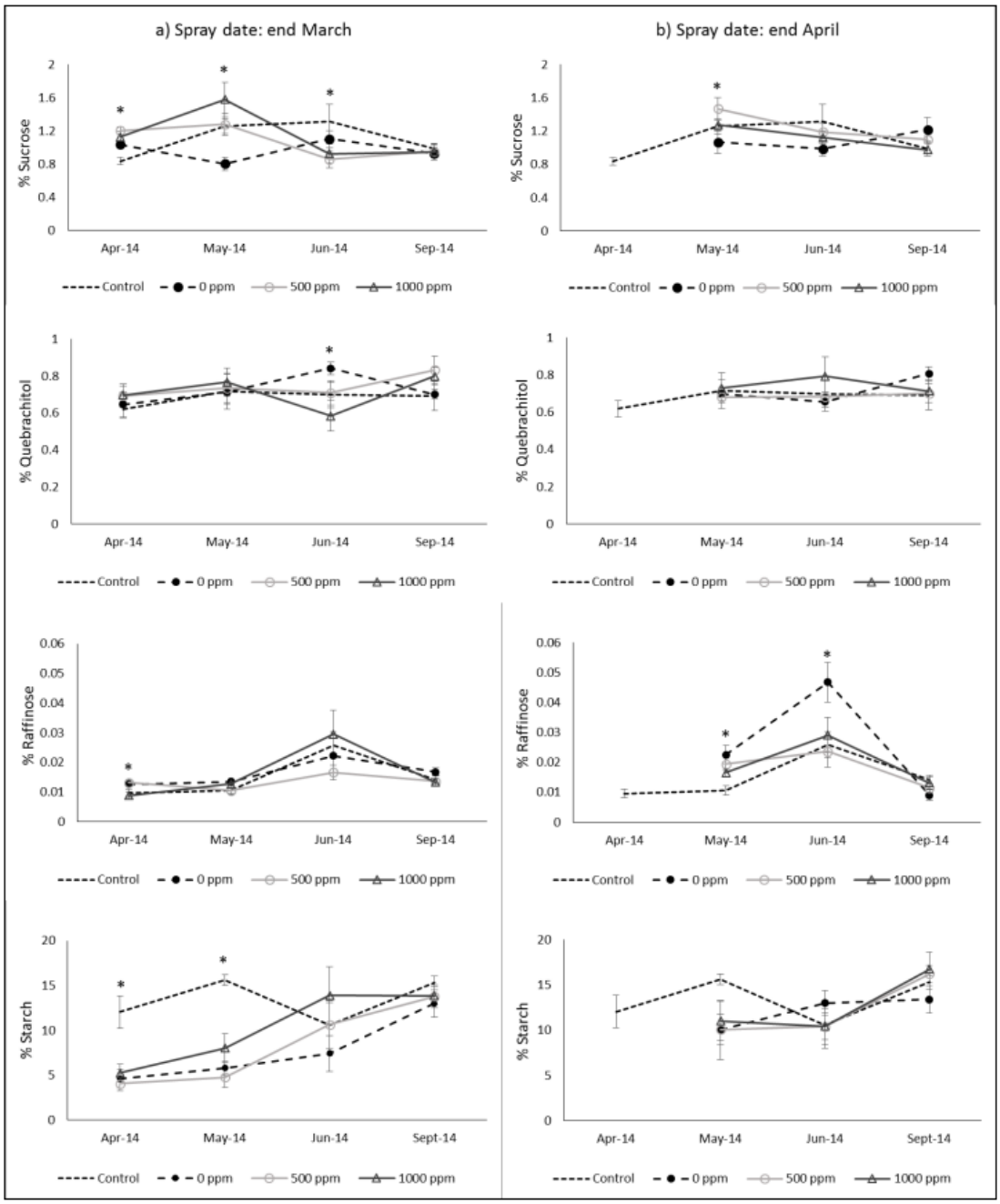

Figure 4. Changes in % sucrose, quebrachitol, raffinose and starch levels of small branches between treatment application and flowering for early (a) and late (b) application times in 2014. Bars indicate value means (n=5) ± standard error. Asterisks above data points denote significant differences between treatments according to Fisher's t-test ($P<0.05$).

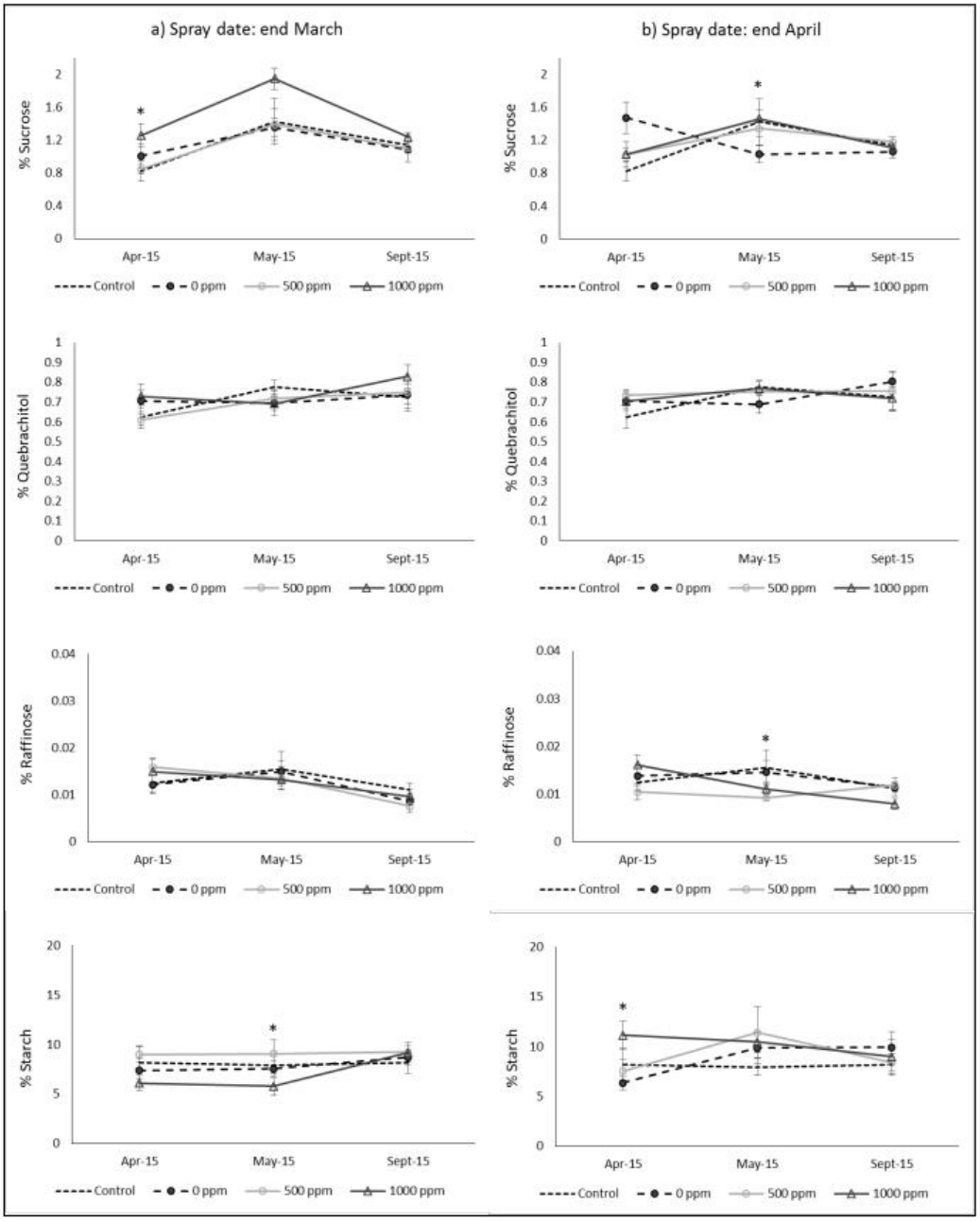

Figure 5. Changes in % sucrose, quebrachitol, raffinose and starch levels of small branches between treatment application and flowering for early (a) and late (b) application times in 2015. Bars indicate value means (n=5) ± standard error. Asterisks above data points denote significant differences between treatments according to Fisher's t-test (P<0.05).

Relationship between carbohydrate reserves and flowering

The relationship between carbohydrate reserves and flowering was studied by means of correlations. A significant positive correlation between sucrose in May and flowering rate was found in 2014 (r^2=0.52473, P=0.0254). In both years, a significant positive correlation between sucrose in May and days to panicle emergence was found (2014: r^2=0.53375,

P=0.0225; 2015: r^2=0.60411, P=0.0171). No significant correlations between starch and flowering were found in this study. Principal component analysis (PCA) was preformed per year and revealed a close association between the percentage sucrose (April, May) and starch (April, May, June) for the 500 and 1000 ppm full cover ethephon applications and flowering (Figure 6). The control treatment was closely associated with starch (April, May) and flowering in 2014, but not in 2015 due to climate effects. The 0 ppm treatment was associated with raffinose, which may indicate that untreated trees are more sensitive to chill temperatures under low temperature conditions and advanced flower development compared with ethephon treated trees. In contrast, this may suggest that ethephon treatment increased the responsiveness to chilling and in combination with higher carbohydrates reserves increased flower induction under less inductive temperatures as could be seen in 2015.

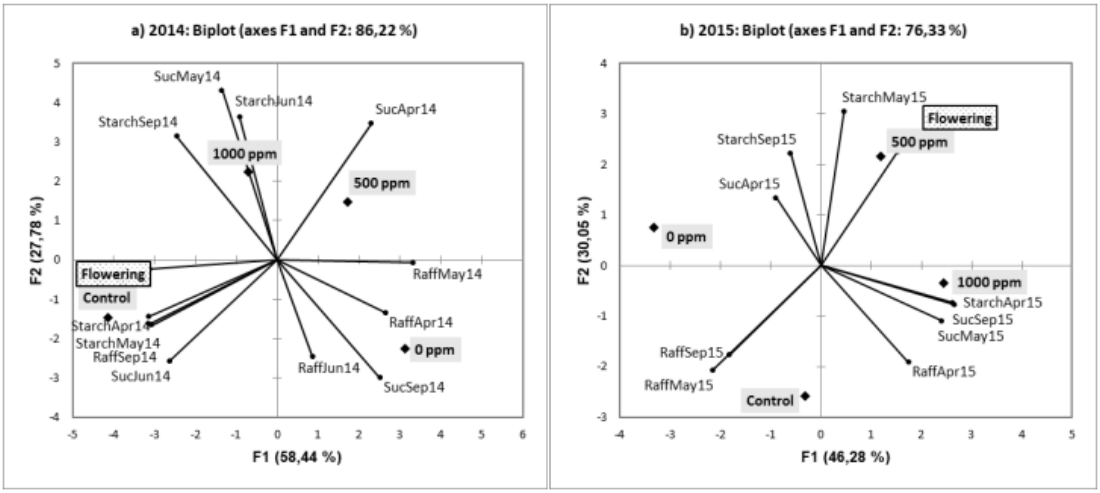

Figure 6. Principle component analysis between flowering, sucrose, raffinose, starch and treatments for both spray dates combined for: a) 2014, b) 2015.

CONCLUSION

The current study investigated the effect of various shoot control strategies on changes in carbohydrate reserves of small branches, also with respect to phenological growth stage and climatic conditions prior to flowering. The results revealed that both vegetative and reproductive growth had a direct influence on carbohydrate reserve dynamics. Full cover ethephon application to fully mature shoots with dormant buds reduced pre-flowering shoot growth most effectively, thereby promoting carbohydrate accumulation, in particular of sucrose and starch, and flowering. Principle component analysis was able to establish an association between carbohydrate accumulation (sucrose, starch) in full cover ethephon applications and flowering. After ethephon uptake and degradation in the plant, the released ethylene appears to arrest bud growth for a certain amount of time, depending on concentration and temperature, which makes this treatment a valuable tool to mitigate the effects of climate change. The current practice of spot spray application to remove already existing young flush remains an option for shoot control but it allows earlier panicle emergence under less inductive conditions and thus development of leafy flower panicles which are less fruitful. Future research will focus on elucidating the interactions between ethylene, carbohydrate reserves and gene expression of dormancy and flowering related genes.

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APPENDIX III

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Foliar application of ethephon induces bud dormancy and affects gene expression of dormancy- and flowering-related genes in 'Mauritius' litchi (*Litchi chinensis* Sonn.)

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ABSTRACT

A previous study showed that foliar application of ethephon to litchi trees with mature shoots and dormant terminal buds during autumn successfully inhibited new vegetative shoot growth prior to floral induction thereby promoting carbohydrate accumulation and flowering. However, the functional mechanisms of ethylene, the breakdown product of ethephon, in the leaves and terminal buds of litchi and its involvement in the flowering process is largely unknown. Therefore, this study aimed to investigate the phenological, physiological and molecular changes underlying ethephon application and its associations with bud dormancy and flowering in litchi. Ethephon was applied as a single full canopy spray at a concentration of 1000 mg·L^{-1} to 'Mauritius' litchi trees with mature vegetative shoots and dormant terminal buds during late autumn of 2018 (mid-April; Southern Hemisphere). Untreated trees served as a control. Phenological characteristics, such as bud dormancy and panicle development, leaf chlorophyll (as an indicator of shoot maturity), ethylene evolution, gene expression levels of flowering- (*LcFT2*, *LcFLC* and *LcAP1*), dormancy- (*LcSVP1* and *LcSVP2*) and ethylene pathway-related (*LcEIN3*) genes and non-structural carbohydrates were determined in terminal buds, leaves and/or shoots. Ethephon application induced bud dormancy, significantly delayed panicle emergence and promoted pure floral panicle development under more favorable inductive conditions. Ethylene evolution increased sharply 2 h after application in both leaves and terminal buds, but decreased rapidly thereafter in the leaves, while remaining high in terminal buds for seven days before gradually declining. Ethephon application significantly increased relative expression of *LcEIN3* and *LcFLC* in terminal buds one day after application, while *LcFT2* expression in leaves and *LcAP1* expression in terminal buds were significantly increased at the bud break stage. Significant treatments differences were also observed for various carbohydrate metabolites in leaves and shoots at the bud break or floral initiation stage. Our study provided evidence that ethephon application plays an important role in the physiological and molecular regulation of bud dormancy of litchi. By influencing the time of bud break, ethephon application can be a useful tool to manage panicle emergence under less inductive conditions.

1. Introduction

Ethephon (2-chloroethylphosphonic acid) has been widely used in agriculture for different applications, such as fruit ripening, flower stimulation and organ abscission (Ju and Chang, 2015). After absorption by the plant tissue, or in the presence of a base, ethephon rapidly degrades to ethylene, phosphate and chloride (Domir and Foy, 1978; Hartley and Kidd, 1983; Warner and Leopold, 1969). By binding to specific ethylene receptors in the membrane of the endoplasmic reticulum, ethylene then influences downstream gene expression of various transcription factors, such as *ETHYLENE INSENSITIVE3* (*EIN3*), which eventually leads to ethylene responses specific to its exogenous or

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endogenous triggers (Bleecker and Kende, 2000; Dubois et al., 2018; Ju and Chang, 2015; Wang et al., 2013). Ethylene is involved in the regulation of various aspects of plant growth and development, such as seed germination, inhibition of cell division in meristems, organ abscission, fruit ripening, as well as stress responses and stress tolerance under suboptimal conditions (Burg, 1973; Dubois et al., 2018; Iqbal et al., 2013; Olsen, 2010; Wang et al., 2013).

