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Trinexapac-Ethyl Dose–Response Curve for Eucalyptus Growth and Hormonal Crosstalk Between Leaf and Shoot Apical Bud

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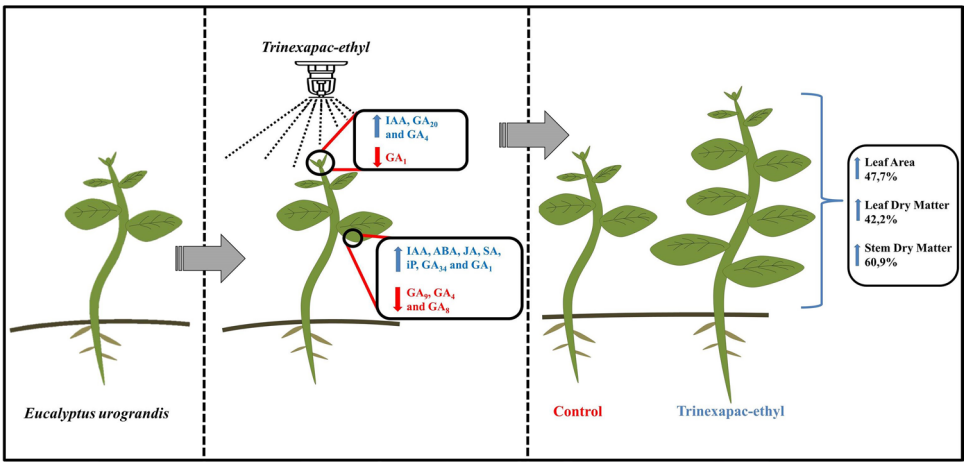
Abstract

Although recent studies have reported stimulatory effect of trinexapac-ethyl (TE) on eucalyptus growth, there is no consensus regarding the best dose to promote this response. Since TE acts in the gibberellin (GA) biosynthesis pathway, the study of hormonal crosstalk between the leaves and the shoot apical bud (SAB) can provide important information for understanding the positive effect previously reported. We evaluate the TE dose–response curve for eucalyptus growth in different soil moisture conditions (well watered—WW and 40% of field capacity—40-FC) and its effects on plant physiology, as well as the hormonal crosstalk between the leaves and SAB. TE caused a 49% increase in WW eucalypt growth, but not to plants under 40-FC. Estimated dose for the greatest stimulatory effect on WW eucalypt plants is 202 g a.i. ha^{-1}. TE did not cause an increase in the plants' photosynthetic characteristics up to 15 days after application (DAA), suggesting a later increase in the eucalypt's primary metabolism. Conversely to what have been reported for monocot crops, TE caused a fivefold increase in leaf GA_1 as a short-term effect (05 DAA), but significantly decreased SAB-GA_1 concentration. Leaf concentrations of indole-3-acetic acid, salicylic acid, abscisic acid and N^6-isopentenyladenine also increased. TE caused changes in both 13-hydroxylated (GA_{20}, GA_1 and GA_8) and non-13-hydroxylated (GA_9, GA_4 and GA_{34}) GA metabolic pathways in an organ-specific manner. Our results provide information to support the use of this plant growth regulator in eucalyptus plantations, as well as insights into the hormonal crosstalk between leaves and SAB in response to TE.

Graphical Abstract



Keywords Plant growth regulator · Gibberellin pathway · Eucalyptus physiology · Stimulatory effect

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Introduction

Given the importance of eucalyptus cultivation worldwide, there are currently more than 20 million hectares of planted forests (Fao 2013), of which 7.5 million hectares are located in Brazil (Ibá 2022). The Brazilian forestry sector has considerable relevance in the country's economy, as it generates around US$ 17.3 billion annually, which represents 1.2% of the national GDP (Ibá 2019). With the highest eucalyptus productivity in the world, Brazil averaged 38.9 $m^3\ ha^{-1}\ year^{-1}$ in 2021. This is due to the development of more efficient management strategies and genetic improvement programs (Pereira et al. 2012; Gonçalves et al. 2013; Ibá 2022).

The initial growth phase of eucalyptus, the first year after crop planting, is when plants are most susceptible to interference caused by stress factors (Nambiar and Sands 1993; Garau et al. 2008). Low water availability is one of the most common and can cause losses of up to 44 and 47% in the production of stem and leaves, respectively (Correia et al. 2014). Under these conditions, the decrease in plant growth occurs due to several physiological, biochemical and molecular changes (Lawlor 2009; Pinheiro and Chaves 2011). As a signaling factor, the production of abscisic acid is stimulated (Correia et al. 2018), which mediates processes that promote stomatal closure, decreasing gas exchange. As a consequence, there is a limitation in the carbon fixation process, thus reducing plant growth and productivity (Bedon et al. 2011; Pinheiro and Chaves 2011; Correia et al. 2014, 2018).

With the expansion of eucalyptus cultivation across one of the most diverse regions in the world (Fao 2013), where there are many edaphic systems with low water availability, studies focusing on alternative ways to assist eucalyptus seedlings to deal with these stress related conditions are needed. The use of trinexapac-ethyl (TE) could be a viable option since recent research reported gains up to 70% for eucalyptus initial growth with its application, along with cultivating plants under adequate irrigation conditions (Pires et al. 2013, 2019; Correia and Villela 2015; Bacha et al. 2017).

The utilization of TE to promote the potential stimulatory effect on eucalyptus seedlings came from experiments examining drift, due to the proximity of eucalyptus and sugarcane cultivation areas in the State of São Paulo, Brazil (Pires et al. 2013). Although several studies have been carried out to examine this affect (Pires et al. 2013, 2019; Bacha et al. 2017, 2018, 2019), all of these used low concentrations of TE and none examined the dose–response that would result in the greatest stimulus to eucalyptus growth, being a notable gap in information.

Trinexapac-ethyl is an acylcyclohexanedione that acts in the final stages of gibberellin biosynthesis (Rademacher 2000). This plant growth regulator (PGR) is frequently used as a ripener in sugarcane cultivation and as a growth reducer in winter cereals where application can reduce lodging. TE causes a reduction in internode elongation in cereal crops (Rademacher 2000; Nascimento et al. 2009; Moddus 2023). At the molecular level, TE reduces the conversion of GA_{20} to GA_1, due to competition between TE and 2-oxogluterate for the Fe^{+2}/ascorbate-dependent dioxygenase cosubstrate (Adams et al. 1992). Despite this, Rademacher (2016) emphasized a possible paradoxical effect of TE on plants, since it is expected to cause a drastic reduction in the level of GA_1 due to the inhibition of the 3β-hydroxylase enzyme (Nakayama et al. 1990) and thus markedly increasing its immediate biosynthetic precursor, GA_{20} (Adams et al. 1992; Hedden 2020). In addition to avoiding hydroxylation at the 3β position, for the formation of GA_1 (Adams et al. 1992), Hisamatsu et al. (1998) observed that TE also inhibits hydroxylation in the 2β position, preventing already existing GA_1 from being transformed into GA_8 (inactive form). Thus, three hypotheses are raised as the causes of the stimulatory effects previously observed in eucalyptus plants, which can also occur simultaneously: (i) the accumulation of GA_1, due to non-conversion to GA_8, has its action prolonged; (ii) the accumulation of GA_{20}, due to its non-conversion to GA_1, causes an overproduction of GA_1 after TE degradation; (iii) the oscillation in the gibberellin biosynthesis pathway, caused by TE, results in a hormonal imbalance which may lead to the overproduction of other hormones (or a synergistic effect between them), resulting in the positive effects observed in previous studies (Pires et al. 2013, 2019; Correia and Villela 2015; Bacha et al. 2017, 2018, 2019).

Therefore, research was conducted to (i) unveil the TE effect on hormonal crosstalk between leaves and shoot apical bud of *Eucalyptus urograndis* (Clone 1407), (ii) investigate the effect of increasing doses of TE on the physiological characteristics of plants under different soil moisture conditions and (iii) establish the dose that causes the greatest stimulatory effect on eucalyptus growth.

Materials and Methods

Trinexapac-Ethyl Dose–Response Curve for Eucalypt Growth

Greenhouse Experiment, Plant Material and Treatments

The first experiment was conducted in greenhouse conditions at the Sao Paulo State University (UNESP/FCAV) located in the municipality of Jaboticabal-SP, Brazil. The climate of the region, according to the Köppen (1948)

classification, is Cwa, subtropical, dry in winter, with summer rains (meteorological data from the experimental period in supplementary data—Table S1). The altitude is 590 m and the geographic coordinates are latitude 21° 15′17″ S and longitude 48° 19′ 20″ W.

The experiment was conducted for 90 days after planting (DAP) of eucalypt plants in 25-l pots. A mixture of Dark Red Latosol and sand in a 2:1 (v/v) ratio was used as substrate (soil physical–chemical characteristics in supplementary data—Table S2).

Commercial *Eucalyptus urograndis* (Clone 1407) seedlings, approximately 100 days old, 33.5 ± 0.91 cm tall, 2.81 ± 0.09 mm in stem diameter and 1.63 ± 0.09 g of total dry matter (mean of eight seedlings), were purchased from Agriflora® (Araraquara-SP, Brazil).

A randomized block experimental design was used, with five replications, with treatments arranged in a 2 × 7 factorial design. Factors constituted two soil moisture conditions of well watered (WW) and at 40% of field capacity (40-FC) and seven doses of TE (Moddus®) at 0 (non-treated control), 15, 30, 60, 120, 150 and 300 g of active ingredient (a.i.) ha^{-1}. To maintain seedlings at 40-FC, substrate moisture monitoring was carried out with the Falker® HidroFarm sensS1or (model HFM 2030), considering values between 20 and 22% for WW plants and 7–10%, which was equivalent to an average of 40% of field capacity, for plants maintained at 40-FC (supplementary data Figure).

Trinexapac-ethyl Application

Before planting, the eucalyptus seedlings (yet in 50 mL tubes) were sprayed with TE at the doses previously mentioned. For this, a backpack CO_2 sprayer was used, working at constant pressure, equipped with a bar with two TT 110.02 tips and adjusted to spray a tank volume of 200 L ha^{-1}. At the time of application, which took place in an experimental spraying room, the air temperature and humidity were 27.3 °C and 71%, respectively.

Twenty-four hours after TE application, all seedlings were planted in the pots with soil moisture already adjusted according to each treatment. The experimental plot consisted of a pot with one eucalyptus seedling, totaling 70 experimental plots.

Assessed Variables

We evaluated the total relative chlorophyll content, gas exchange and the maximum quantum yield of photosystem II (PSII) always at 9 am. Thus, from 9 to 15 DAP, in the third fully expanded leaf, the net CO_2 assimilation rate (A), transpiration rate (E), intercellular CO_2 concentration (C_i) and stomatal conductance (g_s) were measured with an infrared gas analyzer (IRGA mod. LCpro-SD, ADC BioScientific®). To this end, we used working conditions of 19 mmol H_2O mol^{-1}, 398 µmol CO_2 mol^{-1}, flow rate of 400 µmol s^{-1}, atmospheric pressure of 1000 kPa and the photosynthetically active photon flux (quantum) in 1100 µmol m^{-2} s^{-1}. From these data, the water use efficiency (A/E—WUE) and the instantaneous carboxylation rate (A/C_i) were calculated. On the same leaf, the maximum quantum yield of photosystem II—F_v/F_m (fluorimeter, mod. MINI-PAM-II, Walz®) was also measured at 11, 12, 13, 14 and 15 DAP, and the relative total chlorophyll content (chlorophyllometer, mod. ClorofiLog, Falker®) at 14, 15, 19, 20, 26, 27 and 28 DAP.

At 6, 20, 35, 47, 64, 78 and 90 DAP, plant height (cm) and stem diameter (caliper mm) were evaluated. At the end of the experimental period (90 DAP), the plants were cut at the soil surface and leaves were detached for leaf area determination (LiCor®, mod. LI 3100 A). Leaves and stems were dried with a forced air circulation oven (70 °C) for 96 h, and then dry matter mass (g) was evaluated.

Trinexapac-Ethyl Effect on Hormonal Crosstalk Between Leaf and Shoot Apical Bud

Eucalyptus Planting, Treatments and Plant Material Collection

The second experiment was conducted in an open area, at the same location previously described, for 18 DAP in 10-l pots. The same commercial seedlings of *E. urograndis* (Clone 1407) were used. These plants were approximately 100 days old, 31 (± 0.72) cm tall, 2.63 (± 0.07) mm stem diameter and had 1.45 (± 0.12) g of total dry matter (mean of eight seedlings).

A randomized block experimental design was used, with five biological replications, with treatments arranged in a 2 × 2 factorial scheme. The factors constituted of two organ collections (leaves and shoot apical bud—SAB) and two TE doses: 60 g a.i. ha^{-1} and a non-treated control (0 g). Plants were grown under WW conditions with daily irrigation. This experiment was concomitantly done with the dose–response study. The TE dose of 60 g a.i. ha^{-1} was chosen as it had been reported to cause a stimulatory effect on eucalyptus (Pires et al. 2019). Prior to planting, eucalyptus seedlings were treated with TE following the same methodology previously described. Twenty-four hours after TE application, all seedlings were planted in the pots.

Plant samples for hormonal analyses were carried out at 5 and 18 DAP. The collected plant material consisted of the third fully expanded leaf from the main stem, and the SAB (together with the first pair of leaves, measuring up to 25 mm) from the main stem and lateral branches. Plant samples were immediately frozen in liquid nitrogen, homogenized, lyophilized, weighed (about 30 mg) and sent

for hormonal quantification analyses. Three replicates per treatment were used, collected from a pool of five biological replicates.

Hormone Quantification Analyses

The extraction of plant tissue samples collected at 05 and 18 DAP was performed by adding 2.0 mL of the extracting solution (80% methanol, 1% acetic acid and 19% distilled water) to the tubes. The internal standards of the respective hormones (deuterium-labeled hormones, purchased from OlChemim® Ltd, Olomouc, Czech Republic) quantified were added, which consisted of a mixture of 80 μL containing the deuterated hormones: 10 ppb of gibberellins (d_2-GA_1 and d_2-GA_4,) and cytokinins (d_3-DHZ, d_6-iP and d_5-tZ); and 1000 ppb of abscisic acid (d_6-ABA), indoleacetic acid (d_5-IAA) and salicylic acid (d_4-SA). For the samples collected at 18 DAP, only GAs (GA_{19}, GA_{20}, GA_1, GA_8, GA_{24}, GA_9, GA_4 and GA_{34}) were quantified. This mixture remained under stirring (Ika®, VXR basic Vibrax®, 1000 vibrations min^{-1}) for 60 min at 4 °C. After this procedure, the samples were centrifuged (Eppendorf®, mod. 5415R) at 10,000 g for 4 min at 4 °C. The supernatant was removed and placed in a new 2 mL tube and stored for 24 h at − 20 °C for protein precipitation. Afterward, the samples were centrifuged at 10,000*g* for 4 min at 4 °C. The supernatant was transferred to 5 mL glass tubes and concentrated in an evaporator (Thermo Scientific®, Savant SPD1010 SpeedVac Concentrator) for 3 h at ambient temperature. Concentrated samples were made up to 1 mL with 1% acetic acid. After mixing with a vortex, the crude extract (30 mg) was loaded through Oasis HLB® columns (reverse phase). Elution of hormones was performed by applying 1 mL of 95% methanol. The samples were dried in an evaporator and the dried residue was dissolved in 1% acetic acid and the extracts were additionally passed throught an Oasis® MCX (cation exchange). For GAs, IAA, ABA, SA and JA quantification, the dried eluate was eluted with 100% methanol–1% acetic acid, to recover the acid fraction. CKs were eluted with 60% methanol–5% NH_4OH from the Oasis® MCX column to obtain the basic fraction containing cytokinins. Finally, the hormones were separated by ultrahigh performance liquid chromatography (UHPLC) with Accela 11,250 Pump (Thermo Scientific®, Waltham, MA, USA) and detected by a high-resolution mass spectrometer (Q-Exactive Orbitrap mass spectrometer, Thermo Fisher Scientific®, San Diego, CA, USA) (Seo et al. 2011).

Statistical Analysis

The growth and physiological data were submitted to a two-way analysis of variance (ANOVA) using the *F* test.When significant means were compared using the Tukey test at a 5% probability level. A regression analysis was performed for the TE dose–response curve using the second-order polynomial model. To compare the hormonal variation between TE and control, considering both organs separately, the Student's t test was used at the level of 5% probability, with the aid of AgroEstat® software. To enhance eucalyptus' hormonal variation, data from treated plants were transformed into a percentage of the non-treated control (supplementary data Figures S2 and S3). The transformed data were also used for bivariate analysis, represented by Pearson's heatmap matrix correlation.

Furthermore, to identify the effect of TE on hormonal variation in leaves and SAB, a principal component analysis (PCA) was performed. The process was carried out by reducing the multivariate data matrix to an interpretable two-dimensional biplot that explains the greatest proportion of variation in the data obtained in the samples evaluated at 5 DAP. To create the graphics, Origin v. software was used 9.0 (Microcal®).

Results

Trinexapac-Ethyl Dose–Response Curve for Eucalypt Growth

Gas Exchange Parameters

Under WW conditions, none of the TE doses were consistently different from each other ($p > 0.05$—data not shown) for all gas exchange variables evaluated (Fig. 1a–d). Considering the averages of both water conditions, TE at 30 g caused values greater than 150 and 300 g for *E*, and greater than 15 and 150 g for g_s (Table S3).

For plants under 40-FC (Fig. 2), there was an interaction between the factors for *A* at 9 DAP ($p < 0.05$—Table S4) where TE at 30 g resulted in values greater than those in the control and the highest TE doses, 150 and 300 g (Fig. 2a and Table S4). For all other evaluations, there was no difference considering all TE doses tested ($p > 0.05$).

Considering the average of the TE doses, WW plants exhibited higher values of *A*, *E* and g_s in all evaluation days (Fig. 3a–c, respectively). For C_i (Fig. 3d), WW plants exhibited higher values only at 9 DAP. Thus, from 11 DAP onward, plants under 40-FC had higher C_i, probably due to lower g_s (Fig. 3c).

The reduction in *E* and the increase in g_s (Fig. 3b, c, respectively), detected at 11 and 12 DAP for both water conditions, are possibly related to the weather conditions on those days. Skies were partialy covered by dust clouds (due to strong winds). Despite this, *A* was not affected (Fig. 3a), resulting in high WUE values (Fig. 3e).