In South Africa, ethephon is commercially applied to litchi trees as repeated foliar spot sprays to burn off any young vegetative shoots that emerge during autumn and early winter, thereby preventing these new shoots from impeding floral development (Roets et al., 2010). Recent studies suggested that a single full canopy application of ethephon to trees with fully mature shoots and dormant terminal buds can inhibit any new shoot growth occurring during periods of high temperature in autumn and winter, and was more effective than the frequent spot spray applications (Cronje and Ratlapane, 2017, 2018). In these studies, ethephon not only prolonged bud dormancy and delayed panicle emergence to a period with more consistent low temperatures, but also promoted tree carbohydrate accumulation, thereby increasing flowering rate and yield (Cronje and Ratlapane, 2017, 2018). While ethylene, in the form of a commercial ethephon application, directly induced flowering in pineapple (Bartholomew, 1977; Maruthasalam et al., 2010), the exact functional mechanisms of ethylene in the litchi flowering processes are unknown, despite its evident role in flowering alluded to in previous studies (Cronje and Ratlapane, 2017, 2018). Moreover, ethylene evolution and endogenous ethylene regulation have been closely linked to environmental cues, in particular temperature (Galvão and Schmid, 2014; Olien, 1976), which also plays an essential role in litchi floral formation (Batten and McConchie, 1995; Chen and Huang, 2005).

Successful flower induction in litchi is highly dependent on trees being exposed to sufficiently low temperatures as well as on the maturity status of terminal shoots (Batten and McConchie, 1995; Chen and Huang, 2005; Davenport and Stern, 2005; O'Hare, 2002). The signal for flowering, i.e. low temperature in the case of litchi, is perceived in the leaves and is transmitted in the vascular tissue to the shoot apical meristems through a series of signal transduction mechanisms and complex regulatory networks. These regulate the expression of a set of flowering-related genes in the leaves and shoot apical meristems, such as *FLOWERING LOCUS T* (*FT*), *CONSTANS* (*CO*), *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *APETALA1* (*AP1*), while suppressing flowering repressor genes, such as *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*) to prevent floral reversion (Galvão and Schmid, 2014; Hu et al., 2018; Lu et al., 2017; Yang et al., 2014; Zhang et al., 2014).

The maturity status of the vegetative shoots is critical for the chilling responsiveness of crops like litchi and mango (Wilke et al., 2008; Zhang et al., 2016). Since the breaking buds are most responsive to low inductive temperatures in litchi, there is only a narrow window for floral induction and initiation (Batten and McConchie, 1995; Huang and Chen, 2005; Zhang et al., 2016). Thus, preventing untimely vegetative shoot growth prior to floral induction, as well as enabling a sufficient dormancy period is crucial. In deciduous crops, such as peach, pear and apricot, dormancy was found to be regulated by *SVP* genes and the related *DORMANCY ASSOCIATED MADS-BOX* (*DAM*) genes (Zhang et al., 2016). Similarly in litchi, *LcSVP1* and *LcSVP2* genes were correlated with growth cessation and dormancy maintenance, while *LcSVP3* was associated with bud break and growth of vegetative shoots (Zhang et al., 2016). Whereas under floral inductive conditions, all three *LcSVP*'s declined to low levels during floral initiation and panicle development (Hu et al., 2018; Lu et al., 2017).

Apart from ensuring shoot maturity and bud growth at an opportune time, the dormancy period and associated growth check is essential for the accumulation of carbohydrate reserves. Since flowering is a carbon costly process, carbohydrate accumulation during the winter dormancy period is important for successful flowering in litchi (Huang and Chen, 2014). Carbohydrates, in particular soluble sugars, are known to play an important role in signal transduction, and control internal regulators and environmental cues that govern growth (Rolland et al., 2002). Yang et al. (2014) showed that leaves of mature litchi shoots had higher carbohydrate levels, as well as higher gene expression levels of the floral promoters *LcCO* and *LcFT2* and flowering rates, compared with leaves of immature litchi shoots. Various other studies confirmed that carbohydrate reserves increased in the main storage organs of flowering litchi trees during the autumn dormancy period, thus confirming their important role in litchi flowering (Cronje and Mostert, 2009; Cronje et al., 2020; Menzel et al., 1995; Yang et al., 2014). Moreover, Huang and Chen (2014) postulated that sufficient carbohydrate accumulation prior to flower induction might increase the responsiveness of litchi to chill temperatures, which may reduce the chilling period required for flower induction, thus promoting floral formation. Horticultural practices that control vegetative growth prior to floral induction and promote carbohydrate accumulation include the use of ethephon to remove or inhibit new vegetative growth, water stress and girdling (Huang and Chen, 2014; Menzel et al., 1995; Roets et al., 2010; Wilke et al., 2008).

Results from previous reports on the growth inhibiting and possibly flowering promoting effects of ethephon (Cronje and Ratlapane, 2017, 2018; Cronje et al., 2020), as well as studies exploring the flowering mechanisms in litchi (Chen et al., 2016; Hu et al., 2018; Huang and Chen, 2005; Lu et al., 2017, 2020; Shen et al., 2016; Yang et al., 2014; Zhang et al., 2014), suggest that there may be a link between the ethylene and flowering pathways via dormancy regulation. To gain more insight into the regulatory mechanisms of bud dormancy and flowering in litchi as induced by ethephon application, the aim of this study was to investigate the effect of foliar-applied ethephon on physiological and molecular changes in the leaves and shoot apical meristems of 'Mauritius' litchi.

2. Materials and methods

2.1. Experimental site and plant material

The experimental site was located on a commercial farm near Mbombela in the Mpumalanga Province of South Africa (latitude: -25.453, longitude: 30.945; elevation: 680 m; subtropical climate with moderate autumns and cool winters).

All experiments were conducted on 18-year old air-layered 'Mauritius' litchi trees. The trees were planted on sandy soil (84% sand, 13% clay, and 3% silt) at a planting distance of 6.0 × 6.5 m. All experimental trees were selected for uniformity of tree size and phenological growth stage, and were subjected to standard cultural farming practices with regard to fertilization, irrigation (micro irrigation) and pruning.

Weather data for the experimental site was obtained from a nearby weather station. Chilling degree hours (CDHs) were calculated according to the formula provided by Chen et al. (2016) using 20 °C as the base temperature for 'Mauritius', as determined by Batten and McConchie (1995), and incorporating 12-h day and 12-h night mean temperatures instead of hourly temperatures.

2.2. Treatments and experimental design

Ethephon (Ethapon SL; Plaaskem (Pty) Ltd, Witfield, South Africa; 480 g·L^{-1} a.i.) was applied on April 12, 2018 at a concentration of 1000 mg·L^{-1} as a single full canopy spray to the point of run-off to trees with fully hardened, mature shoots and dormant terminal buds, using a motorized sprayer equipped with hand lances and a 2.5 mm nozzle, at a pressure of 15 bar. A wetting agent (Sanawett 90-940 SL; Dow AgroScience (Pty) Ltd, Bryanston, South Africa; 940 g·L^{-1} a.i.) was added at 10 mL·100 L^{-1} water. Untreated trees served as a control.

The experiment was laid out in a randomized complete block design with two treatments and five block replicates (i.e. five experimental

units per treatment). Each experimental unit consisted of five trees and was separated from other experimental units by buffer trees.

2.3. Phenological observations

Six representative terminal shoots with fully mature leaves were selected per tree at a height of approximately 1.5–1.8 m above soil level and tagged. Tree responses between spray application and anthesis were monitored at weekly or bi-weekly intervals. Developmental stages for bud and flower development were identified as described by Wei et al. (2013) and the time of their appearance recorded accordingly. The most critical developmental stages considered for this study were the pre-induction dormancy period, the bud break stage, the floral initiation stage (i.e. panicle emergence), as well as anthesis. Dormant buds (Fig. 1A) were characterized by brown coloured rudimentary leaves tightly enclosing the growing point, while breaking buds (Fig. 1B) were greenish and slightly swollen (Zhang et al., 2016). The floral initiation stage (Fig. 1C), also known as the "white millet" stage, was defined as inflorescence primordia becoming visible in the form of millet-like whitish hairy buds appearing in the leaf axils (Huang and Chen, 2005). The interval between application and bud break or floral initiation stage was calculated as the number of days between application and the visible appearance of each stage. The days to bud break (DTBB) and days to floral initiation stage (DTFI) served as demarcation points for the duration of the dormancy period and defined floral initiation, respectively. The proportion of vegetative shoots during floral induction (May), flowering shoots and leafy panicles was calculated from the number of tagged shoots. At anthesis, the number of panicles per shoot (including those arising from axillary buds) was counted and the length of the longest panicle per shoot was measured. The number of fruit per panicle was recorded at fruit set and at harvest.

2.4. Leaf chlorophyll measurements

One leaflet from the compound leaf closest to the terminal bud of each tagged shoot was used to measure the Chlorophyll Content Index (CCI) at the dormancy, bud break and floral initiation stage as well as at anthesis using a portable chlorophyll meter (CCM-200, Opti-Sciences) (Fu et al., 2014).

2.5. Determination of ethylene evolution

The protocol for the quantification of ethylene evolution in tomato leaves developed by Kim et al. (2016) was adapted for litchi. For each sample, two mature leaflets (one leaflet each from the compound leaf closest to the terminal bud from two shoots) and up to 20 terminal buds (4–8 mm in length, depending on bud stage and size) (Fig. 1), respectively, were randomly selected from all trees within a replicate 24 h prior to treatment, 2 h after treatment and over a 70-day period following treatment. Two sub-samples per replicate were collected. The harvested leaflets and buds were inserted into 20 mL and 2 mL glass vials, respectively, and allowed to equilibrate for 10 min to release possible wound ethylene before incubating the samples for 3 h at ambient temperature. Volumes aspirated were 1 mL and 0.5 mL from the 20 mL and 2 mL vials, respectively. All samples were analyzed using a gas chromatograph (Trace GC Ultra, Thermo Scientific, USA) with flame ionization detection and equipped with a TG-BOND Q+ column. The analyses were performed at 60 °C with helium as carrier gas at a flow rate of 3.5 mL·min^{-1}. Quantification was achieved based on a standard curve derived from an external standard gas calibration with certified ethylene gas.

2.6. Leaf and shoot carbohydrate analysis

Sixteen leaflets (one leaflet from the compound leaf closest to the terminal bud per sampled shoot) and mid-stem sections of 12 terminal shoots (xylem and phloem; ~50 mm in length) were randomly selected from all trees within a replicate and split in two sub-samples per replicate. The samples were collected one day after application (representing the dormancy stage), at the bud break and floral initiation stage, as well as at anthesis. After collection, the leaves were washed with tap water. Both leaf and shoot samples were oven-dried at 60 °C and thereafter ground to a fine powder using a laboratory grinder fitted with a 0.5 mm mesh (Fritsch, Germany).

Soluble sugars were extracted according to the method described by Chow and Landhäusser (2004), with slight modifications. The samples were analyzed using an HP 1100 LC system (Agilent Technologies, California) with refractive index detection and equipped with a Rezex RCM Monosaccharide Ca^{+2} column (Phenomenex, California). The analyses were performed on 20 µL injection volume with deionized water as eluent, at a flow rate of 0.5 mL·min^{-1}. The column temperature was kept at 80 °C. Quantification was achieved based on external standard solution calibration.

Leaf and shoot starch were extracted and analyzed according to the iodine colorimetric method as described by Xu et al. (1998). The starch content was determined from the changes in absorbance of the starch-iodine complex at 620 nm using a UV–Vis spectrophotometer (Shimadzu UV-1800, Japan). The concentration of starch was calculated based on a standard curve derived from an external standard calibration with analytical grade soluble starch.

2.7. RNA sample collection and extraction

Leaf sections of eight mature leaflets from the compound leaf closest to the terminal bud (~10 mm^2, avoiding the mid rib), and 30 to 40 terminal buds (depending on bud size) were randomly collected from all

Fig. 1. Morphology of terminal buds at different developmental stages between dormancy and floral initiation in 'Mauritius' litchi. Subfigures specify the developmental stages used for sample collection for ethylene and gene expression analysis: (A) dormant bud stage, (B) bud break stage, and (C) floral initiation stage.

trees within a replicate, at two days before application, one day after application, during the dormancy stage (21 days after application), as well as at the bud break and floral initiation stage (Fig. 1). On collection, all samples were frozen in liquid nitrogen and stored at -80 °C until further processing. Samples collected prior to treatment application were pooled for each replicate. Immediately prior to analysis, the leaf and bud samples were finely ground in liquid nitrogen, where after total RNA was extracted using an RNAprep Pure Kit (Tiangen, China) according to the manufacturer's instructions. The RNA samples were treated with RNase-free DNase I (Tiangen, China) to remove genomic DNA. In addition, for bud samples, 2% PVP-40 (w/v) was added to the SL buffer of the RNA kit in the first clean up step to remove contaminating polyphenols.

The RNA integrity was assessed using 1% (w/v) agarose gel electrophoresis (80 V for 20 min). The quantity of the isolated RNA was measured using an ultraviolet spectrophotometer (NanoDrop UV–Vis, Thermo Scientific, USA). Only RNA samples with a 260/280 nm ratio of higher than 1.8 were used for subsequent analyses.

2.8. cDNA synthesis and generation of standard curves

First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SensiFast cDNA synthesis kit (Meridian Bioscience, UK) in a final volume of 20 µL, following the manufacturer's instructions. The undiluted cDNA samples were stored at -20 °C until further use. Prior to analysis, the cDNA samples were diluted 50 times. For the generation of standard curves of all reference and target genes, 1 µL of each cDNA sample was pooled and a five-fold dilution series was prepared from the cDNA sample mix.

2.9. Target and reference gene selection

The genes of interest included the dormancy genes *LcSVP1* and *LcSVP2*, the ethylene pathway gene *LcEIN3* and the floral genes *LcFLC*, *LcFT2* and *LcAP1*. Primer sequences for the studied reference and target genes are displayed in Supplementary Table 1. The primers were synthesized by Integrated DNA Technologies (IDT; Whitehead Scientific, South Africa). The *LcActin* and *LcGAPDH* genes were selected as reference genes due to their stability in qRT-PCR analyses across different tissue types, developmental stages, and treatments in litchi (Zhong et al., 2011). The stability of reference gene expression in leaf and bud samples was assessed using the Excel-based application BestKeeper (Pfaffl et al., 2004).

2.10. Gene expression analysis with qRT-PCR

Quantitative RT-PCR analysis was performed on a Rotor Gene Q instrument (Qiagen, Germany) with three technical replicates for each biological replicate, using the SensiFast SYBR No-Rox kit (Meridian Bioscience, UK) in a final volume of 15 µL, following the manufacturer's instructions. The analyses were run with the following program: hot start at 95 °C for 2 min, followed by 45 cycles of 10 s at 95 °C, 10 s at 54–58 °C (depending on primer annealing temperature), and 15 s at 72 °C. The Rotor-gene Q software 2.3.1 (Qiagen) was utilized to calculate PCR efficiency, quantification cycle (Cq)-, and quantification values for all samples.