At 10 DAP and forward, WW plants had higher WUE than those at 40-FC (Fig. 3e). As for A/C_i, the response

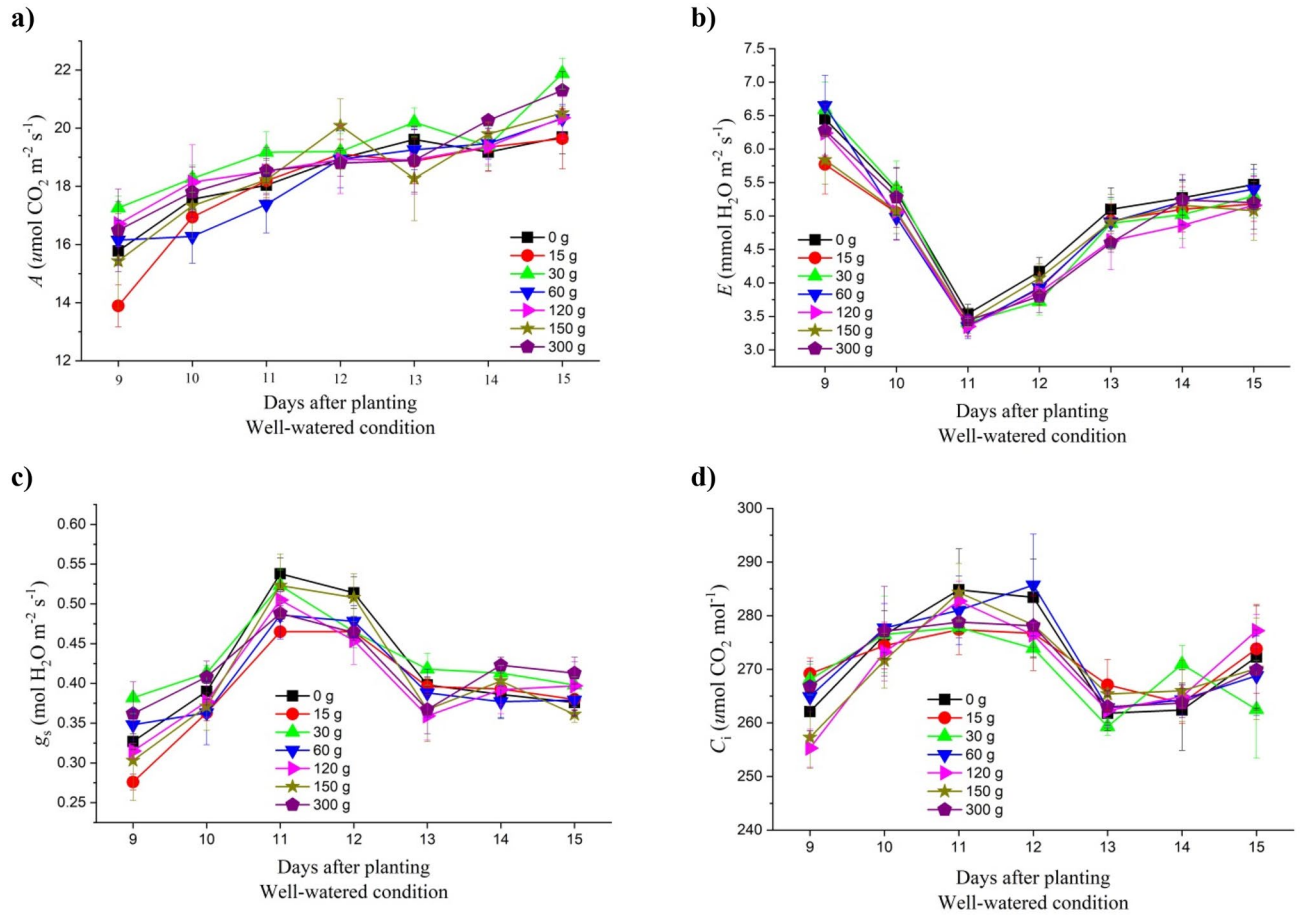

Fig. 1 Net CO_2 assimilation rate (A—**a**), transpiration rate (E—**b**), stomatal conductance (g_s—**c**) and internal CO_2 concentration (C_i—**d**) of *Eucalyptus urograndis* (Clone 1407) when plants were treated with increasing doses of trinexapac-ethyl (TE) and cultivated under well-watered conditions. 0, 15, 30, 60, 120, 150 and 300 g are equivalent to the doses in the active ingredient (a.i.) ha^{-1} of TE (Moddus®). $N = 5$

pattern was similar to that observed for A, with a significant difference on all evaluation days (Fig. 3f).

Maximum Quantum Yield of Photosystem II and Total Relative Chlorophyll Content

For both F_v/F_m and total relative chlorophyll content, no differences were noted comparing all TE doses ($p > 0.05$—Tables S5 and S6).

F_v/F_m, differences between water conditions were noted at 11 and 13 DAP, in which WW eucalyptus trees had the highest values (Fig. 4a). For total relative chlorophyll content, which was evaluated for a longer period, an inversion in the values recorded was observed comparing both water conditions. Up to 19 DAP, WW plants exhibited higher values (Fig. 4b), while at 20 DAP, no difference was observed. From then on, plants under 40-FC accumulated greater amounts of chlorophyll in their leaves (Fig. 4b).

Eucalypt Growth

For WW eucalyptus height, from 35 to 78 DAP, plants treated with TE at 150 g showed higher values than 0, 15, 30 and 60 g ($p < 0.05$—data not shown). At 90 DAP, only the treatment with TE at 30 g exhibited statistically lower values compared to that at 150 g (Fig. 5a).

For stem diameter, TE at 150 g provided greater growth stimulation, differing from the control from 78 DAP ($p < 0.05$—data not shown) (Fig. 5b). The other TE doses were not different from that of the control ($p > 0.05$), despite a tendency to obtain higher values (Fig. 5b).

For plants under 40-FC, 300 g provided greater growth in height, differing from the control at 78 DAP ($p < 0.05$—data not shown) (Fig. 5c). There was no difference between treatments for stem diameter throughout the experimental period ($p > 0.05$—Fig. 5d). At the end of the experiment, considering the average of all TE doses, WW plants

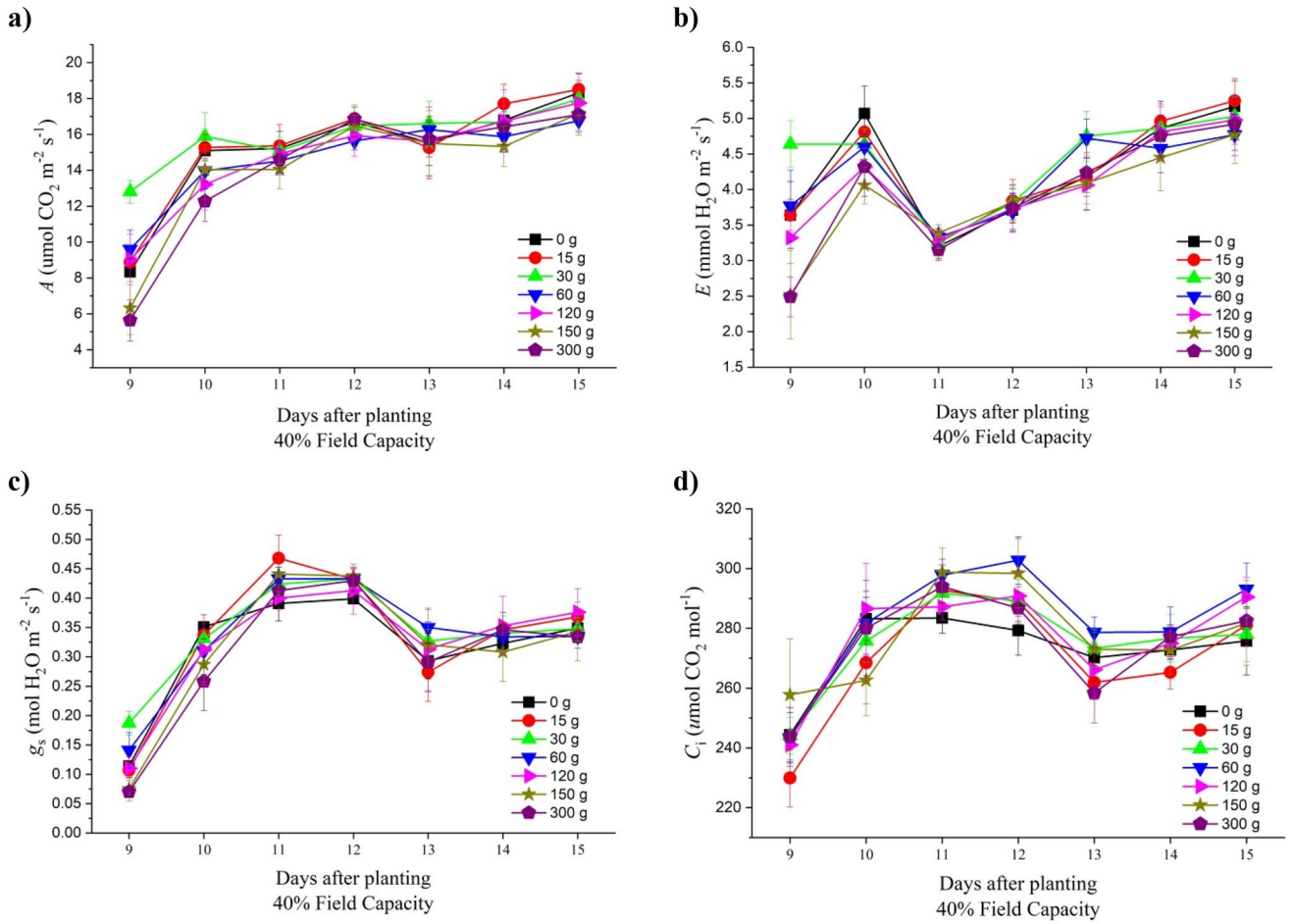

Fig. 2 Net CO_2 assimilation rate (A—**a**), transpiration rate (E—**b**), stomatal conductance (g_s—**c**) and internal CO_2 concentration (C_i—**d**) of *Eucalyptus urograndis* (Clone 1407) when plants were treated with increasing doses of trinexapac-ethyl (TE) and cultivated under 40% of field capacity. 0, 15, 30, 60, 120, 150 and 300 g are equivalent to the doses in the active ingredient (a.i.) ha^{-1} of TE (Moddus®). $N = 5$

exhibited height and stem diameters of 11.7 and 15.4% greater than those under 40-FC, respectively (Table S3).

For the variables leaf area, leaf dry matter, stem dry matter and total dry matter, the same response pattern was observed, in which the second-order polynomial regression curves crossed at TE doses lower than 60 g and tended to diverge between 120 and 240 g (Fig. 6). The smallest difference between both water conditions was observed for the leaf area (Fig. 6a).

Although the growth curves from plants at 40-FC showed a response pattern with the concavity facing upward, none of the values were statistically lower than that of the control for all variables ($p > 0.05$—Table S4). These data indicate that TE was not toxic to plants. Water was a limiting factor and was probably the reason why TE could not provide the extra growth to eucalyptus plants (Fig. 6 and Table S4).

Considering the second-order polynomial model for the total dry matter from WW plants, the dose that would provide the greatest stimulatory effect on eucalyptus growth would be 202 g a.i. ha^{-1} (Fig. 7a). For the total dry matter data transformed to the percentage of non-treated control, the three highest doses of TE provided an average increase of 45% in eucalyptus growth and were significantly greater than that of the non-treated control ($p < 0.05$—Fig. 7b).

Trinexapac-Ethyl Effect on Hormonal Crosstalk Between Leaf and Shoot Apical Bud

The concentrations of phytohormones in eucalyptus leaves at 5 DAA were positively ($p < 0.05$) changed for abscisic acid (ABA), salicylic acid (SA) and indoleacetic acid (IAA) with an increase between 65 and 83% compared to the non-treated control (Fig. 8a, c, d, respectively). N^6-isopentenyladenine

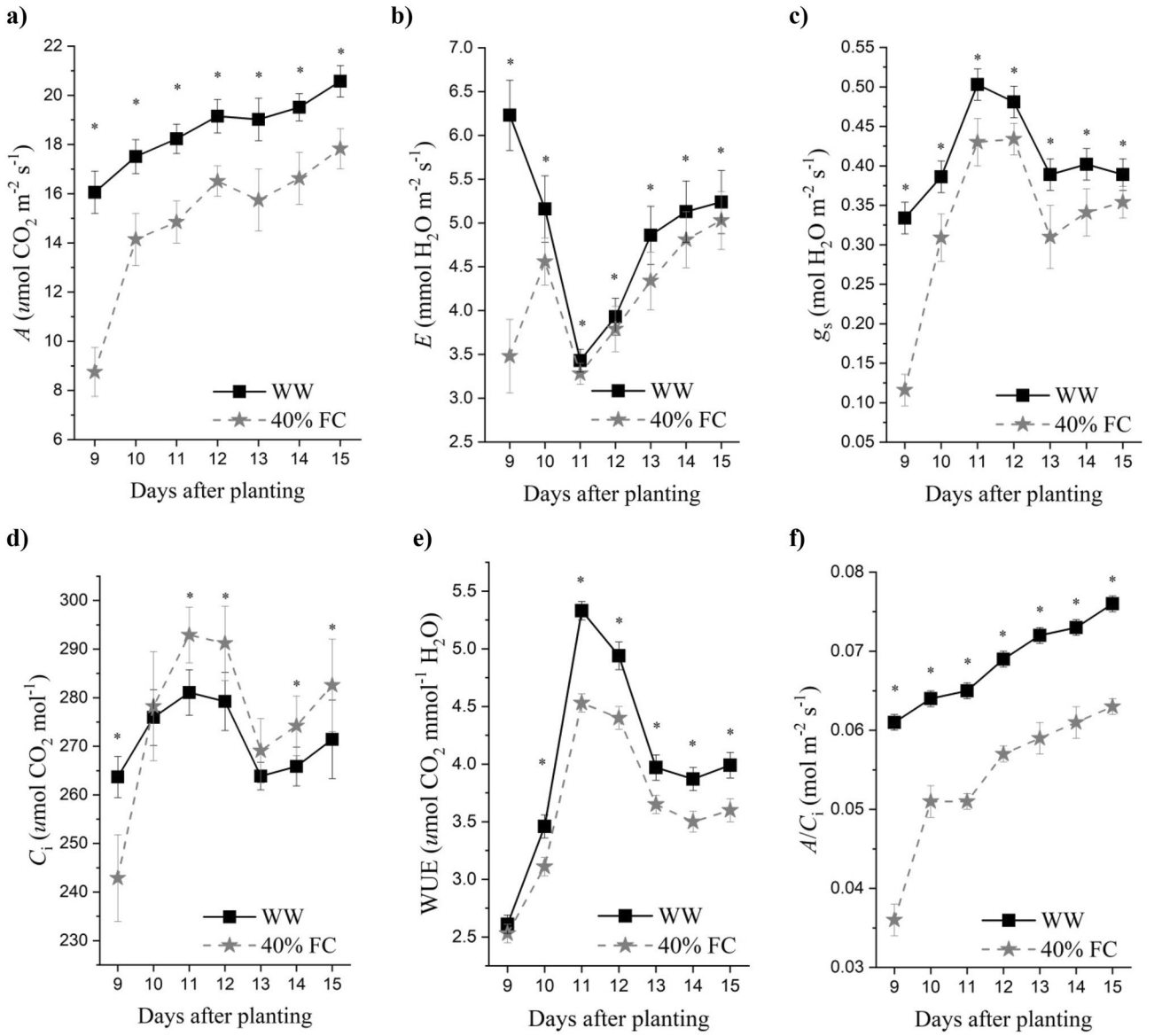

Fig. 3 Net CO_2 assimilation rate (*A*—*a*), transpiration rate (*E*—*b*), stomatal conductance (g_s—**c**), internal CO_2 concentration (C_i—**d**), water use efficiency (WUE—**e**) and instantaneous carboxylation efficiency (A/C_i—**f**) of *Eucalyptus urograndis* (Clone 1407) when plants were treated with increasing doses of trinexapac-ethyl (TE) and cultivated under well-watered (WW) and 40% of field capacity (40% FC) conditions. The values were obtained from the average of the TE doses. The asterisk indicates a significant difference (*F* test—$p < 0.05$) between water conditions for each evaluation period. $N = 5$

(iP) concentration increased nearly fourfold (Fig. 8f and Figure S2a). In SAB, the only hormone affected by TE was IAA, increasing its concentration by almost 35% compared to the control (Figs. 8d and S2b).

Regarding leaf gibberellins (leaf GA) evaluated at 5 DAA, the greatest changes occurred for compounds from the 13-hydroxylation pathway, especially for gibberellin A_8 (GA_8) (-493%—Fig. 9c) and GA_1 (+443%—Fig. 9b). At 18 DAA, both molecules showed an average concentration of + 129% compared to the control (Fig. 10b, c), while GA_{20} showed little change (Fig. 10a). For GAs from the non-13-hydroxylation pathway, the greatest changes were detected in the levels of leaf GA_9 (-62%), GA_4 (-110%) and GA_{34} (+81%) at 5 DAA (Fig. 9d–f, respectively, and Figure S3a).

The responses of SAB-GA_1 and GA_4 were opposite to that from the leaves at 05 DAA, decreasing by -72% for the first and an increasing 42% for the later, compared to the control (Fig. 9b, e, respectively, and Figure S4b). At 18 DAA, SAB-GA_1 and GA_{34} were upregulated by + 199 + 41%, respectively. It is worth noting that GA_8 concentration did

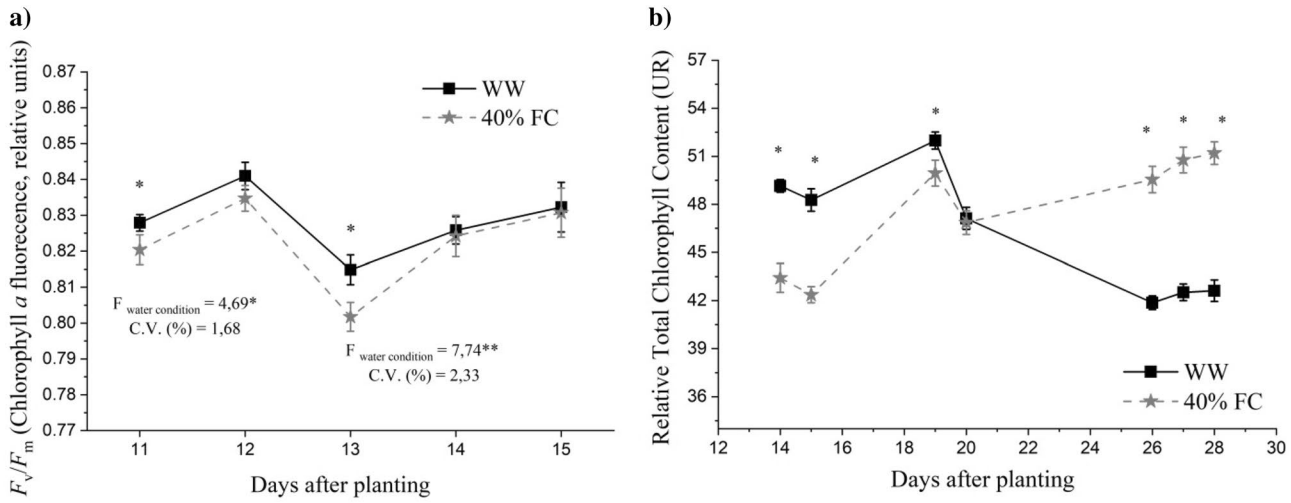

Fig. 4 Maximum quantum yield of photosystem II (F_v/F_m—**a**) and total relative chlorophyll content (**b**) of *Eucalyptus urograndis* (Clone 1407) plants sprayed with increasing doses of trinexapac-ethyl (TE) and cultivated under well-watered (WW) and 40% of field capacity (40% FC) conditions. The values were obtained from the average of TE doses. The asterisk indicates a significant difference (F test—$p < 0.05$) between water conditions for each evaluation period. $N = 5$

not change compared to the non-treated control, in contrast to what occured in the leaves (Fig. 10c).