2.11. Statistical analysis

All data were subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Data collected in a repeated measures design was subjected to repeated measures analysis of SAS software with treatment, developmental stage and treatment × developmental stage as fixed factors. The Shapiro-Wilk and Levene's tests confirmed normality of the data and homogeneity of variances, respectively. Bartlett's test for homogeneity of variances was used where outliers had to be removed, to ensure consistent reliability of ANOVA data. Where ANOVA indicated significant treatment effects at $P < 0.05$, Fisher's Least Significant Difference (LSD) test was performed at a 5% significance level to compare treatment means for main effects and interactions. Pearson's product-moment correlations were performed separately for each studied plant organ (leaves, buds, shoots) to identify significant relationships ($r > 0.5$) between dormancy- and carbohydrate-related parameters, respectively, and the relative expression levels of each gene for a given developmental stage, using the correlation procedure (PROC CORR) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA) at a 5% significance level, unless indicated otherwise. Principal component analysis (PCA) was performed to investigate the relationship between treatments and the different variables using XLSTAT (Version, 2015.1.03.15485, Paris).

3. Results

3.1. Ethylene evolution in leaves and terminal buds following ethephon application

Minimal ethylene was detected from leaves and terminal buds prior to the application of ethephon (Fig. 2). However, 2 h following the ethephon treatment, ethylene evolution rapidly increased to 111 $\mu L \cdot kg^{-1} \cdot hr^{-1}$ in treated leaves, where after it dropped sharply to almost one-tenth of the initial peak within 7 days after application. Ethylene evolution from treated terminal buds peaked at 64 $\mu L \cdot kg^{-1} \cdot hr^{-1}$ 2 h after treatment, and remained elevated for a period of 7 days after application, where after it deceased gradually. Ethylene evolution from treated leaves dropped to below 5.0 $\mu L \cdot kg^{-1} \cdot hr^{-1}$ from day 35 onwards, whereas in the treated buds, ethylene evolution only decreased to below the 5.0 $\mu L \cdot kg^{-1} \cdot hr^{-1}$ threshold level after 63 days. Ethylene levels for untreated leaves and terminal buds stayed below 1.4 and 3.0 $\mu L \cdot kg^{-1} \cdot hr^{-1}$, respectively, throughout the observation period (Fig. 2).

Temperature appeared to have an effect on ethylene evolution in treated buds, particularly between day 2 and day 7 and again around day 42, showing slight increases in ethylene release as temperatures increased and vice versa (Fig. 2). Pearson's correlations performed on the data between day 2 and 42 revealed a significant positive correlation between temperature and ethylene release from treated buds ($r = 0.82448$, $p = 0.0225$), whereas no significant correlation could be found

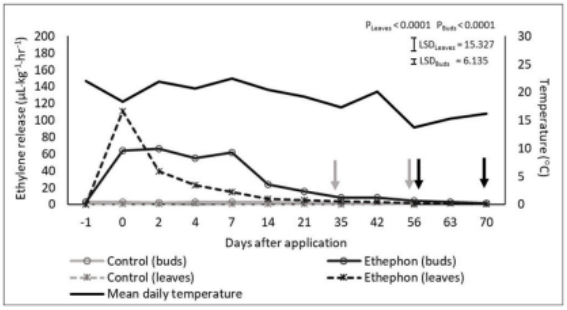

Fig. 2. Ethephon application increased ethylene evolution in leaves and terminal buds of 'Mauritius' litchi. Data was collected one day prior to application and over a 70-day period following ethephon treatment at 1000 $mg \cdot L^{-1}$ (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Detached leaves and terminal buds were incubated for 3 h prior to analysis with gas chromatography. Grey arrows indicate the bud break (day 33) and floral initiation stage (day 56) of the control trees. Black arrows indicate the bud break (day 56) and floral initiation stage (day 70) of the ethephon treated trees. Data points are mean values (n = 10). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$), for leaf and bud data individually. Statistical significance of treatment x time interaction is indicated as LSD bar according to Fisher's LSD test ($p = 0.05$).

for treated leaves ($r = 0.63404$, $p = 0.1262$) during the same period.

3.2. Treatment effect on tree phenology

Ethephon treatment increased the number of days to bud break, as well as delayed panicle emergence (floral initiation stage) by 22 days compared with the control (Table 1). However, the time between bud break and the floral initiation stage was 5 days shorter for treated trees compared with control trees. Furthermore, the accumulated chilling degree hours (CDHs) were higher for treated trees than for control trees due to the delayed bud break. This resulted in an increase of 1374 and 2057 accumulated CDHs for treated trees at the bud break and floral initiation stage, respectively, compared with untreated trees (Table 1). The application of ethephon influenced various phenological parameters compared with the control (Table 2). No vegetative shoot growth occurred in ethephon treated trees during floral induction compared with the 13.3% flushing incidence in the control trees. The flowering rate was high for both treatments with no statistical differences between them. The percentage leafy panicles was significantly decreased, while the number of flower panicles per shoot was significantly increased by the ethephon treatment. Ethephon application reduced panicle length and increased the initial and final number of fruit set per panicle, albeit not significantly (Table 2). Furthermore, the ethephon treatment was associated with higher ($P > 0.05$) leaf chlorophyll content at the bud break stage compared with the untreated control (Fig. 3), while leaf chlorophyll content at dormancy, floral initiation and anthesis was similar for both treatments.

3.3. Leaf and shoot carbohydrate concentrations

Changes in starch and soluble sugar concentrations were observed in response to ethephon application as well as over time (Fig. 4). Both leaf and shoot sucrose concentrations were significantly increased in ethephon treated trees at bud break, but were significantly lower at the floral initiation stage compared with the control trees (Fig. 4A and E). Glucose concentrations in leaves of treated trees were significantly higher at bud break and significantly lower at the floral initiation stage compared with the control, while the treatment effect was inverse in the shoots (Fig. 4B and F). Fructose concentrations in both leaves and shoots were below the level of detection and are therefore not presented. Quebrachitol was found in similar high concentrations as sucrose in both leaves and shoots (Fig. 4C and G). Quebrachitol concentrations in leaves did not differ between treatments, but were significantly lower in shoots of ethephon treated trees at bud break and the floral initiation stage compared with the control treatment. Starch concentrations in the leaves of both treatments fluctuated throughout the trial period and were considerably lower than in the shoots (Fig. 4D and H). In the leaves, starch concentrations of ethephon treated trees were significantly increased at the floral initiation stage and significantly decreased at anthesis compared with the control trees (Fig. 4D). In the shoots, starch concentrations increased over time in both treatments, but no significant treatment differences were found (Fig. 4H).

3.4. Expression levels of ethylene pathway and dormancy-related genes

Gene expression levels of *LcEIN3* were generally lower in leaves compared with terminal buds (Fig. 5A and D). Ethephon application did not influence *LcEIN3* expression levels in the leaves, however, it significantly increased *LcEIN3* expression in terminal buds one day after application and at dormancy, where after it declined to the same low level recorded for the control.

Expression levels of *LcSVP1* in the leaves were increased at dormancy and again at the floral initiation stage but no significant differences between treatments or interactions were observed (Fig. 5B). In terminal buds, *LcSVP1* showed similar expression levels for both treatments throughout the observation period, with a significant reduction in expression levels one day after application and at dormancy and a subsequent significant increase during floral formation (Fig. 5E). At bud break, expression levels of *LcSVP1* were significantly increased, while at the floral initiation stage they were significantly reduced in ethephon treated trees compared with the control trees (Fig. 5E). Relative gene expression of *LcSVP2* in leaves remained stable throughout the observation period, except at bud break where ethephon treated trees had lower ($P > 0.05$) *LcSVP2* expression levels compared with the control trees and when compared with the other developmental stages (Fig. 5C). In the terminal buds, *LcSVP2* expression levels decreased after application, similar to *LcSVP1*, and remained stable thereafter in both treatments, except for bud break where *LcSVP2* expression was increased in ethephon treated trees (Fig. 5F). However, no significant treatment × stage interactions were found.

3.5. Expression levels of flowering-related genes

Relative expression of the flowering antagonist *LcFLC* remained stable in leaves for both treatments throughout the observation period with a slight, but non-significant increase in treated trees at bud break (Fig. 6A). In terminal buds, however, *LcFLC* expression levels increased significantly in treated trees one day after application. Thereafter, *LcFLC* expression levels declined to the same level as the control, and no further treatment differences were observed (Fig. 6D).

Expression levels of *LcFT2* in leaves of both treated and untreated trees remained low between pre-application and dormancy (Fig. 6B). At bud break, *LcFT2* expression significantly increased in treated trees compared with the control, while at the floral initiation stage, *LcFT2* expression of both treatments reached the same level. In terminal buds, *LcFT2* expression levels of both treatments remained low until dormancy, but significantly increased at bud break and again at the floral initiation stage. However, no significant differences between treatments were observed (Fig. 6E).

Relative gene expression of *LcAP1* in leaves was stable throughout the observation period for both treatments with no significant treatment differences (Fig. 6C). In terminal buds, *LcAP1* expression levels before and one day after ethephon application were low, but gradually increased thereafter in both treatments and exhibited the similar *LcAP1* expression levels at the floral initiation stage (Fig. 6F). However, while the control trees displayed significantly higher *LcAP1* expression levels at dormancy, *LcAP1* expression levels in ethephon treated trees were significantly higher at bud break.

3.6. Associations between phenology, carbohydrates and gene expression

Pearson's correlations confirmed the growth inhibiting effects of

Table 1
Growth response time and accumulated chilling degree hours (CDHs) between treatment application and the bud break (BB) or floral initiation (FI) stage in response to ethephon treatment at 1000 mg·mL^{-1} (Ethephon) or no treatment (Control) of 'Mauritius' litchi trees with mature shoots and dormant terminal buds. Data are mean values ($n = 25$).

Treatment	Days to bud break stage (BB)	Days to floral initiation stage (FI)	Days between BB to FI	Accumulated CDHs to BB	Accumulated CDHs to FI	CDH difference between stages/ treatments
Control	31	53	22	2072	3446	1374
Ethephon	53	70	17	3446	5503	2057

Table 2
Phenological parameters for vegetative and reproductive growth in response to ethephon treatment at 1000 mg·mL^{-1} (Ethephon) or no treatment (Control) of 'Mauritius' litchi trees with mature shoots and dormant terminal buds. Data are mean values (n = 150). Statistical analysis of data was assessed with randomized block design ANOVA ($P < 0.05$). Different letters within columns indicate significant differences between treatments according to Fisher's LSD test ($p = 0.05$).

Treatment	Vegetative shoots during floral induction (%)	Flowering (%)	Leafy panicles (%)	Panicles/shoot (incl. axillary) (no.)	Panicle length (cm)	Fruit/panicle at fruit set (no.)	Fruit/panicle at harvest (no.)
Control	13.3 a	80.0 a	38.7 a	1.7 b	23.2 a	8.6 a	5.3 a
Ethephon	0.0 a	74.7 a	4.7 b	2.7 a	21.5 a	11.0 a	6.1 a
P value	*0.1324*	*0.5803*	*0.0017*	*0.0035*	*0.1388*	*0.0614*	*0.1524*

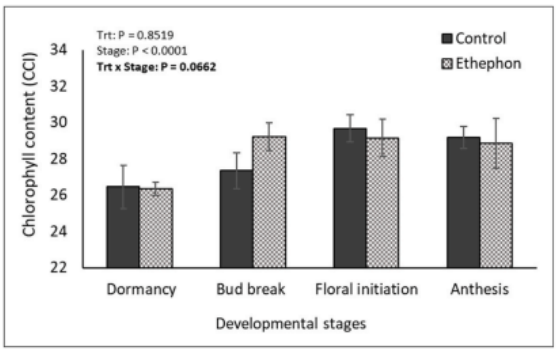

Fig. 3. Chlorophyll content of 'Mauritius' litchi leaves was increased at bud break in response to ethephon treatment. Leaf chlorophyll content from a leaflet of the compound leaf closest to the terminal bud was determined at the indicated developmental stages following ethephon treatment at 1000 mg·L^{-1} (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Columns indicate mean values ± SE (n = 150). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$).

ethephon one day after application by significant positive correlations between *LcEIN3* and *LcFLC* expression in buds ($r = 0.85219$; $p = 0.0312$), and between *LcEIN3* expression and the number of days to bud break (DTBB) ($r = 0.90229$, $p = 0.0139$). Expression levels of *LcEIN3* were also negatively correlated with flushing incidence at the dormancy stage ($r = -0.87720$; $p = 0.0507$). The influence of ethephon on bud dormancy was further highlighted by a positive correlation between *LcFLC* and *LcSVP2* expression in buds one day after application ($r = 0.79067$, $p = 0.0611$), albeit only significant at 10%. In addition, at dormancy, *LcSVP1* expression in buds was significantly negatively correlated with *LcFT2* and *LcAP1* expression in buds ($r = -0.79691$, $p = 0.0019$; $r = -0.75153$, $p = 0.0048$, respectively).

The role of dormancy in carbohydrate accumulation was established by significant positive correlations between *LcSVP1* expression in buds and leaf sucrose concentration at the dormancy stage ($r = 0.89314$, $p = 0.0165$), as well as between DTBB and sucrose concentration in leaves and shoots at bud break ($r = 0.87545$, $p = 0.0009$ and $r = 0.70281$, $p = 0.0234$, respectively). The importance of shoot maturity in carbohydrate accumulation was emphasized at the dormancy stage by a significant positive correlation between shoot sucrose and leaf chlorophyll content (as a shoot maturity indicator) ($r = 0.69163$, $p = 0.0267$). Furthermore, at bud break, leaf sucrose concentration was significantly positively correlated with leaf chlorophyll content ($r = 0.66274$, $p = 0.0368$). An association between carbohydrates and flowering was established at bud break by leaf and shoot sucrose concentrations being significantly positively correlated with *LcFT2* expression in leaves ($r = 0.69283$, $p = 0.0263$) and *LcAP1* expression in buds ($r = 0.78387$, $p = 0.0026$).

Processes governing flowering were identified at the floral initiation stage by a strong significant negative correlation between *LcAP1* and *LcSVP2* expression in buds ($r = -0.90010$, $p = 0.0145$), as well as by a significant positive correlation between *LcAP1* expression and flowering rate ($r = 0.66581$, $p = 0.0181$). Significantly positive correlations between *LcFT2* expression in leaves and *LcAP1* expression in buds ($r = 0.80049$, $p = 0.0054$) were only observed at bud break, but not at the floral initiation stage.