To consider all hormones evaluated at 05 DAA, a principal component analysis (PCA) was performed to compare treatments (Fig. 11a). It was possible to separate the treatments into four distinct groups: the first is composed of leaves treated with TE, located in negative PC2 and directly correlated to GA_1 and iP; in positive PC2 and negative PC1, there was a grouping of control leaf, directly correlated to GA_8, GA_9 and DHZ (pink dot); SAB had the lowest variation among the organs studied, being grouped into PC1 and PC2 positive. The hormones directly correlated with these treatments were IAA, SA, JA, ABA, tZ, and GA_{20}. Although it is possible to distinguish SAB-TE from SAB-control samples, the grouping of both SAB treatments in this quadrant showed that this organ was less responsive to TE compared to the leaf (Fig. 11a).

The correlation of hormonal variation caused by TE in both organs is represented in the heatmap matrix (Fig. 11b). The hormones that showed the most negative correlations were DHZ and GA_4; however, only the latter had a significant correlation ($p < 0.05$) with GA_1, ABA, JA and SA. Among the other compounds, SA (correlated with GA_1, ABA, JA and iP), GA_1 (correlated with ABA, SA and iP) and iP (correlated with GA_1, SA and IAA) were those that showed the most directly proportional interactions ($p < 0.05$) (Fig. 11b).

Discussion

Trinexapac-Ethyl Effect on GAs Homeostasis and Organ-Specific Response for Both GAs Biosynthetic Pathways

In monocotyledonous crops, TE has been reported as a growth retardant due to the decrease in GA_1 concentration, one of the main bioactive gibberellins (Rademacher 2000; 2016; Ervin and Zhang 2007; Krishnan and Merewitz 2015; van Heerden et al. 2015; Hedden 2020). Conversely, the fivefold increase in leaf GA_1 concentrations found at 05 DAP (Fig. 9b) may be one of the justifications for the stimulatory effect observed here (Fig. 7) and in other studies with eucalyptus (Pires et al. 2013, 2019; Correia and Villela 2015; Bacha et al. 2017, 2018, 2019, 2024). In a woody species *Pinus densiflora*, Park et al. (2015) reported that a significant increase in endogenous GA_{20}, GA_1 and GA_8 was accompanied by increased stem growth. The GAs effect in stimulating cell elongation has been well studied in GA-deficient (Willige et al. 2007; Weier et al. 2014) and GA-overexpressing mutants (Eriksson et al. 2000; Park et al. 2015). In this sense, Liu et al. (2018) reported that GA_3 promoted root and shoot elongation accompanied by significant increase of xylem cell size and that response was directly related to GA20ox gene overexpression. Reports from the past decades have shown that the upregulation of GA20ox genes increases endogenous GA concentrations, enhancing shoot growth in herbaceous and woody plants (Huang et al. 1998; Coles et al. 1999; Carrera et al. 2000; Eriksson et al. 2000; Israelsson et al. 2003; Fagoaga et al. 2007; Mauriat

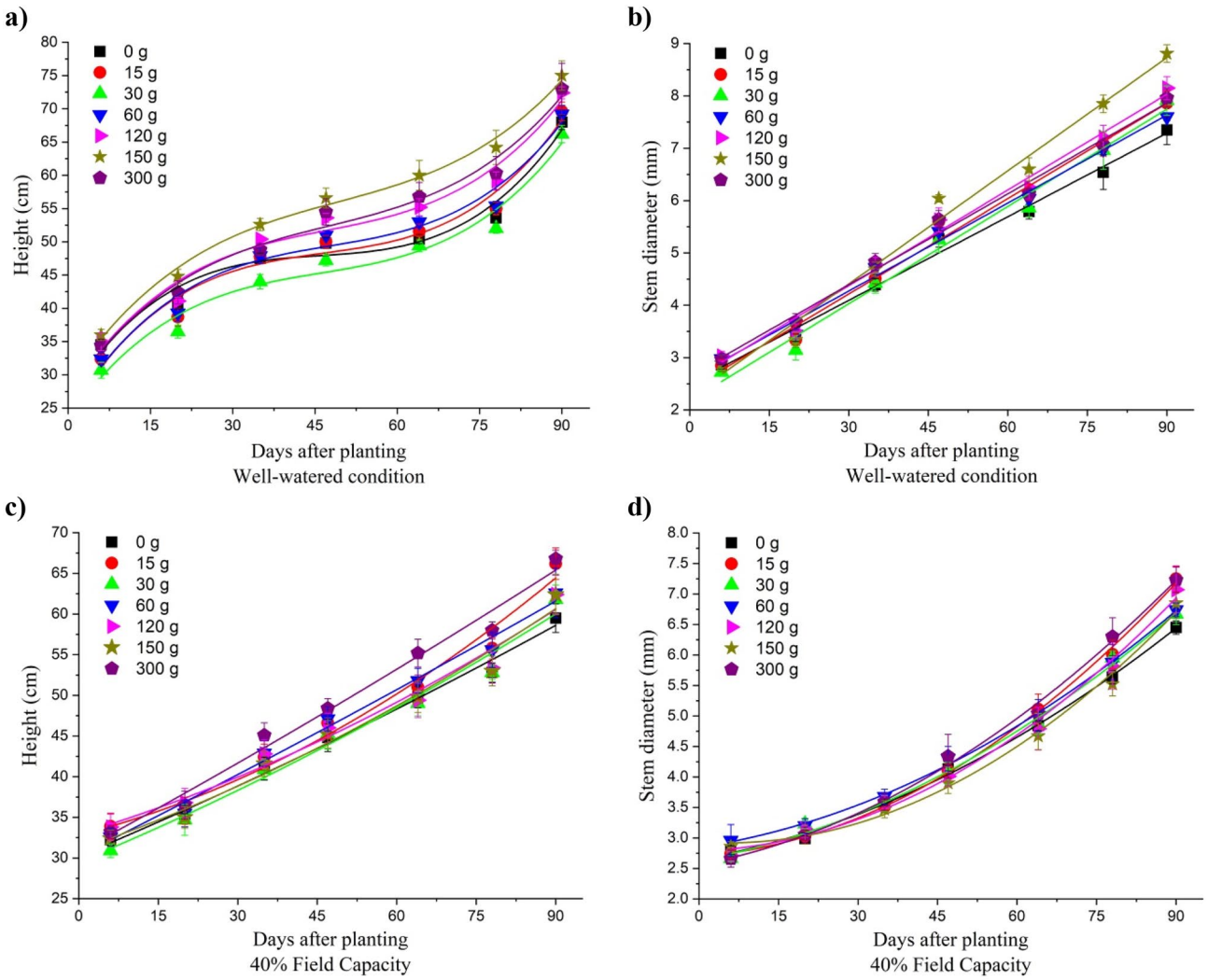

Fig. 5 Height (**a**) and stem diameter (**b**) of *Eucalyptus urograndis* (Clone 1407) plants sprayed with increasing doses of trinexapac-ethyl (TE) and cultivated under well-watered conditions; and height (**c**) and stem diameter (**d**) of plants grown under 40% of field capacity. 0, 15, 30, 60, 120, 150 and 300 g are equivalent to the doses in the active ingredient (a.i.) ha^{-1} of TE (Moddus®). $N = 5$

and Moritz 2009; García-Hurtado et al. 2012; Voorend et al. 2016; Nam et al. 2017).

Plant GA homeostasis is achieved by altering the biosynthetic reactions (controlling the expression levels of the GA20ox and GA3ox genes) or the GA inactivation (involving the expression level of GA2ox) (Hedden 2020). The disturbance of the GAs biosynthetic pathway would lead to increasing or decreasing levels of the products of these reactions, comprising GA_{20}, GA_1 and GA_8 for the 13-hydroxylation pathway, and GA_9, GA_4 and GA_{34} for the non-13-hydroxylation pathway (Hedden 2020). Our results suggest that both of these pathways were altered due to TE application in an organ-specific manner (Figs. 9, 10). In the leaves, at 5 DAP, the accumulation of GA_1 due to the inhibition of GA_8 conversion suggests the inhibition of the GA2oxidase enzyme (while the control concentration increased almost 5-fold) (Fig. 9b, c). Since GA homeostasis acts upon the transcription of 2-oxoglutarate-dependent dioxygenases (2-ODD) genes (Yamaguchi 2008; Hedden and Thomas 2012), possibly this accumulation of leaf GA_1 caused an increase in *GA2ox* expression up to 18 DAP through a feed-forward mechanism, suggesting an upregulation of GA2oxidase expression and increase in GA_8 concentration at this time. However, there was still a greater concentration of GA_1 in the TE-treated plants compared to non-treated control (Fig. 10b, c). Considering the non-13-hydroxylation pathway in the leaf, the effect was the opposite. There was a total conversion of GA_4 to GA_{34} at 5 DAP (Fig. 9e, f), suggesting high GA2oxidase activity. The concentration of leaf GA_9 was also reduced (Fig. 9d), possibly due to inhibition of GA20oxidase, once the level of GA_{24} increased during this period (Figure S4b). At 18