The principal component analysis (PCA) was limited to key developmental stages and variables for flower initiation. These included leaf chlorophyll content as an indicator of shoot maturity, shoot starch concentration as an indicator of reserve carbohydrates, leaf sucrose concentration as a signaling molecule, accumulated CDHs, DTBB and DTFI as factors relating to temperature influences and duration of dormancy, as well as the expression of certain genes involved in dormancy and flowering regulation. The principal components F1 and F2 explained 92.77% of the variation in the data (Fig. 7). The variables contributing the most to F1 were leaf chlorophyll content, leaf sucrose concentration, shoot starch concentration, *LcFT2* and *LcFLC*, while CDHs and DTBB contributed the most to F2. The variables in F1 and F2 can thus be considered as indicators for flowering and dormancy, respectively. Highly positive correlations between leaf chlorophyll content, leaf sucrose, shoot starch concentrations, *LcFT2* and *LcAP1* were found. These variables were also highly associated with the ethephon treatment at the bud break and floral initiation stage, whilst *LcEIN3* and *LcFLC* were associated with the ethephon treatment at the dormancy stage. Furthermore, DTBB, DTFI, and their corresponding accumulated CDHs were closely associated with the ethephon treatment. Flowering was associated with the control treatment at bud break, however, no association between the ethephon treatment and flowering was detected.

4. Discussion

4.1. Post-ethephon treatment ethylene release patterns, and associated temperature effects

Ethylene release revealed distinct pattern in leaves and terminal buds (Fig. 2). The high peak and subsequent rapid decline in ethylene evolution observed in the leaves immediately after ethephon application suggests that ethephon absorption by the leaves was limited, most likely due to the thick wax layer of the leaf cuticle. Therefore, ethylene evolution detected in leaves may have primarily been the result of a chemical breakdown reaction of ethephon on the leaf surface rather than ethylene release after absorption by the plant tissue itself (Domir and Foy, 1978; Hartley and Kidd, 1983). In contrast, ethephon appeared to have been absorbed by the bud tissue, where it subsequently degraded to ethylene as the active component over an extended period and could then exert its influence on a physiological and molecular level.

It was also observed that ethylene evolution followed similar variations as the mean daily temperatures recorded on the day of observation (Fig. 2). Olien (1976) reported that ethylene evolution from ethephon in cherry occurred at an exponential rate for temperatures between 10 °C and 34 °C, and was higher when temperatures changed from lower to higher temperatures as compared with a change from higher to lower temperature. Although not performed under controlled temperature conditions, our results confirmed similar trends. The effect of environmental conditions, such as ambient temperature in reference to ethephon applications, is an important aspect to consider when evaluating the effectiveness of a spray application. For the current study, this suggests that intermittent temperature increases may have contributed

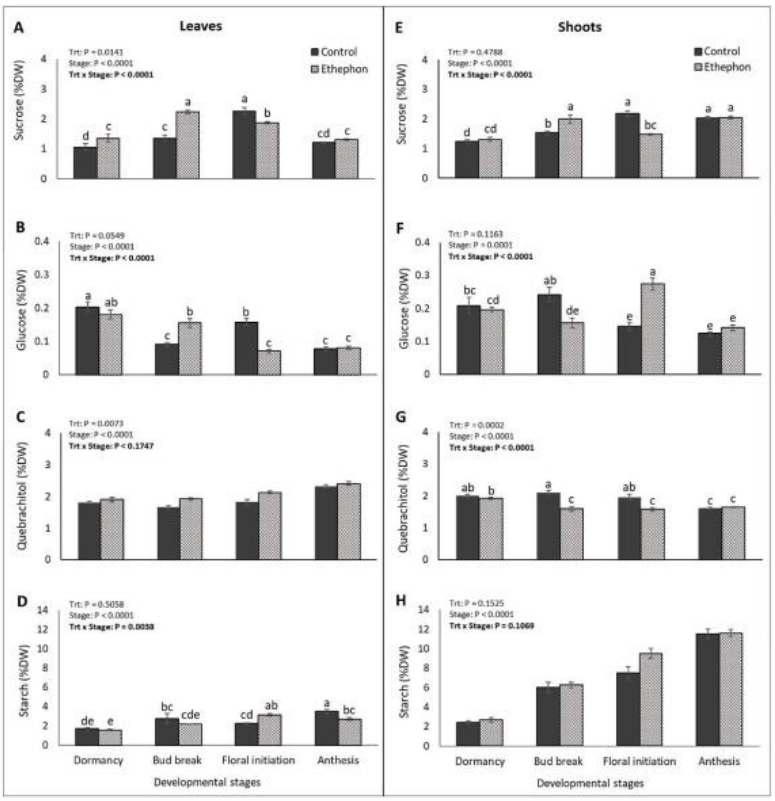

Fig. 4. Ethephon application increased carbohydrate concentrations in leaves and terminal shoots of 'Mauritius' litchi at specific developmental stages. Figures A to D display changes in carbohydrate concentrations in leaves for A) sucrose, B) glucose, C) quebrachitol and D) starch. Figures E to H display changes in carbohydrate concentrations in terminal shoots for E) sucrose, F) glucose, G) quebrachitol and H) starch. Data was collected from a leaflet of the compound leaf closest to the terminal bud, as well as from terminal shoots at the indicated developmental stages following ethephon treatment at 1000 mg·L^{-1} (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Columns represent mean values ± SE (n = 10). DW denotes dry weight. Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Different letters above columns indicate significant differences for treatment × stage interactions according to Fisher's LSD test ($p = 0.05$).

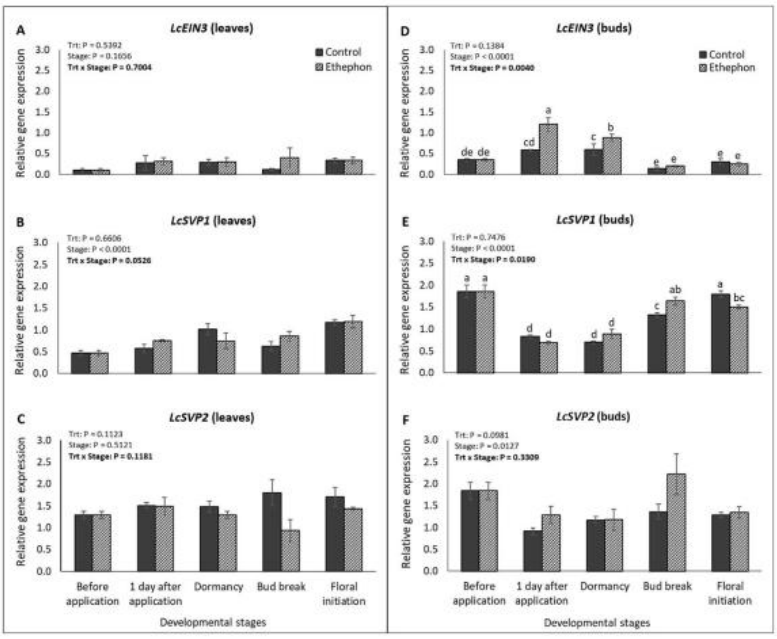

Fig. 5. Relative expression levels of ethylene- and dormancy-related genes in leaves and terminal buds of 'Mauritius' litchi in response to ethephon treatment (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Figures A to C display changes in relative gene expression in leaves for A) *LcEIN3*, B) *LcSVP1* and C) *LcSVP2*. Figures D to F display changes in relative gene expression in terminal buds for D) *LcEIN3*, E) *LcSVP1* and F) *LcSVP2*. Data was collected from a leaflet of the compound leaf closest to the terminal bud, as well as from terminal buds at the indicated developmental stages. Relative gene expression was determined with qRT-PCR. Columns represent mean values ± SE (n = 3). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Different letters above columns indicate significant differences for treatment × stage interactions according to Fisher's LSD test ($p = 0.05$).

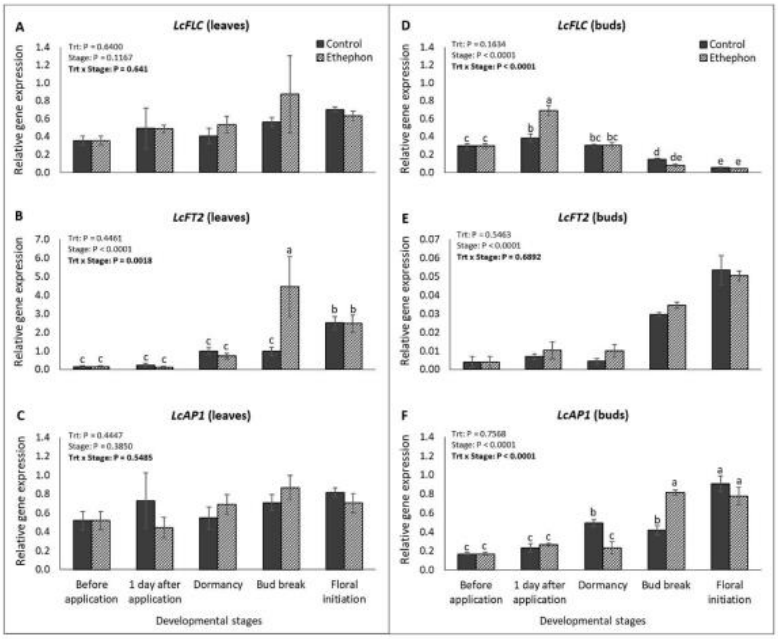

Fig. 6. Relative expression levels of flowering-related genes in leaves and terminal buds of 'Mauritius' litchi in response to ethephon treatment (Ethephon) or no treatment (Control) of trees with mature shoots and dormant terminal buds. Figures A to C display changes in relative gene expression in leaves for A) *LcFLC*, B) *LcFT2* and C) *LcAP1*. Figures D to F display changes in relative gene expression in terminal buds for D) *LcFLC*, E) *LcFT2* and F) *LcAP1*. Data was collected from a leaflet of the compound leaf closest to the terminal bud, as well as from terminal buds at the indicated developmental stages. Relative gene expression was determined with qRT-PCR. Columns represent mean values ± SE (n = 3). Statistical analysis of data was assessed with repeated measures ANOVA ($P < 0.05$). Different letters above columns indicate significant differences for treatment × stage interactions according to Fisher's LSD test ($p = 0.05$).

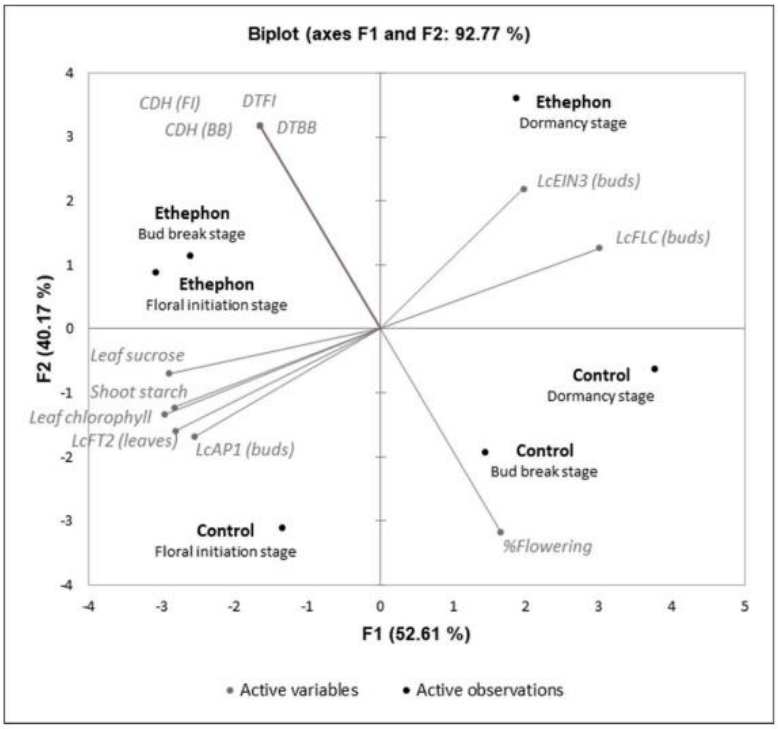

Fig. 7. Principle Component Analysis (PCA) revealed close associations between ethephon treatment and key regulatory genes involved in dormancy (*LcEIN3*, *LcFLC*) and flowering (*LcFLC*, *LcFT2*, *LcAP1*), shoot maturity (leaf chlorophyll) and carbohydrates (leaf sucrose, shoot starch) in 'Mauritius' litchi. PCA was calculated from data collected at the indicated developmental stages (dormancy, bud break and floral initiation stage) in response to ethephon treatment (Ethephon) or no treatment (Control). *LcEIN3*, *LcFLC*, *LcAP1* represent relative gene expression in terminal buds. *LcFT2* represents relative gene expression in leaves. CDH indicates chilling degree hour accumulation at the bud break (BB) or floral initiation stage (FI). DTBB indicates days to bud break. DTFI indicates days to floral initiation.

to an extended ethylene release in the terminal buds.

4.2. Role of ethylene in bud dormancy and subsequent growth events

In litchi, floral induction and initiation can only take place when the terminal buds are exposed to floral-inducing chilling temperatures (Batten and McConchie, 1995; Huang and Chen, 2005). For buds to be released from dormancy, the leaves of the terminal shoot should be fully mature (Fu et al., 2014; Zhang et al., 2016). Previous studies have shown that ethephon applications to trees with fully mature shoots delayed bud break to a time when temperatures were more conducive to floral induction by inhibiting new shoot growth during warmer periods prior to floral induction (Cronje and Ratlapane, 2017, 2018). However, the exact functional mechanisms were not elucidated and remained unclear. It is known from other crops that ethylene binds to the relevant receptors within a plant cell and exerts its influence on physiological and molecular processes within the cell, such as the inactivation of cell growth in the bud apical meristem (Burg, 1973; Ju and Chang, 2015; Dubois et al., 2018). Ethylene and ethephon treatment were also associated with a delay in bud growth and flower development (Abeles et al., 1992; Hansen and Grossmann, 2000). Quantification of ethylene evolution in our study revealed an extended presence of ethylene in the terminal buds of litchi, which induced bud dormancy and subsequently inhibited new flush growth whilst promoting shoot maturity (i.e. leaf chlorophyll accumulation). These observations were also confirmed by increased *LcEIN3* expression levels in terminal buds one day after application and during dormancy, as well as by *LcEIN3* expression being positively correlated with DTBB and being negatively correlated with flushing incidence. In addition, ethephon application and the subsequent ethylene release maintained bud dormancy irrespective of temperature increases during the pre-induction and induction period (at around day 42). Consequently, this prevented shoot growth during a warmer period.

The subsequent delay in panicle emergence under more favorable inductive conditions significantly reduced the percentage of leafy panicles, and increased the number of panicles per shoot in ethephon treated trees (Tables 1 and 2; Fig. 2). Despite delayed bud break and panicle emergence, panicle development in ethephon treated trees was accelerated, as was indicated by a shorter period between the bud break and floral initiation stage (Table 1), leading to anthesis at the same time as in the control trees (data not shown). Most of the changes in tree phenology in response to ethephon application were consistent with previous reports on ethephon used for shoot control and delay of bud break in litchi (Cronje and Ratlapane, 2017, 2018). Our study did not find significant differences in percentage flowering (Table 2), possibly because temperatures at the time of flower panicle emergence in untreated trees were sufficiently low to achieve high flower induction and initiation success. Nevertheless, and despite lacking significant treatment differences, the higher number of fruit set per panicle (Table 2) indicates that the ethephon treatment in our study showed the potential to improve the crop, similar to that reported by Cronje and Ratlapane (2017, 2018).