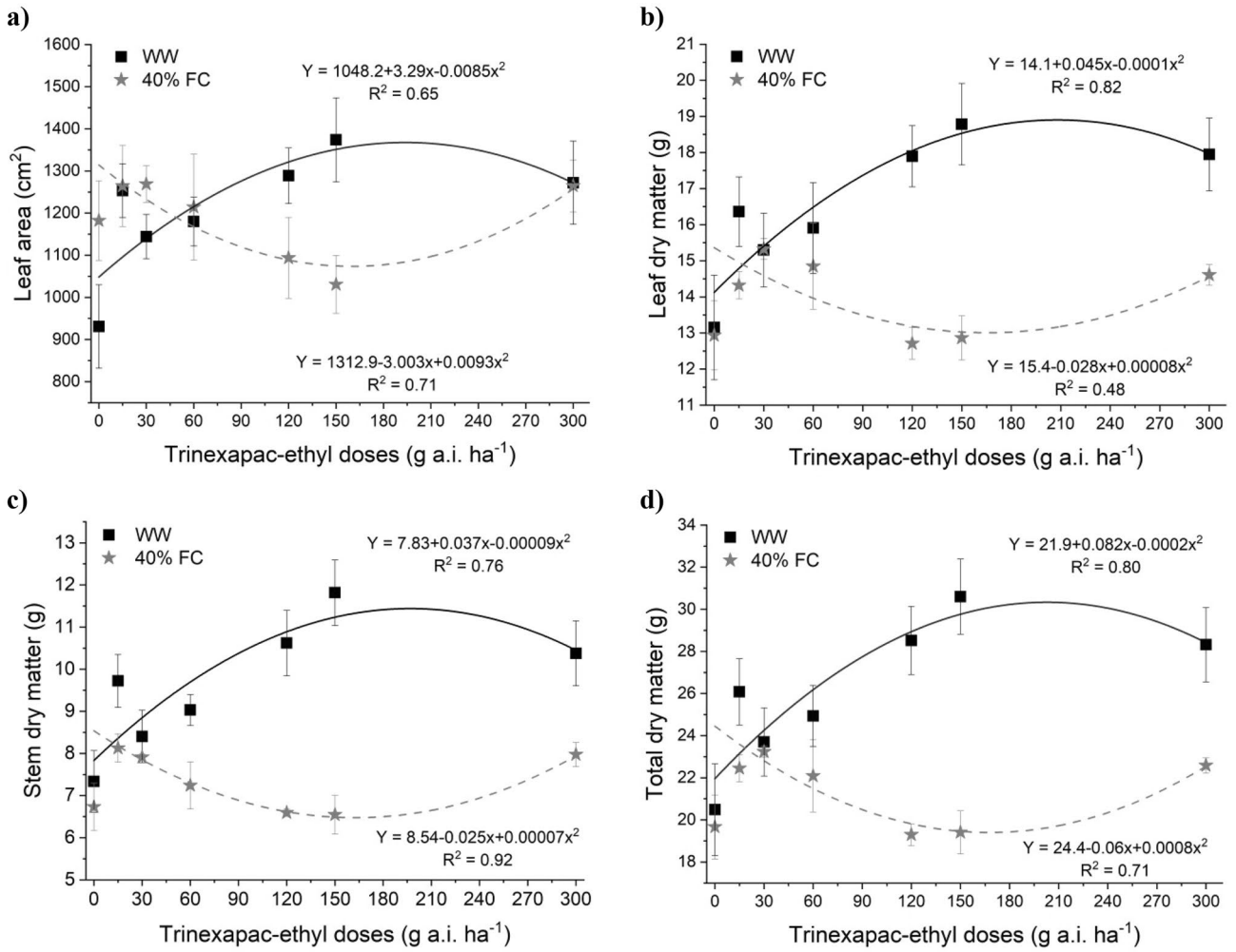

Fig. 6 Effect of increasing doses of trinexapac-ethyl (TE) on *Eucalyptus urograndis* (Clone 1407) leaf area (**a**), leaf dry matter (**b**), stem dry matter (**c**) and total dry matter (**d**) after 90 days of cultivation under well-watered (WW) conditions and 40% of field capacity (40% FC). The values were obtained from the average of TE doses. $N = 5$

DAP, there was a tendency for leaf GA_9 accumulation (almost a 3-fold increase—$p > 0.05$—Fig. 10d), possibly after TE degradation and a decrease in the previous inhibition of GA20oxidase, which started converting GA_{24} to GA_9 again. Further analysis of the determination of the inhibition constants for TE in the GA20ox-, GA3ox- and GA2ox-enzyme families should help clarify how this chemical actually acts on the GAs biosynthetic pathways in eucalyptus, since it appears to be in the opposite way to those reported in monocots (Rademacher 2000, 2016; Ervin and Zhang 2007; Krishnan and Merewitz 2015). In the SAB, at 05 DAP, the effect was the opposite compared to the leaf, for the 13-hydroxylation pathway. There was an inhibition of GA_{20} to GA_1 conversion reaction (and possibly GA3oxidase), causing an accumulation of GA_{20} and decreasing GA_1 concentration, compared to the control (Fig. 9a, b). At 18 DAP, there was a superconversion of SAB-GA_{20} to GA_1, causing accumulation of GA_1 (Fig. 10b), suggesting an increase in GA3oxidase activity after TE degradation. For the non-13-hydroxylation pathway, there was no effect of TE at 5 DAP (Fig. 9d–f). At 18 DAP, a greater accumulation of SAB-GA_{34} was noted (Fig. 10f), possibly due to the trend toward greater concentrations of GA_4 ($p > 0.05$—Fig. 10e). Despite recent reports indicating that disturbance in GA homeostasis caused by TE remains for at least 42 days after application (Bacha et al. 2024), information regarding the duration of TE action in plants is still incipient in the literature and should be the focus of future research. This is especially important when considering TE use in perennial crop cultivation. Although GAs can affect cell elongation, these molecules can also induce mitotic activity in the subapical region of the stem (Sauter et al. 1995). In meristematic tissues, it has been suggested that GAs extend the elongation

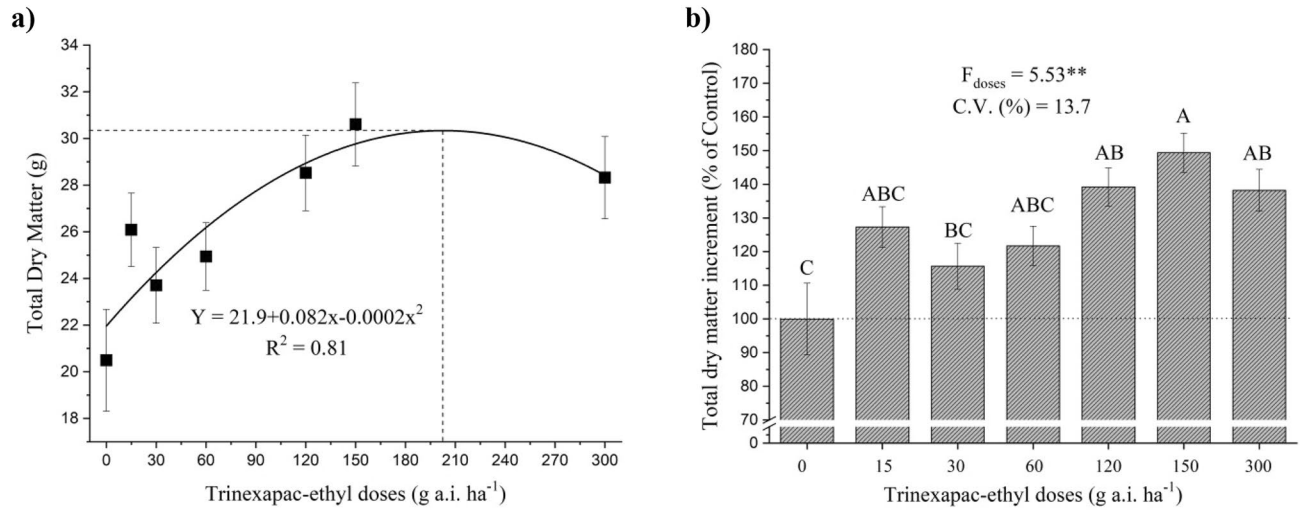

Fig. 7 Dose–response curve for total dry matter of *Eucalyptus urograndis* (Clone 1407) plants sprayed with increasing doses of trinexapac-ethyl and cultivated for 90 days under well-watered conditions (**a**). Data transformed to percentage from non-treated control (**b**). Means followed by the same letter do not differ from each other by the Tukey's test at 5% probability. ** = significant values at 1% probability level by the *F* test. C.V. = coefficient of variation. $N = 5$

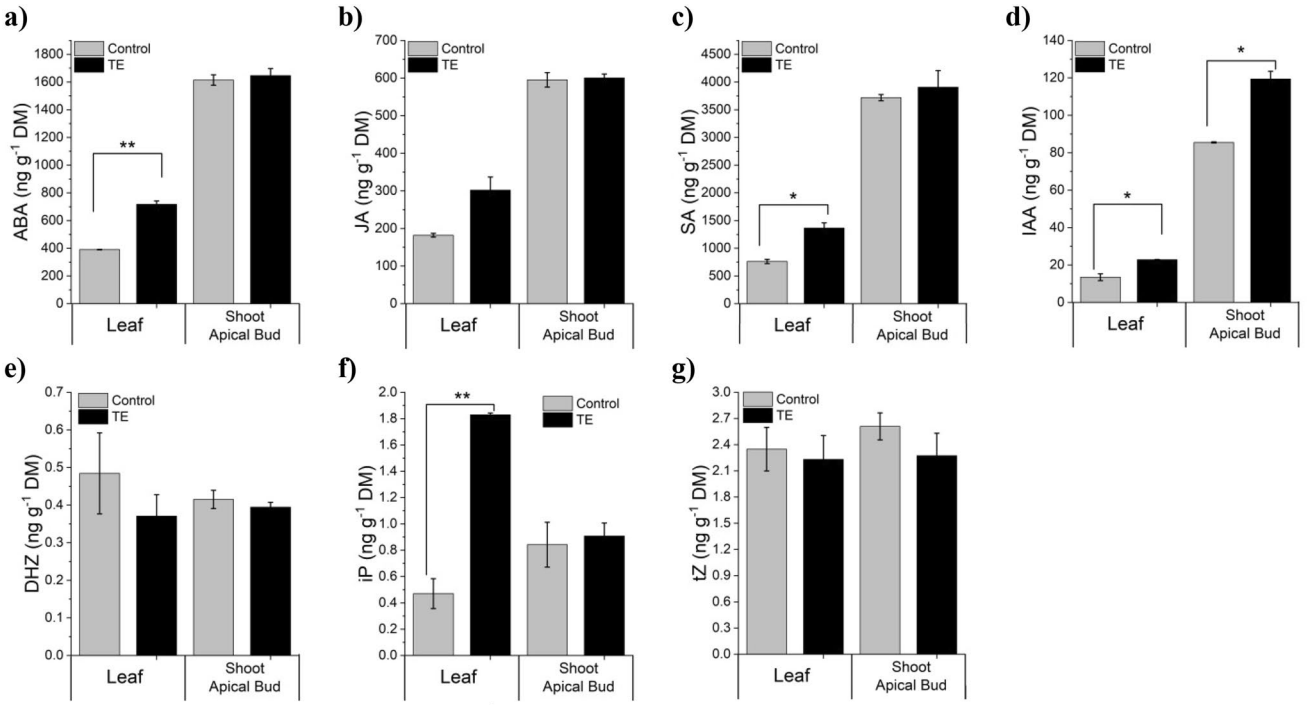

Fig. 8 Hormone concentration [ng g^{-1} of dry matter (DM)] in leaves and shoot apical bud of *Eucalyptus urograndis* (Clone 1407) plants at 5 days after application of 60 g a.i. ha^{-1} of trinexapac-ethyl (TE) and cultivated under well-watered conditions. * and ** = significant at 5% and 1% probability level by Student's t test, respectively. $N = 3$, collected from a pool of five biological replicates. **a** ABA = abscisic acid; **b** JA = jasmonic acid; **c** SA = salicylic acid; **d** IAA = indole-3-acetic acid; **e** DHZ = dihydrozeatin; **f** iP = N^6-isopentenyladenine; **g** tZ = *trans*-zeatin

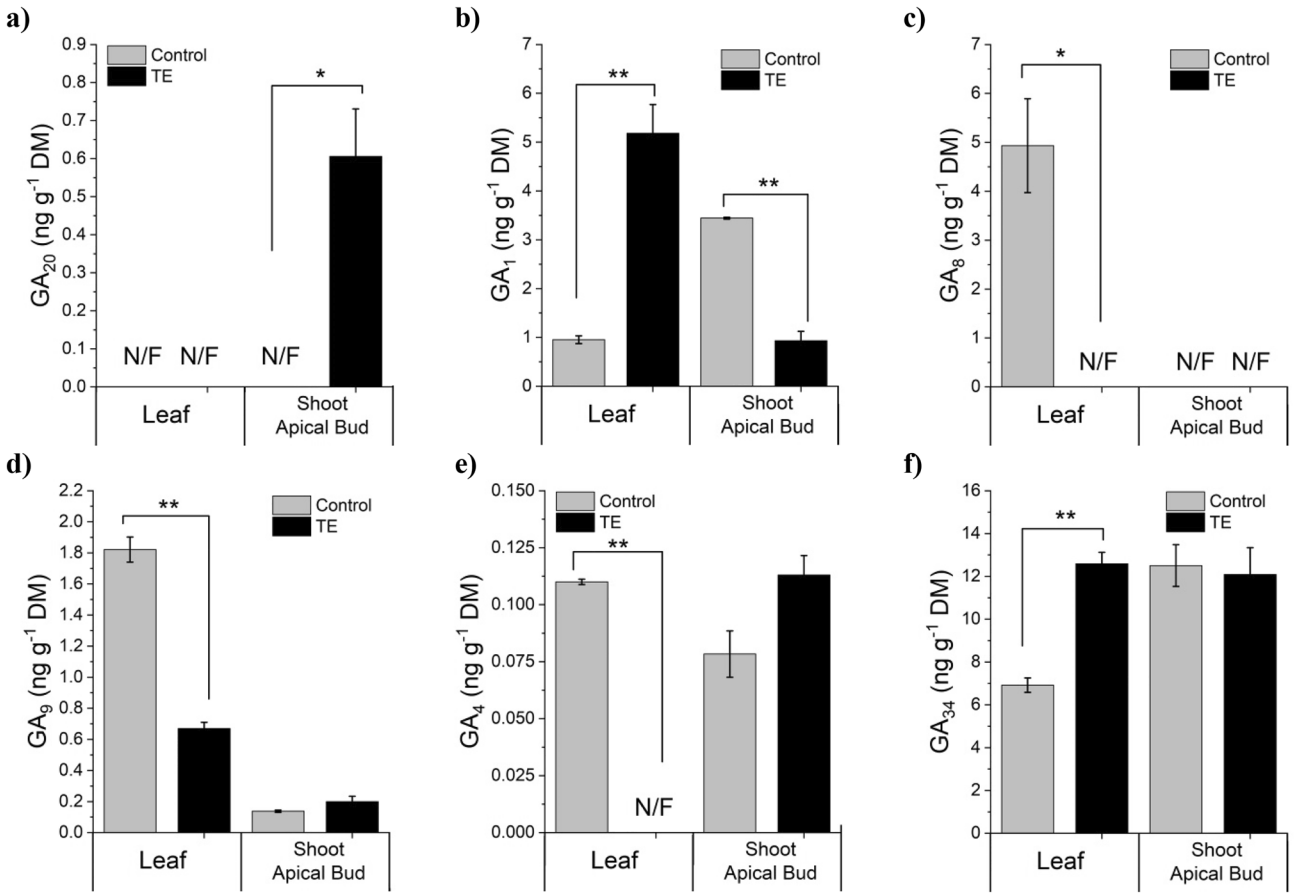

Fig. 9 Gibberellin (GA) concentration [ng g^{-1} of dry matter (DM)] in leaves and shoot apical bud of *Eucalyptus urograndis* (Clone 1407) plants at 5 days after application of 60 g a.i. $ha.^{-1}$ of trinexapac-ethyl (TE) and cultivated under well-watered conditions. * and ** = significant at 5% and 1% probability level by Student's t test, respectively. $N = 3$, collected from a pool of five biological replicates. **a** GA_{20} = gibberellin A_{20}; **b** GA_1 = gibberellin A_1; **c** GA_8 = gibberellin A_8; **d** GA_9 = gibberellin A_9; **e** GA_4 = gibberellin A_4; **f** GA_{34} = gibberellin A_{34}. N/F = not found (under the limit of detection)

zone of the organ being formed (Sauter et al. 1993; Sakamoto et al. 2001; King et al. 2008). Taken together, these results emphasize the TE influence on eucalyptus growth by altering GA homeostasis in an organ-specific manner.

However, despite the great importance of GAs homeostasis alterations for the eucalyptus growth reponse, we cannot attribute this effect only to the changing levels of GAs. Plant growth often occurs in response to a complex interaction among hormones (Gao et al. 2011), and altered GA levels can influence transport, signaling and accumulation of other hormones (Oh et al. 2007; Willige et al. 2011; Wang et al. 2015; Duan et al. 2019), such as ABA, SA, IAA and iP (Figs. 8, 11b).

Trinexapac-Ethyl Effect on Hormone Crosstalk

Regarding the crosstalk between GA and IAA in eucalyptus plants, the auxin-related genes EgrSUR2 and EgrPIN1 expression were found to be upregulated by GA treatment (Liu et al. 2018). Auxin transporter PIN1 was reported by Xu et al. 2005 to be involved in auxin-dependent adventitious root emergence and was superexpressed in *E. grandis* by exogenous GA application, mediating adventitious root elongation in eucalypt plants (Liu et al. 2018). This suggests that GAs may interact with IAA through the auxin transport pathway (Li et al. 2015), which could be another idicator for the extra growth caused by TE in eucalypt plants observed here (Fig. 7a, b). The increase in GA_1 caused by TE (Fig. 9b) may have similarly affected the expression of these genes. However, further studies with transcriptome analysis should be carried out to confirm such hypothesis.

Previous studies have reported that IAA can also act as a messenger from the apical bud to stimulate GA biosynthesis, resulting in stem elongation (Ross and O'Neill 2001; O'Neill and Ross 2002). This process occurs by activating the GA3ox and GA20ox genes and deactivating GA2ox, as seen in *Arabidopsis thaliana*, rice and pea (Frigerio et al. 2006; Yin et al. 2007; O'Neill et al. 2010) and can