4.3. Role of ethylene in carbohydrate accumulation

Carbohydrates are considered to play an important role in the regulation of flowering in litchi (Cronje and Mostert, 2009; Menzel et al., 1995; Yang et al., 2014) and were found to be correlated with floral formation in litchi (Zhang et al., 2017). In contrast, initial shoot growth is known to consume carbohydrate reserves and may deplete necessary reserves for floral development if it occurs prior to flowering (Cronje and Mostert, 2009; Cronje et al., 2020; Huang and Chen, 2014; Menzel et al., 1995). By inhibiting shoot growth and extending dormancy (Table 1), ethephon application in our study provided a prolonged opportunity to accumulate carbohydrates which led to increased concentrations of sucrose, glucose, quebrachitol and starch in leaves and shoots, mainly at bud break and the floral initiation stage, compared with untreated trees (Fig. 4). Furthermore, shoot maturity played an important role in carbohydrate assimilation, which was highlighted by leaf chlorophyll content being significantly positively correlated with shoot and leaf sucrose concentration at dormancy and bud break, respectively. Fu et al. (2014) showed that CO_2 assimilation and photochemical efficiency was highest when leaves of the litchi cultivars 'Baili' and 'Heiye' reached full maturity, which corresponded to 90% of the maximum chlorophyll concentration. Furthermore, Yang et al. (2014) found that leaf chlorophyll and carbohydrate concentration was higher for dark-green mature leaves compared with yellowish-green or yellowish-red leaves of three different litchi cultivars during the floral induction period. In our study, the role of dormancy in carbohydrate accumulation was particularly evident in shoot starch concentrations, which significantly increased over time in both treatments (Fig. 4G). Significant positive correlations between DTBB and sucrose concentrations in leaves and shoots, as well as between *LcSVP1* expression and leaf sucrose concentration during the dormancy stage further accentuated the link between dormancy and carbohydrate accumulation.

While both starch and sucrose are important non-structural carbohydrates, sucrose is a primary product of photosynthesis and generally the principle form of translocated carbon in vascular plants (ap Rees, 1984). Sucrose also plays an important role in the transition from the vegetative to the reproductive phase (Cho et al., 2018), and was found to act as a primary messenger in signal transduction during floral induction in *Arabidopsis thaliana* (Rolland et al., 2002). Cho et al. (2018) discussed various studies where an increase or mobilization of sugar and/or starch concentration in leaves, phloem sap or shoot apex in response to different treatments, such as changes in photoperiod, irradiation, exogenous sucrose application or by use of transgenic plants like *A. thaliana*, promoted floral induction and the expression of regulatory genes involved in flowering and carbohydrate metabolism. Similarly in our study, significantly increased concentrations of soluble sugars (sucrose, glucose and quebrachitol) in the leaves of ethephon treated trees at bud break corresponded with significantly higher expression levels of *LcFT2* in leaves and *LcAP1* in buds at bud break (Figs. 4 and 6). Furthermore, PCA analysis highlighted strong associations between ethephon treatment and shoot maturity, carbohydrates and flowering-related genes at bud break and the floral initiation stage, and confirmed that shoot maturity and leaf sucrose are key factors for the expression of flowering related genes (Fig. 7). The decrease of leaf sucrose and glucose concentrations of ethephon treated trees at the floral initiation stage suggests the possibility of higher sugar demand for respiratory processes at bud break (Zhang et al., 2016) to facilitate the accelerated panicle development (ap Rees, 1984, Table 1). Similarly, increased quebrachitol concentrations in the leaves and decreased concentrations in the shoots of ethephon treated trees at bud break and the floral initiation stage (Fig. 4C and G) suggest that quebrachitol was possibly mobilized from the shoots to meet the high sugar demand for sucrose and glucose for floral development in the ethephon treated trees. Quebrachitol was previously reported to have a unique function in litchi as transportable photosynthate under reduced sucrose conditions (Wu et al., 2018). It represents about 50% of soluble sugars in mature litchi leaves and occurs in similar high concentrations as sucrose (Liu et al., 2022; Wu et al., 2018).

4.4. Role of ethylene in modulating gene expression

ETHYLENE INSENSITIVE 3 (*EIN3*) is a transcription factor in the ethylene pathway (Wang et al., 2013) and regulates the expression of ethylene-responsive genes (*ERF*s), which in turn are directly responsible for a wide range of ethylene induced (stress) responses (Bleecker and Kende, 2000; Wang et al., 2013). In our study, ethephon application and the associated ethylene release had a direct effect on *LcEIN3* expression levels in terminal buds immediately after application and during the dormancy period (Fig. 5). This led to specific responses, such as shoot inhibition due to extended bud dormancy, as mentioned earlier, and

corresponded directly with the data on ethylene evolution (Fig. 2). In support of our results, research on the gene expression of ethylene-related genes in litchi fruitlets following ethephon application revealed that several key regulatory genes in the ethylene pathway, such as *ERF*s and *EIN3/EIL*, were highly expressed one day after application (Li et al., 2015). The overall low *LcEIN3* expression levels in treated leaves throughout the observation period compared with treated buds (Fig. 6) further provide evidence in support of the view that ethephon may not have been absorbed by the leaves, but that the peak in ethylene evolution (Fig. 2) can be attributed to a chemical breakdown reaction of the applied ethephon on the leaf surface.

SHORT VEGETATIVE PHASE genes (*SVP*s) are transcription factors that act as repressors of flowering during the vegetative phase as well as play an important role during floral meristem specification in *A. thaliana* (Gregis et al., 2013; Hu et al., 2018). The *SVP* genes also control flowering time under ambient temperature changes in *A. thaliana* by repressing the expression of the flowering gene *FLOWERING LOCUS T* (*FT*) (Lee et al., 2007). In litchi, *LcSVP1* regulates growth cessation and dormancy maintenance, while *LcSVP2* regulates the entrance into dormancy and flowering (Zhang et al., 2016). In our study, pre-treatment expression levels of both *SVP*s were high in terminal buds, possibly indicating that the buds might have been in a late stage of growth cessation and dormancy entrance. The dormancy maintenance function of *LcSVP1* can be supported by *LcSVP1* expression being significantly negatively correlated with *LcFT2* and *LcAP1* expression at the dormancy stage. However, *LcSVP1* gene expression data (Fig. 5B and E) did not reveal significant treatment differences one day after application or at dormancy. The increased, albeit not significantly, expression levels of *LcSVP2* in buds of treated trees one day after application and at bud break (Fig. 5F), could indicate a possible interaction between *LcSVP2* and the ethylene and/or flowering pathways in litchi. It has been proposed that some genes in the *SVP* family may be regulated by ethylene, abscisic acid and gibberellin (Gregis et al., 2013; Liu et al., 2018).

The *FLOWERING LOCUS C (FLC)* gene is a flowering repressor that integrates the autonomous flowering and vernalization pathways (Zhang et al., 2014). Overexpression of *FLC* was associated with the downregulation of the flowering genes *FT* and *AP1*, as well as with a delay in flowering in *A. thaliana* and litchi (Lu et al., 2017; Zhang et al., 2014). Similar interactions between *LcFLC* and the flowering genes, *LcFT2* and *LcAP1*, in terminal buds were found in our study for the period between application and bud break (Fig. 6D and F). Generally, *FLC* is expressed under conditions that are not conducive for flowering, such as high temperatures (Liu et al., 2018). However, in our study, overexpression of *LcFLC* one day after ethephon application may have been in direct response to the ethephon treatment or been regulated by *LcEIN3*, and may have contributed to the extended dormancy period and delayed bud break (Table 1). A possible interaction between *LcFLC* and *LcSVP2* expression, as indicated by the positive correlation between these two genes one day after ethephon application, may also have played a role in bud dormancy of ethephon treated trees, since some genes in the *SVP* family in *A. thaliana* were found to interact with the flowering repressor *FLC* to downregulate flower promoters, such as *FT* (Gregis et al., 2013). Due to the delay in flower panicle emergence, ethephon treated trees initiated floral development at lower temperatures (~4 °C difference to control, Fig. 2) and had accumulated more CDHs at the bud break and floral initiation stage compared with control trees at the same phenological stages (Table 2, Fig. 2). The stronger chill effect on ethephon treated trees was associated with significantly increased expression levels of *LcFT2* in leaves, which is primarily associated with low-chilling temperature in litchi (Lu et al., 2017; Shen et al., 2016), as well as with significantly increased expression levels of *LcAP1* in buds at the bud break stage, compared with control trees (Fig. 6B and F). However, similarly high *LcAP1* expression levels in both treatments at the floral initiation stage (Fig. 6F) indicate that temperatures were still sufficiently low for successful flower differentiation in the control trees, hence leading to equally high flowering rates in both treatments (Table 2). The interdependence of *FT* and *AP1* genes in response to cold was also described in other studies on *A. thaliana* and litchi (Gregis et al., 2013; Lu et al., 2017). The stronger chill effect on ethephon treated trees can also explain their significantly lower percentage leafy panicles compared with control trees (Table 2), because under low temperature conditions, the mixed floral buds of litchi, which consist of leaf and panicle primordia as well as rudimentary leaves, abort their rudimentary leaves and develop into pure leafless flower panicles (Huang and Chen, 2005; Zhou et al., 2008).

Reactive oxygen species (ROS) may also possibly be involved in ethephon responses that cause abscission of young leaves in litchi and inhibit bud growth in other crops (Abeles et al., 1992; Cronje and Ratlapane, 2017, 2018; Roets et al., 2010; Sexton, 1997). In our study, the prolonged presence of ethylene in the bud tissue as a result of slow ethephon degradation in the shoot apex most likely generated ROS (Lu et al., 2014; Zhou et al., 2013). Indeed, Zhou et al. (2013) detected ROS in apical buds as well as in petioles and stems of abscising rudimentary leaves after applying ethephon to leafy panicles for the purpose of promoting pure panicle development in litchi. Lu et al. (2014) found that treating litchi shoots with the ROS-generating chemical methyl viologen dichloride hydrate (MV) increased expression levels of several genes in the ethylene signalling pathway, among them *EIN3*, similar to ethephon application in our study. Furthermore, the most recent study on genome-wide transcriptomic analysis of oxidative stress-induced flowering genes in litchi leaves revealed that ROS, such as those induced by MV, significantly upregulated *LcFT1* and *LcFT2* and even promoted litchi flowering under less inductive temperatures (Lu et al., 2020). In an earlier study by the same research group, Lu et al. (2017) also discovered an overexpression of *LcAP1* in low temperature-treated as well as in medium temperature plus MV-treated trees. The MV application was associated with an increase of hydrogen peroxide as well as an increase in the expression of the floral gene *LcLFY*, which interacts with *LcAP1* to establish floral meristem identity. Although applied at a different time, ethephon application and the subsequent breakdown to ethylene in our study may also have generated ROS, which, together with the ethylene effect, could have triggered a cascade of physiological and molecular processes. This may explain the observed changes in the expression of related genes, such as *LcFLC*. Apart from promoting a higher percentage of pure floral panicles, the application of ethephon may hold potential to induce litchi flowering under less inductive conditions similar to MV in the above-mentioned study.

Based on our results, we developed a conceptual model that illustrates the effect of ethephon-derived ethylene on key regulatory genes primarily involved in the regulation of bud dormancy, and the ensuing effects on, and interactions between shoot maturity, carbohydrate accumulation, chill accumulation and floral gene expression, which eventually lead to successful floral formation in litchi (Fig. 8).

5. Conclusion

This study aimed to investigate the effect of ethephon application on physiological and molecular mechanisms governing shoot maturity, bud dormancy, carbohydrate accumulation and expression of key regulatory genes involved in dormancy and flowering in 'Mauritius' litchi. Our study has provided evidence that ethephon application to trees with mature shoots and dormant terminal buds during autumn resulted in an upregulation of ethylene- and dormancy-related genes, particularly in terminal buds. Consequently, this led to an extended dormancy with an increased accumulation of carbohydrates as well as a subsequent delay in bud break and panicle emergence, which promoted pure floral panicle development under more favorable inductive conditions, thereby indirectly influencing phenological, physiological and molecular changes associated with flowering in litchi. Our study confirmed that ethylene plays an important role in the physiological and molecular regulation of bud dormancy in litchi, and that ethephon application can be a useful

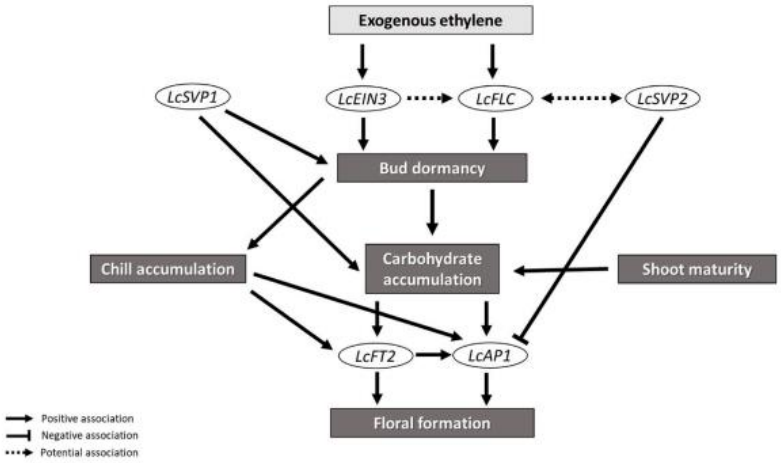

Fig. 8. Conceptual model depicting interactions between phenological, physiological and molecular processes affecting floral formation in litchi in response to exogenous ethylene. Exogenous ethylene (via ethephon application) stimulates expression of *LcEIN3* and *LcFLC* in terminal buds, which, in possible association with *LcSVPs*, enforces and prolongs bud dormancy. The extended dormancy promotes shoot maturity and carbohydrate accumulation. The subsequent delay in bud break and panicle emergence stimulates upregulation of flowering related genes (*LcFT2*, *LcAP1*) under more favorable inductive conditions with higher chill accumulation leading to successful floral panicle development. Arrows with solid lines indicate positive associations; solid lines ending with a bar indicate negative association; arrows with dotted lines indicate potential, but unconfirmed associations.

tool to prevent panicle emergence under less inductive conditions. The role of ROS in this regard, however, warrants further research.

With the current knowledge, future studies should focus on finding strategies that increase the responsiveness of litchi trees to induce flowers under less inductive temperatures or that may reduce their chill requirements for flowering as an approach to mitigate unfavorable seasonal climatic changes, which otherwise would lead to partial or complete flower and crop failure.

CRediT authorship contribution statement

Regina B. Cronje: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing, Visualization, Supervision of experimental work, Project administration, Funding acquisition. **Elliosha Hajari:** Methodology, Writing – review & editing. **Arnold Jonker:** Methodology, Investigation, Validation. **Innocent M. Ratlapane:** Investigation. **Xuming Huang:** Conceptualization, Resources, Visualization, Writing – review & editing. **Karen I. Theron:** Writing – review & editing. **Eleanora W. Hoffman:** Resources, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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APPENDIX IV

bio-protocol

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Quantification of Ethylene Production in Leaf and Bud Tissue of the Subtropical Tree Crop Litchi (*Litchi chinensis* Sonn.) Using Gas Chromatography and Flame Ionization Detection)

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Abstract

Ethylene is an important plant hormone that is involved in the regulation of numerous processes in plant development. It also acts as a signaling molecule in response to biotic and abiotic stress conditions. Most studies have investigated ethylene evolution of harvested fruit or small herbaceous plants under controlled conditions, but only a few explored ethylene release in other plant tissues, such as leaves and buds, particularly those of subtropical crops. However, in light of increasing environmental challenges in agriculture (such as temperature extremes, droughts, floods, and high solar radiation), studies on these challenges and on potential chemical treatments for mitigating their effects on plant physiology have become more and more important. Thus, adequate techniques for the sampling and analysis of tree crops are needed to ensure accurate ethylene quantification. As part of a study on ethephon as a mitigating agent to improve litchi flowering under warm winter conditions, a protocol was developed for ethylene quantification in leaf and bud tissue of litchi following ethephon application, taking into account that these plant organs release lower ethylene concentrations than fruit. At sampling, leaves and buds were placed in glass vials of appropriate sizes for the respective plant tissue volumes and allowed to equilibrate for 10 min to release possible wound ethylene before incubating the samples for 3 h at ambient temperature. Thereafter, ethylene samples were aspirated from the vials and analyzed using a gas chromatograph with flame ionization detection, the TG-BOND Q+ column for separation of ethylene, and helium as the carrier gas. Quantification was achieved based on a standard curve derived from an external standard gas calibration with certified ethylene gas. This protocol will also be appropriate for other tree crops with similar plant materials as study foci. It will enable researchers to accurately determine ethylene production in various studies investigating the role of ethylene in general plant physiology or stress-induced plant responses following a range of treatment conditions.

Keywords: Ethylene, Gas chromatography, Flame ionization detector, Headspace, Leaves, Apical buds, Subtropical crops

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Background

Ethylene is a gaseous plant hormone that plays an important role in the regulation of numerous physiological and developmental plant processes, such as seed germination, root and shoot development, cellular growth regulation, carbon assimilation, senescence of leaves and flowers, organ abscission, and fruit ripening (Abeles et al., 1992; Pierik et al., 2006; Olsen, 2010; Wang et al., 2013; Dubois et al., 2018). Ethylene also acts as a signaling molecule in responses to biotic and abiotic environmental stresses, such as temperature extremes, droughts, floods, shading, radiation, nutrient deficiency, and mechanical and chemical damage (Yang and Hoffman, 1984; Iqbal et al., 2013; Dubois et al., 2018). The amount of ethylene production depends on the plant species, developmental stage of the plant, and organ type (Cristescu et al., 2013). Apart from endogenous or stress-induced ethylene production, production in plant organs can also be induced by exogenous application of the plant growth regulator ethephon (2-chloroethylphosphonic acid). Ethephon has been widely used in agriculture for different applications, including fruit ripening and coloration, organ abscission to aid harvesting, bloom delay, and suppression of vegetative growth (Nickell, 1994).

Accurate quantification of ethylene evolution from ethephon as well as endogenous ethylene production following different treatments is necessary to correlate production with respective plant responses and elucidate the functions and role of ethylene. According to Cristescu et al. (2013), there are three main methods to detect ethylene in plants: gas chromatography detection, electrochemical detection, and optical detection. These can be used for periodic or continuous measurement depending on the type of plant material and experimental design. Gas chromatography (GC) is most widely used for separation and analysis of ethylene, due to its small sample requirement, high selectivity, and fast analysis. Although more sensitive detection techniques have been developed, such as a laser-based sensing technique (Cristescu et al., 2013; Gwanpua et al., 2018), GC detection is still the most applicable method for tree crops, since non-destructive and/or continuous sampling is not possible. This applies particularly to studies that focus on trees in orchard systems and that are subject to climatic effects.

In fruit tree crops, the quantification of ethylene has mainly been used to assess the effect of endogenous ethylene production in developing fruit, e.g., on fruit coloration (Yin et al., 2001; Chervin et al., 2005; Wang et al., 2007), or in harvested fruit, e.g., on fruit quality and shelf life after harvest (Tseng et al., 2000; Gwanpua et al., 2018). However, few studies investigated ethylene production in leaves, buds, flowers, or other vegetative material from tree crops. Examples are GC detection of ethylene in leaves of citrus (Tudela and Primo-Millo, 1992), in shoot portions of apple (Sanyal and Bangerth, 1998), and in floral buds and leaves of mango (Bindu et al., 2017), peach (Liu et al., 2021) and litchi (Cronje et al., 2022) trees. However, most of these studies only provided limited information on the sampling and analysis techniques used to successfully reproduce the techniques for application in other crops with similar plant organs. For this purpose, we developed a protocol to easily determine ethylene evolution in leaves and apical buds of litchi as part of a recent study, which used ethephon to induce bud dormancy and delay panicle emergence for more consistent floral initiation in litchi (Cronje et al., 2022). To determine the mode-of-action of ethylene inside the leaf and bud tissue, as well as the downstream processes, such as relative expression of ethylene-, dormancy- and flowering-related genes, ethylene concentration needed to be quantified reliably and accurately in these plant organs to correlate the results with those obtained from corresponding biochemical and molecular analyzes (Cronje et al., 2022). The sampling technique and incubation period was adapted specifically for litchi to account for low overall ethylene production and wound ethylene release after detaching of the respective plant organs. The quantification protocol was derived from a protocol for the quantification of ethylene evolution in tomato leaves developed by Kim et al. (2016) and used a porous layer open tubular (PLOT) TG-Bond Q+ column (Thermo Scientific) for direct separation of ethylene. While several related studies used packed alumina (Al_2O_3) columns that are recognized for the separation of hydrocarbons from C1 to C5, the use of the mentioned PLOT column is considered advantageous since it is specifically developed for selective separation of acetylene (C_2H_2), ethylene (C_2H_4) and ethane (C_2H_6) to baseline. Additionally, the use of a capillary column in conjunction with a flame ionization detector has proven to be highly sensitive with a linear calibration range of 3 orders of magnitude, i.e., 0.08 nL to 80 nL of injected C_2H_4. Although the protocol was specifically developed for leaves and buds of litchi, it can be equally applied to plant material from other fruit tree crops by making appropriate changes to container sizes.

Materials and Reagents

1. 100 µL gas tight syringe with needle: 50 mm length, 23 gauge, point style 5, side hole (Thermo Scientific, catalog number: 36520050)
2. 2500 µL gas tight syringe with needle: 65 mm length, 23 gauge, point style 5, side hole (Thermo Scientific, catalog number: 365Q2131)
3. 20 mL crimp top headspace vials (Thermo Scientific, catalog number: CHCV20-14)
4. 20 mm Si/PTFE septa (Cronus, catalog number: VCS-2004-1000)
5. 20 mm Al crimp cap (Cronus, catalog number: VCC-2002CS-1000)
6. 20 mm magnetic crimp cap (Cronus, catalog number: VCC-2002BM-500)
7. 20 mm hand crimper (Cronus, catalog number: VTC-20)
8. 1.8 mL, 9 mm screw top vials (Cronus, catalog number: VZS-0209C-100)
9. 9 mm PTFE/red rubber screw thread closure (Cronus, catalog number VKB-0203-09CB-5000)
10. 51 mm, 23 gauge Hamilton needles, point style 5, side hole (Hamilton, catalog number: 7729-06)
11. 17 mm injection port septum (Thermo Scientific, catalog number: 31303211)
12. 3 ml disposable plastic syringes (generic)
13. Plastic 2-way valves with luer locks and needle adaptor for syringes (Vernier, catalog number: PS-2WAY)
14. High purity helium (He) gas, 99.999% (Afrox, Baseline 5.0, catalog number: 524203-SE-C)
15. 79 $\mu L \cdot L^{-1}$ C_2H_4 gas standard, balance nitrogen (N_2) gas (Afrox, catalog number: GOC mix 3292)
16. TG-BOND Q+ porous layer open tubular capillary column (Thermo Scientific, catalog number: 26005-6030)
17. 0.32 mm graphite encapsulated ferrules (Thermo Scientific, catalog number: 29053487)
18. 5 mm glass split liner (Thermo Scientific, catalog number: 45350030)
19. Graphite liner seal (Thermo Scientific, catalog number: 29033406)

Equipment

1. Long nose pliers (generic)
2. Straight Iris scissors (generic)
3. Gas chromatograph with a flame ionization detector (Thermo Scientific, model: Trace GC Ultra)
4. Autosampler (Thermo Scientific, model: Triplus RSH)
5. Headspace tool (Thermo Scientific, catalog number: 1R77010-1125)
6. Air generator (Peak Scientific, model: Precision Zero Air)
7. N_2 generator (Peak Scientific, model: Precision Nitrogen)
8. H_2 generator (Peak Scientific, model: Precision Hydrogen)
9. 2-stage gas regulator with gauges and inlet stem with 5/8" left hand BSP nut (Afrox, catalog number: W019220)
10. Analytical balance (Adam Precision, model: PW184)

Software

1. Thermo Xcaliber 3.0.63.3 (Thermo Fisher Scientific Inc.)
2. Excel (Microsoft Office 2016)

Procedure

A. Hardware setup of gas chromatograph (GC)

1. Follow the manufacturer's instruction manual to perform the following setup of the GC:
   a. Install a 5 mm glass split liner together with a graphite seal into the split/splitless injector.
   b. Place a 17 mm coated septum between the septum support and septum holder and hand tighten the injector cap. A diagram together with a photograph of the layout and components that form part of the injector is presented in Figure 1.

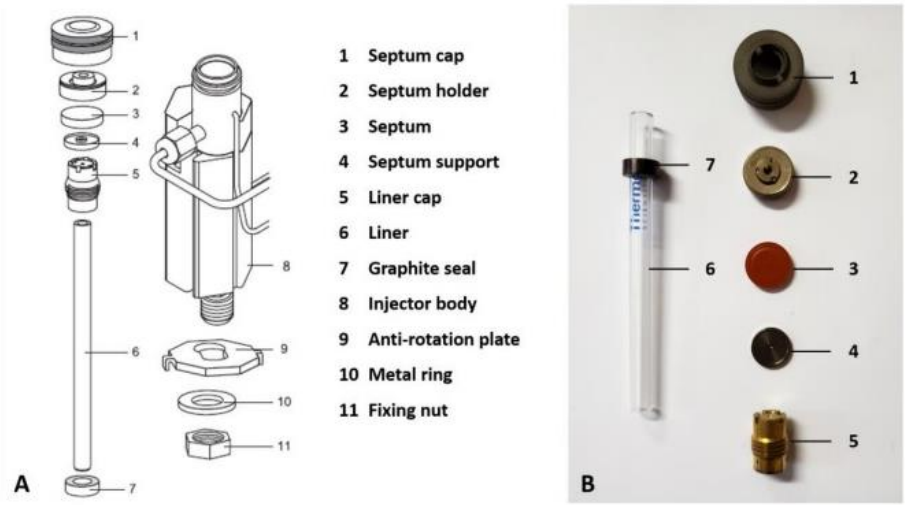

Figure 1. Components and layout of the GC's split/splitless injector. A. Diagram reprinted from Thermo Scientific (2010). B. Photograph of components.

   c. Connect the TG-BOND Q+ capillary column between the flame ionization detector (FID) and split liner using 0.32 mm graphite encapsulated ferrules. The column insertion depths are 40 mm and 94 mm into the split liner and FID respectively. Figure 2 illustrates the installed column inside the oven, as well as how to determine the correct column insertion depths into the injector and FID.

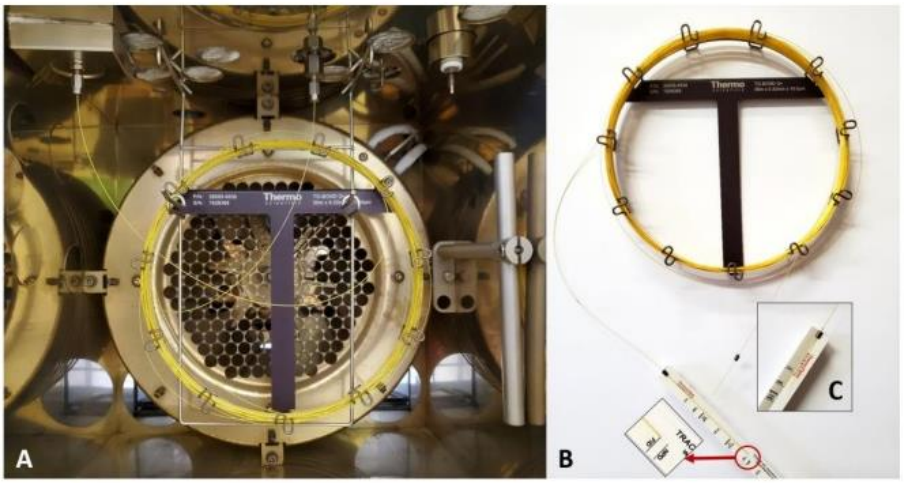

Figure 2. Installation of the TG-BOND Q+ capillary column. A. View of the column as installed in the GC's oven between the split/splitless injector and the FID. B. Measuring the insertion depth of the column for the FID. C. Details of the position of the ferrule when gauging the column insertion depth for the injector.

d. Confirm that a supply of high purity He gas at a pressure of 800 kPa is connected to the GC.
e. Check operation of the Peak Scientific gas generators for providing fuel and make up gas to the FID at pressures and flow rates as specified in Table 1.

Table 1. Pressure and flow specifications of gas supply from gas generators to FID

Gas	Pressure (kPa)	Flow range ($mL \cdot min^{-1}$)
H_2	420	30–50
Dry air	420	300–600
N_2	420	10–60

f. Follow Table 2 for a summary of chromatography measurement parameters to be configured for the GC in the "Instrument Setup" page of the Xcaliber software.

Table 2. List of GC measurement parameters

Variable	Unit	Value
Carrier gas	-	He
Inlet mode	-	Split
Injector base temperature	°C	100
Liner	mm	5
Split flow	$mL \cdot min^{-1}$	40
Septum purge	$mL \cdot min^{-1}$	5
Carrier mode	-	Constant pressure
Pressure	kPa	140
Column	-	TG-BOND Q+
Stationary phase	-	Divinyl benzene homopolymer
Column length	m	30
Column diameter	mm	0.32
Column film thickness	µm	10
Oven starting temperature	°C	60
Heating rate	$°C \cdot min^{-1}$	0

Oven final temperature	°C	60
FID base temperature	°C	200
H_2 flow rate	mL·min^{-1}	35
Air flow rate	mL·min^{-1}	350
N_2 flow rate	mL·min^{-1}	30
FID range	-	1
FID gain	-	1
Analog filter status	-	On
Sampling depth	mm	25
Syringe filling speed	mL·min^{-1}	50
Injection speed	mL·min^{-1}	50
Injection depth	mm	50
Needle penetration speed	mm·s^{-1}	25

B. Configuration of a processing setup in Thermo Xcaliber software

1. Use the "Processing Setup" option to import a ".raw" file for peak identification. The ".raw" file should represent a GC trace from a gas injection consisting of a low concentration C_2H_4 gas.
2. Follow Table 3 for the set of variables to be configured into the software.

Table 3. Parameters for the GC's software processing in terms of peak identification

Page	Variable	Value
Identification	Detector type	Analog
	Peak detect	ICIS
	Expected time (min)	1.64
	Window (sec)	5.00
	View width	0.75
Detection	Smoothing points	1
	Baseline window	60
	Area noise factor	5
	Peak noise factor	10
	ICIS peak detection	Highest peak
	Minimum peak height (S/N)	3.0
Calibration	Component type	Target compound
	Weighting	Equal
	Calibration curve	Linear
	Units	nL
	Origin	Ignore
	Response	Area
Levels	1	0.075
	2	0.150
	3	0.301
	4	0.752
	5	1.505
	6	3.010
	7	8.809
	8	16.709
	9	24.609
	10	44.359
	11	83.859

3. An example of a part of the processing setup is depicted in Figure 3 illustrating a retention time of 1.64 min.

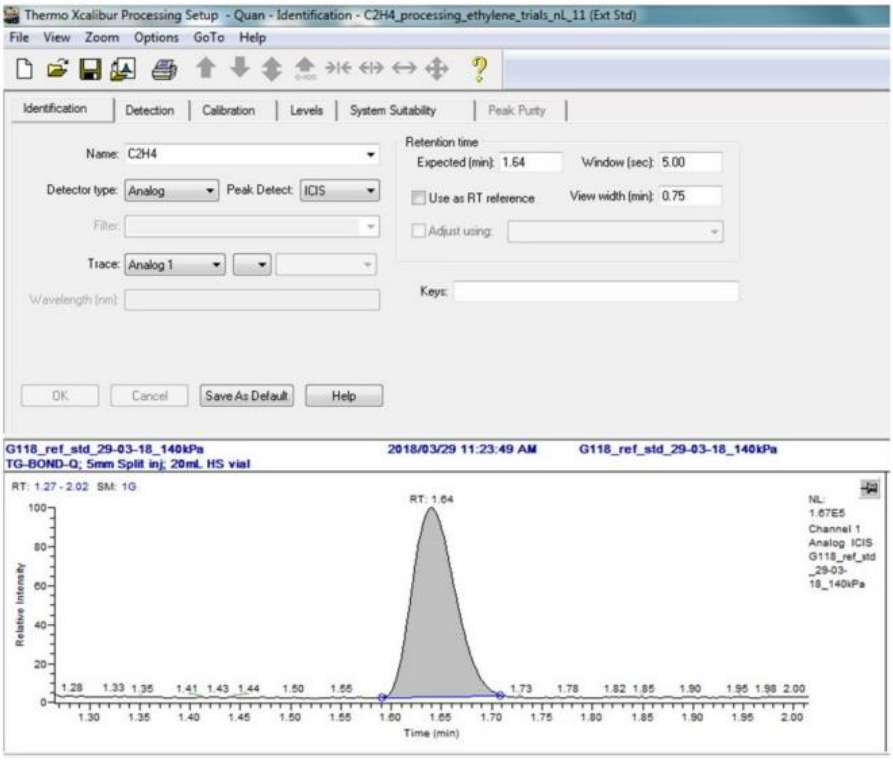

Figure 3. Example of the Xcaliber Processing Setup for peak identification

C. Creation of sequence setups in Xcaliber software for calibration standards and samples

1. Create a sequence file using the "Sequence Setup" window in Xcaliber consisting of 11 entries for the calibration standards. It is important to reference the instrument method file under heading "Inst Meth" and the processing method under "Proc Meth".
2. Give a file path and name for the calibration file in the sequence table under the heading "Cal File".
3. Create a sequence file for the planned number of samples and enter the name of the calibration file to be used under the heading "Cal File".
4. Note that the tray holder and slot position is only important for calibration standards 1 to 6 since the remainder of injections are done manually.
5. Details to create sequence files are further illustrated in Figure 4.

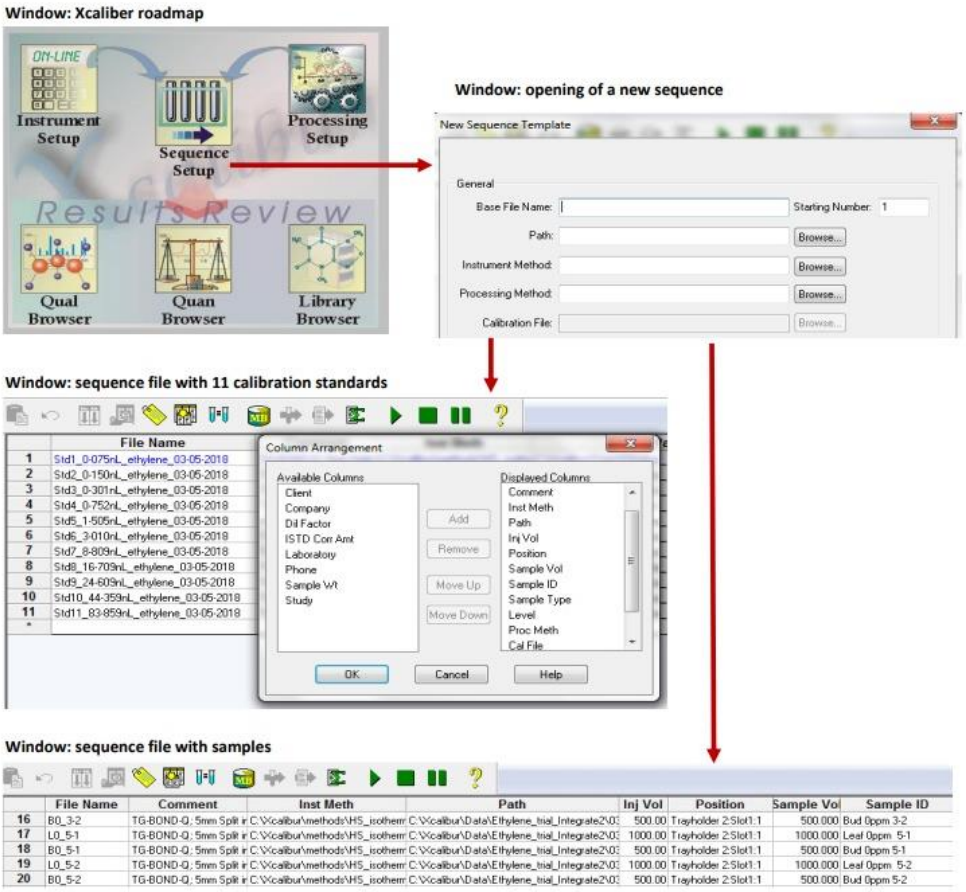

Figure 4. Steps to follow in the Xcaliber software to create sequence files

D. Collection of plant material

1. Detach 2 leaflets (one leaflet each) from the compound leaf closest to the terminal bud by manually breaking them off at the natural abscission layer. Remove up to 20 terminal buds (4–8 mm in length, depending on bud stage and size) with straight sharp Iris scissors. Insert the leaflets and buds into the 20 mL and 2 mL vials, respectively, as illustrated in Figure 5. This will yield minimum masses for buds and leaves of 0.2 g and 1.1 g respectively. The actual mass of plant material is not critical because the calculated rate of C_2H_4 release is normalized to unit mass of plant material. Refer to equation 5.

Figure 5. Leaves and buds sealed in 20 mL (left) and 2 mL (right) vials, respectively

2. Document the time of harvesting of each individual sample as reference for the standardization of the 10 min duration required for the release of wound ethylene (see next step).
3. Leave vials open for 10.0 ± 0.5 min after insertion to allow for release of wound ethylene. This can readily be achieved at the location of harvesting under ambient conditions.
4. After the 10-min equilibration time, seal the 20 mL vials with 20 mm Si/PTFE septa and 20 mm aluminum crimp caps using the 20 mm hand crimper.
5. Ensure that the septa inside the crimp caps have the PTFE face downwards as illustrated in Figure 6, i.e. the PTFE face will contact the beveled edge of the headspace vial.

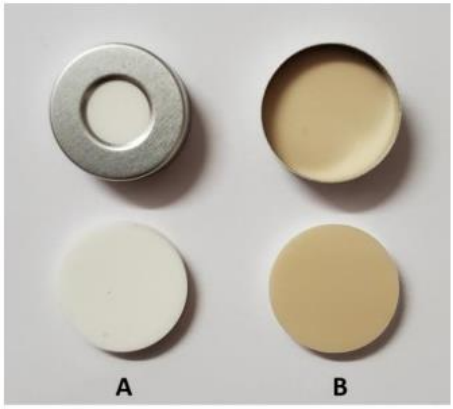

Figure 6. Position of a Si/PTFE septum inside a crimp cap. A. Top view. B. Bottom view.

6. Close the screw caps on the 2 mL vials hand-tight following the 10-min equilibration time.
7. Document the time when each individual vial is sealed as starting time of the 3-h incubation period (see step H1).

E. Preparation and GC measurements of C_2H_4 calibration standards by headspace vial dilution

1. For calibration standards 1 to 6, seal 6 × 20 mL headspace vials containing ambient air with 20 mm magnetic crimp caps and 20 mm Si/PTFE septa.
2. With a 2-stage regulator connected to a 79 $\mu L \cdot L^{-1}$ C_2H_4 gas cylinder, seal the low-pressure outlet with a custom sized Si/PTFE septum inside a nut.
3. Set the outlet pressure of the regulator to 50 kPa. It is important not to exceed 100 kPa as it presents a danger to equipment and personnel due to the nature of the configuration of the outlet. The initial pressure setting is done as follows:
   a. The cylinder shut-off valve is in the closed position.
   b. Turn the control knob on the regulator fully counterclockwise to have the regulator outlet in the closed position.
   c. Open the cylinder shut-off valve and close it immediately again, this will charge the inlet stem and regulator with the cylinder gas.
   d. The reading on the regulator's high-pressure gauge now indicates the cylinder pressure.
   e. Turn the regulator's control knob slowly clockwise until the low-pressure gauge reads 50 kPa.
4. Purge the regulator three times with the analytical gas from the valve side to the low-pressure outlet of the regulator as follows:
   a. It is important to have the cylinder shut-off valve close at the start of the procedure.
   b. Insert a 23 gauge needle through the septum on the low-pressure outlet.
   c. Monitor the pressure on the high-pressure gauge of the regulator until it decreases close to the zero reading. Remove the needle just prior to the gauge reaching zero. This implies that the needle should be removed before the low-pressure gauge decreases from 50 kPa. A small decrease in outlet pressure from the 50 kPa setting can be tolerated as long as the gauge pressure remains positive to prevent air from flowing back into the outlet of the regulator.
   d. Open the cylinder valve and close it again to charge the inlet stem and regulator once more.
   e. Insert the needle through the septum again and repeat steps 5a to 5d three times. The necessity of purging in triplicate is to ensure that all air inside the inlet stem and regulator is replaced by gas from the cylinder and thereby ensuring that the actual analytical gas is aspirated when preparing standards.
5. Use the 100 µL and 2,500 µL syringes to aspirate volumes of analytical gas from the low-pressure outlet of the regulator that is connected to the 79 $\mu L \cdot L^{-1}$ cylinder. Figure 7 presents the technique for extracting gas from a cylinder using a gastight syringe.

Figure 7. Aspirating a volume of C_2H_4 through a septum from a pressure regulator

6. The required volumes, $V_{syringe}$, for injection into the 20 mL headspace vials are specified in column 3 of Table 4, with a photographic illustration of the use of a 100 µL syringe given in Figure 8. A needle insertion

depth of 30 mm is sufficient.

Figure 8. Use of a 100 µL syringe to dilute C_2H_4 gas in a headspace vial

7. The syringe technique should consist of a volume extraction that exceeds the specified volume. The syringe plunger can then be pushed back to the required volume to eliminate pressure effects inside the syringe.
8. Inject 1 mL volumes of standards 1 to 6, V_{inj}, into the GC using the Triplus autosampler with the configurations as described for the instrument method, as well as processing and sequence setups. An automated injection step using a headspace tool is presented by the photograph in Figure 9.

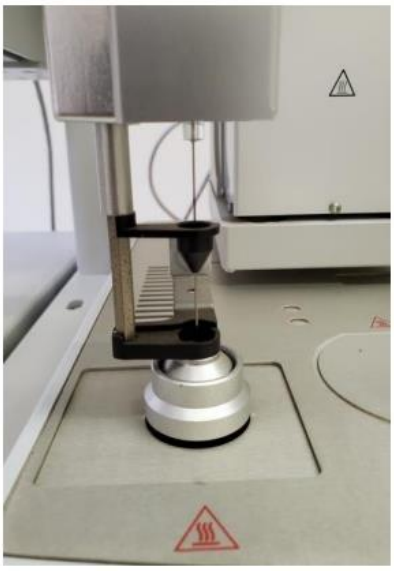

Figure 9. Automated injection with a headspace tool

9. Since a FID is a mass sensitive detector, the calibration is performed based on the quantity of analytical gas, i.e., C_2H_4, injected into the split liner, V'_{inj}. The values presented in Table 4 can be obtained by means of equations 1 to 3:

$$v' = \frac{C_{cylinder} \cdot V_{syringe}}{1 \times 10^6} \quad \text{.....(1),}$$

$$C'_{vial} = \frac{C_{cylinder} \cdot V_{syringe}}{V_{vial}} \quad \text{.....(2),}$$

$$V'_{inj} = \frac{C'_{vial} \cdot V_{vial} \cdot V_{inj}}{(V_{vial} + V_{inj}) \cdot (1 \times 10^3)} \quad \text{.....(3).}$$

Definition of the variables are as follows:
$C_{cylinder}$ = concentration of C_2H_4 in the gas cylinder ($\mu L \cdot L^{-1}$),
V_{vial} = volume of headspace vial (mL),
$V_{syringe}$ = volume aspirated into syringe (µL),
v' = volume C_2H_4 in the syringe (µL),
C'_{vial} = concentration C_2H_4 in headspace vial ($nL \cdot L^{-1}$),
V_{inj} = total volume gas injected into the GC liner (mL), and
V'_{inj} = volume of C_2H_4 injected into the GC liner (nL).

Table 4. Required vial and syringe volumes to prepare standards 1 to 6 in headspace vials

$C_{cylinder}$ ($\mu L \cdot L^{-1}$)	V_{vial} (mL)	$V_{syringe}$ (µL)	v' (µL)	C'_{vial} ($nL \cdot L^{-1}$)	V_{inj} (mL)	V'_{inj} (nL)
79	20	20	1.580×10^{-3}	79	1	0.075
79	20	40	3.160×10^{-3}	158	1	0.150
79	20	80	6.320×10^{-3}	316	1	0.301
79	20	200	1.580×10^{-2}	790	1	0.752
79	20	400	3.160×10^{-2}	1580	1	1.505
79	20	800	6.320×10^{-2}	3160	1	3.010

F. Preparation and GC measurements of C_2H_4 calibration standards by in-situ syringe dilution

1. Standards 7 to 11 are prepared by means of in-situ dilution in a 2,500 µL gastight syringe.
2. Aspirate volumes of gas, $V_{syringe}$, from the cylinder as indicated in column 1 of Table 5. Apply again the technique of extracting volumes in excess of the required and then moving the plunger to the required volume.

Table 5. Required volumes to prepare standards 7 to 11 using in-situ syringe dilution

$V_{syringe}$ (µL)	V_{dead} (µL)	$C_{cylinder}$ ($\mu L \cdot L^{-1}$)	$V'_{inj\text{-}IS}$ (nL)
50	61.5	79	8.809
150	61.5	79	16.709
250	61.5	79	24.609
500	61.5	79	44.359
1000	61.5	79	83.859

3. Thereafter "dilute" the analytical gas with air by setting the plunger to the 1000 µL mark.
4. Inject 1 mL of standards 7 to 11 manually into the GC during a programmed calibration sequence in the Xcaliber software.
5. Limit the insertion depth of the needle into the split liner to 50 mm.
6. Observe that a dead volume, V_{dead}, of the 2,500 µL syringe that contributes to the volume of analytical gas is indicated in Table 5. This value is syringe-specific.
7. Calculate the volume of C_2H_4, $V'_{inj\text{-}IS}$, injected into the liner in nL units by means of equation 4:

$$V'_{inj-IS} = \frac{(V_{syringe}+V_{dead}) \cdot C_{cylinder}}{1 \times 10^3} \quad \text{.....(4).}$$

G. Preparation and GC measurements of blanks and C_2H_4 quality control (QC) standards

1. For the 20 mL headspace QC vials, seal 4 × 20 mL headspace vials, containing only ambient air, with 20 mm aluminum crimp caps and 20 mm Si/PTFE septa.

2. For the 1.8 mL screw top QC vials, close the screw tops of the 4 × 20 mL vials. These vials also only contain ambient air.
3. For the C_2H_4 QC standards, use a 100 µL gastight syringe to aspirate 20 µL and 80 µL of the C_2H_4 standard to prepare 3 technical replicates of the 2 mL and 20 mL vials, respectively.
4. To sample the respective gas volumes for QC samples, aspirate from the regulator in excess of the required volume and move the plunger back to the exact required volume.
5. Refer to Table 6 for data on the prepared C_2H_4 QC samples.

Table 6. Required vial and syringe volumes to prepare C_2H_4 QC samples

$C_{cylinder}$ ($\mu L \cdot L^{-1}$)	V_{vial} (mL)	$V_{syringe}$ (µL)	v' (µL)	C'_{vial} ($nL \cdot L^{-1}$)	V_{inj} (mL)	V'_{inj} (nL)
79	2	20	1.580×10^{-3}	790	0.5	0.316
79	20	80	6.320×10^{-3}	316	1	0.301

6. The total number of prepared QC vials consists of a 1 × 2 mL blank (containing ambient air), a 1 × 20 mL blank (containing ambient air), a 3 × 2 mL C_2H_4 QC standard (containing 20 µL of C_2H_4 standard), and a 3 × 20 mL C_2H_4 QC standard (containing 80 µL of C_2H_4 standard).
7. Perform gas sampling from the QC vials using disposable 3 mL syringes with luer valves, each connected to a 23 gauge needle of 51 mm length. An example of a syringe with a luer valve can be seen in Figure 10.

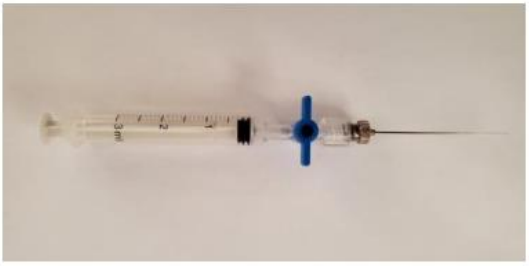

Figure 10. Disposable 3 mL syringe with luer lock and attached needle

8. Aspirate 1 mL and 0.5 mL from the 20 mL and 2 mL vials, respectively.
9. An advisable syringe technique is to cycle the required volume 2 times with the needle stationary through the septum prior to aspirating the required volume.
10. Take care to close the luer valve while holding the plunger in position for the required aspiration volume. This is specifically relevant when sampling 0.5 mL from a 2 mL vial, i.e., air pressure will restore the plunger to a volume less than extracted, which is prevented by ensuring that the valve is closed prior to releasing the syringe plunger. The technique to achieve this is demonstrated by the sequence of photographs in Figure 11.

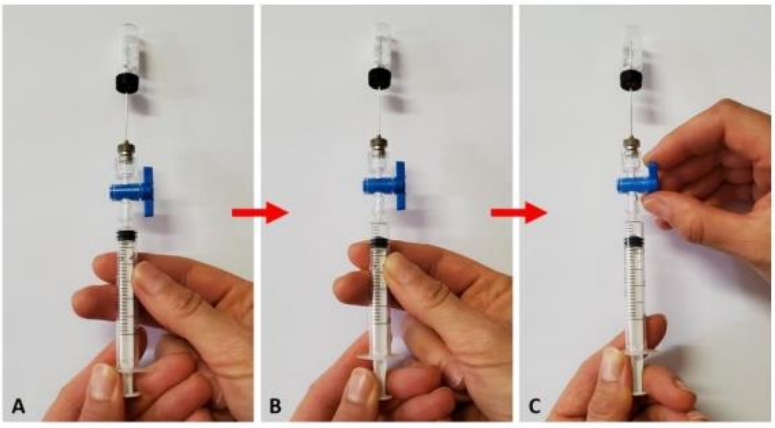

Figure 11. Technique to aspirate a gas sample from a small volume (2 mL) vial with a disposable syringe. A. Positioning the needle through the septum with the luer lock open. B. Withdrawing of a gas sample by moving the plunger outwards. C. Closing the valve's luer lock while holding the plunger in position at the required volume.

11. Use a clean 3 mL syringe to flush needles that are alternating between vials to ensure any C_2H_4 gas is expelled from the needles.
12. Inject the QC samples manually into the GC liner during a programmed sequence.
13. The syringe can be inserted with its full length through the injector's septum, i.e., 51 mm, to agree with the programmed injection depth setting of 50 mm. The manual injection process is illustrated by the sequence of photographs in Figure 12.

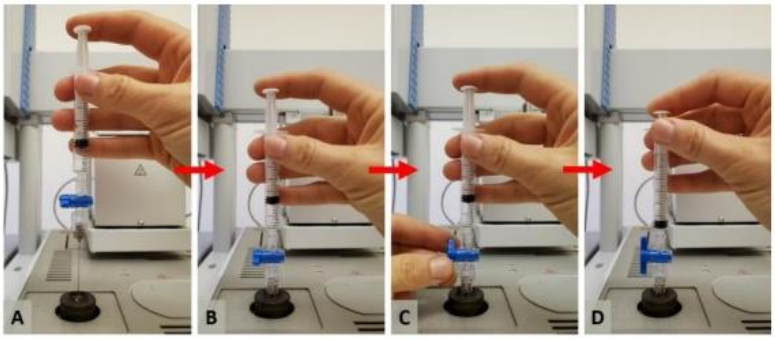

Figure 12. Sequence of steps for manual injection. A. Positioning the needle on top of the injector. B. Inserting the needle through the septum. C. Opening the valve's luer lock while maintaining control of the syringe's plunger to prevent the head pressure from pushing the plunger upwards. D. Pressing the plunger down to inject the gas sample into the injector.

H. GC measurements of C_2H_4 evolved from leaves and buds

1. Perform gas sampling 3.00 ± 0.05 h after sealing vials using disposable 3 mL syringes with luer valves connected to 23 gauge needles of 51 mm length. The 3-h incubation period for each vial consists of the total time the plant material is allowed to release C_2H_4 under sealed conditions. It includes time between sealing of the vials at the location of collection (orchard), transport to the laboratory, and monitored time

on the laboratory bench pending gas sampling. The 3-h incubation period is effectively terminated once gas is sampled from a vial using a syringe with a luer valve.

2. An advisable syringe technique is to cycle the required volume twice with the needle stationary through the septum prior to aspirating the final required volume.
3. The volumes to aspirate are 1 mL and 0.5 mL from the 20 mL and 2 mL vials, respectively. Piercing of the plant material should not occur. In the case of leaves, this is prevented by the technique with which the leaves are inserted into the vial, i.e., predominantly against the wall of the vial. In the case of the buds, the insertion of the needle is visibly controlled to avoid contact with the plant material. Moreover, the needle has a dome tip with a side hole.
4. Take care to close the luer valve while holding the plunger in position for the required aspiration volume as described under G10.
5. Document the sampling time of each vial.
6. Use a clean 3 mL syringe to flush needles that are alternating between sample vials to ensure any C_2H_4 gas is expelled from the needles.
7. Once a headspace volume is aspirated into a syringe and locked, the syringe can be stored for the duration of the sequence of chromatography measurements.
8. Inject gas samples manually into the GC in accordance with a programmed sequence file.
9. The needle can be inserted with its full length through the injector's septum as described under G13.

I. Weighing of plant material

1. Following chromatography analysis, remove the 20 mL vial caps with a pair of pliers and unscrew the caps of the 2 mL vials for weighing the vials individually with their contents.
2. Remove the contents and weigh the masses of the empty 20 mL and 2 mL vials as associated with each sample number.

Data analysis

1. Use the "Quan Browser" function in the Xcaliber software to retrieve quantitative reports of calibration standards and unknown samples as exemplified in Figures 13 and 14. Note from Figure 14 that the peak status of the "blank" is indicated as "not found" by the software. This confirms that C_2H_4 is absent, hence no retention time can be identified or peak area calculated.

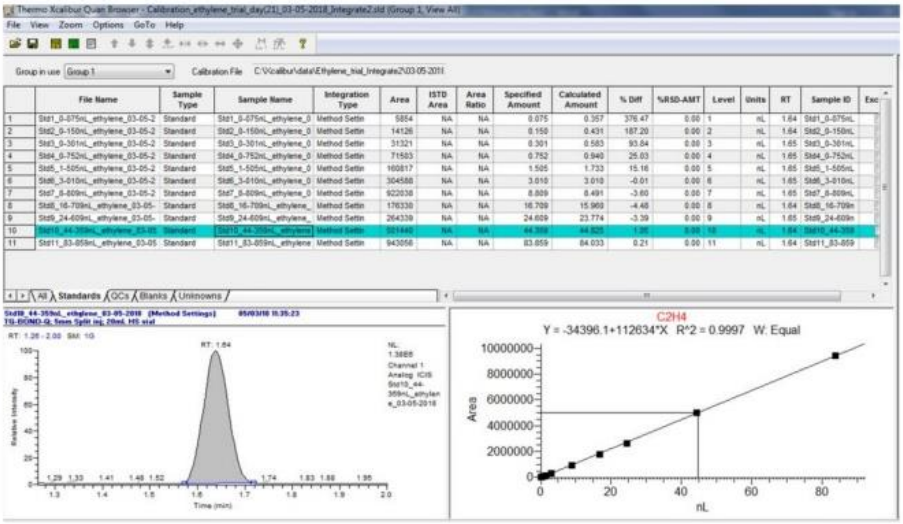

Figure 13. Example of quantitative data of calibration standards from the Xcaliber "Quan Browser" software

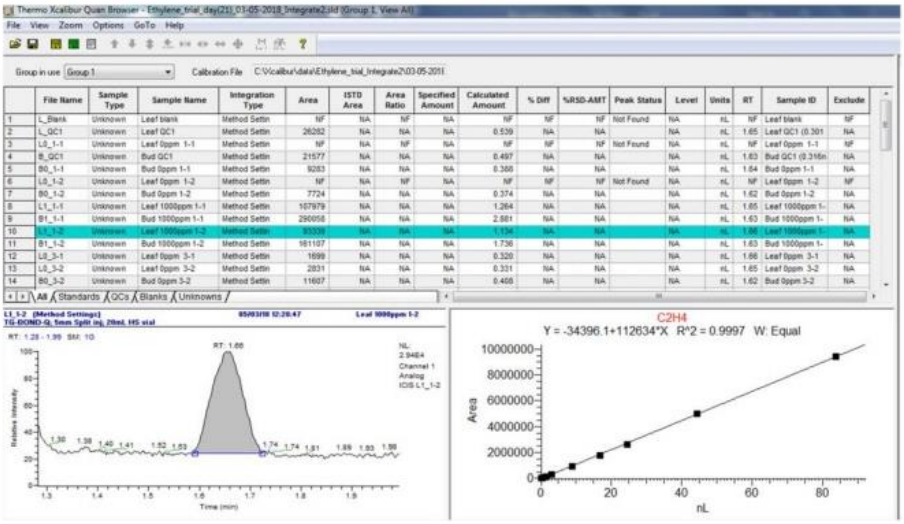

Figure 14. Example of quantitative data of unknown samples from the Xcaliber "Quan Browser" software

3. In the "Quan Browser" window, use the file menu "Export data to Excel" to create an Excel document with the measured data for individual sequences.
4. Perform data processing in Excel to calculate the specific C_2H_4 production rate, R, in $\mu L \cdot kg^{-1} \cdot h^{-1}$, using equation 5. Assignment of variables is as follows:
   GC_{C2H4} = GC analyzed C_2H_4 volume, i.e., calculated amount in Quan Browser (nL),
   V_{vial} = volume of vial (mL),
   V_{inj} = total volume of gas injected with syringe (mL),

m_{vc} = mass of vial and contents (g),
m_v = mass of empty vial (g) and
t = difference between time of sealing vial and time of gas aspirated from vial (h).

$$R = \frac{\frac{GC_{C2H4}\cdot\left(V_{vial}+V_{inj}\right)}{\left(\frac{V_{inj}}{1\times10^3}\right)}\cdot\left(\frac{1}{1\times10^6}\right)}{\left(\frac{m_{vc}-m_v}{1\times10^3}\right)\cdot(t_a-t_s)} \quad \text{.....(5).}$$

Notes

1. Depending on the size of the plant material to be analyzed, the number of plant organs and/or vial sizes can be adjusted.
2. A minimum of 5 biological replicates, with sub-samples per replicate, is advisable as considerable variation in C_2H_4 readings can be expected after exogenous C_2H_4 application, e.g., through ethephon application.
3. The duration for wound ethylene release and incubation period can differ when plant tissues of other crops and vial volumes, respectively, are used as study material. It is advisable to determine these periods before the experiment is conducted.
4. As a cautionary point, it is important to perform timing accurately to ensure that the incubation time is the same for all samples.
5. It is advisable to perform ad-hoc qualitative chromatography of typical samples prior to systematic quantitative measurements to assess the expected maximum and minimum levels of C_2H_4. The observed peak areas can serve as a guide to the required calibration ranges.
6. Table 3 lists the retention time of C_2H_4 as 1.64 min. Slight deviation from the expected 1.64 min may still occur even if the protocol is reproduced in its totality. The retention time for C_2H_4 (or any compound of interest) can be identified or confirmed by an increase in peak area following the measurement of GC traces for a sequence of standards with increasing C_2H_4 concentration.
7. Calibration curves with a correlation coefficient > 99.0% can be easily attained with the described technique.
8. It is worthwhile to include blank and QC injections intermittently during a sequence to calculate precision data and to validate the measurements of unknowns.

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Competing interests

The authors declare no financial and non-financial competing interests.

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