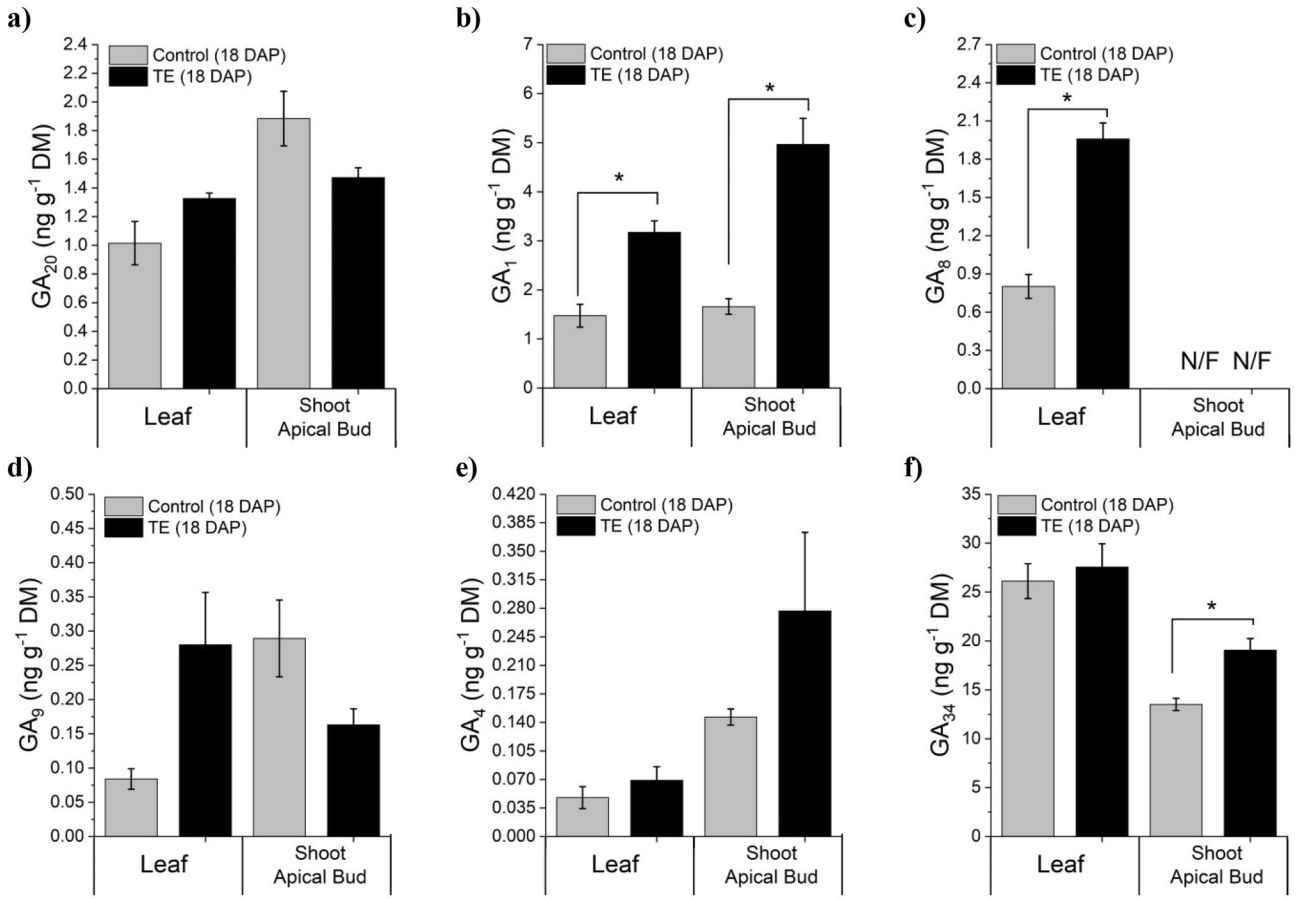

Fig. 10 Gibberellin (GA) concentration [ng g^{-1} of dry matter (DM)] in leaves and shoot apical bud of *Eucalyptus urograndis* (Clone 1407) plants at 18 days after application of 60 g a.i. $ha.^{-1}$ of trinexapac-ethyl (TE) and cultivated under well-watered conditions. *=significant at 5% probability level by Student's t test. $N=3$, collected from a pool of five biological replicates. **a** GA_{20}=gibberellin A_{20}; **b** GA_1=gibberellin A_1; **c** GA_8=gibberellin A_8; **d** GA_9=gibberellin A_9; **e** GA_4=gibberellin A_4; **f** GA_{34}=gibberellin A_{34}. N/F=not found (under the limit of detection)

occur either through a DELLA-independent pathway or via DELLA proteins (Weiss and Ori 2007). On the other hand, gibberellins could also modulate auxin-related genes, depending on the specific set of GA-induced auxin response factors (ARFs) (Oh et al. 2014; Zhang et al. 2019). For poplar trees, Park et al. (2015) reported that increased stem growth was associated with increased IAA and endogenous GAs contents (from both biosynthetic pathways). Simmilarly, Björklund et al. (2007) found that GAs increased IAA levels by stimulating its polar transport, and both hormones have a common transcriptome in *Populus tremulaxtremuloides*, including transcripts related to cell growth. In this sense, our results suggest that leaf GA_1 may have influenced the signaling route of IAA biosynthesis (Ross et al. 2003; Hu et al. 2018, 2022), since the transport of GAs also occurs via the phloem (Regnault et al. 2015; Binenbaum et al. 2018), and the increase in the concentration of leaf GA_1 (+5-fold—Fig. 9b) occurred simultaneously with the increase in SAB-IAA ($p<0.05$—Fig. 8d). Furthermore, at 18 DAA, there was a marked increase in theconcentration of SAB-GA_1(+3-fold—Fig. 10b), which possibly also contributed to the increase in eucalyptus growth observed (Fig. 7). Although a clear relationship between both hormones cannot be stated, as they share a positive influence on some aspects of development, they seem to present a synergistic response (Castro-Camba et al. 2022).

Considering the crosstalk between GAs and cytokinin (CK), we found a positive correlation between both hormones (Fig. 11a, b). Despite controversial responses regarding the synergistic or antagonistic effect between both (Jasinski et al. 2005; Yaarit et al. 2005; Yanai et al. 2005; Wang et al. 2015; Sugiura et al. 2015; Duan et al. 2019), in *E. grandis*, Liu et al. (2018) reported that GAs activate ISOPENTENYL TRANSFERASE 3 (IPT3), which is involved in CK biosynthesis (Frébort et al. 2011). In *Polygonum cuspidatum* the levels of endogenous CKs and GAs increased under high nitrogen conditions, indicating that both hormones act synergistically in the regulation of

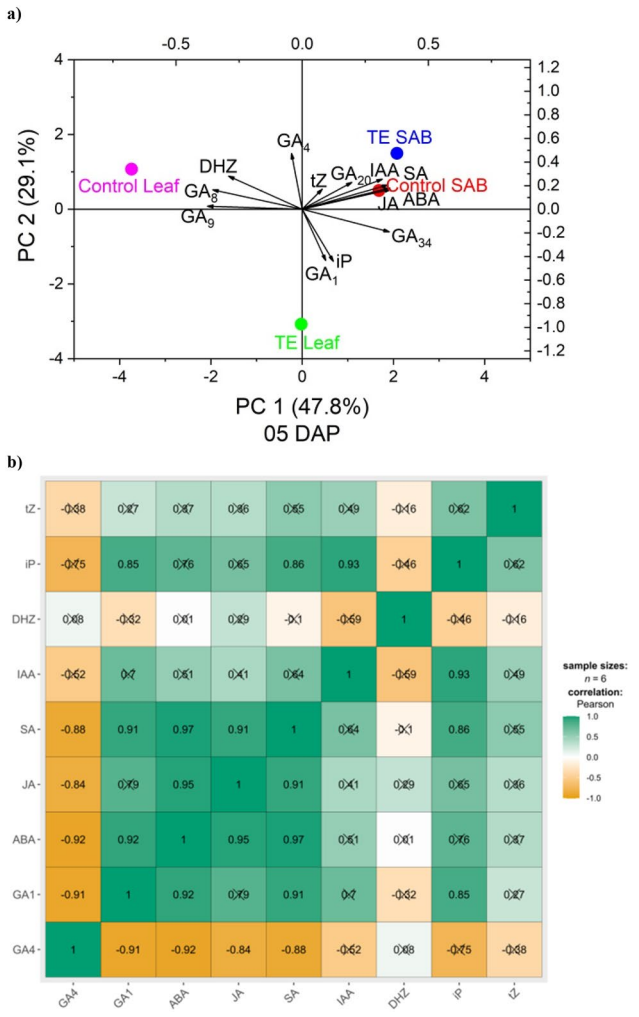

Fig. 11 Principal component analysis (**a**) and Pearson's heatmap correlation matrix (**b**) of hormonal data from leaves and shoot apical buds (SAB) of *Eucalyptus urograndis* (Clone 1407) plants at 5 days after application of 60 g a.i. ha^{-1} of trinexapac-ethyl (TE) and cultivated under well-watered conditions. ABA = abscisic acid; JA = jasmonic acid; SA = salicylic acid; IAA = indole-3-acetic acid; DHZ = dihydrozeatin; iP = *N*.^{6}-isopentenyladenine; tZ = *trans*-zeatin; GA_{20} = gibberellin A_{20}; GA_1 = gibberellin A_1; GA_8 = gibberellin A_8; GA_9 = gibberellin A_9; GA_4 = gibberellin A_4; GA_{34} = gibberellin A_{34}. X = non-significant at $p < 0.05$

morphological and physiological traits (Sugiura et al. 2015). Transcriptome analysis revealed that genes related to auxin and cytokinin were altered in response to exogenous GA_3 application. These results suggest that GAs crosstalk with other hormones regulate the expression of secondary cell wall biosynthesis genes and trigger *E. grandis* shoot and root growth (Liu et al. 2018). This may partially explain the stimulatory effect caused by TE on eucalyptus plants found here (Fig. 7), once all these three hormones were increased at 05 DAP (Figs. 8d, f, 9b).

Regarding the crosstalk between GAs and ABA, previous studies reported antagonistic effects from both of these hormones (Oh et al. 2007; Zentella et al. 2007; Duan et al. 2019). The expression level of ABA-related gene ABAH1 was reduced in celery leaves due to exogenous GA_3 treatment (Duan et al. 2019). In contrast, the transcription level of GA2ox1 gene was reduced by ABA treatment at the seedling stage (Zentella et al. 2007). These results differ from those observed here, in which the 5-fold increase in leaf GA_1 (Fig. 9b) occurred concomitantly with the almost 2-fold increase in leaf ABA ($p < 0.01$—Fig. 8a), showing a direct correlation between both hormones (Fig. 11b). However, this answer may be species specific. How the application of TE would affect ABA- and GA-related genes still needs to be clarified.

Previous studies reported that alterations in GA biosynthesis induced changes in the concentration or expression of hormone-related genes (Wang et al. 2015; Duan et al. 2019), suggesting that GAs may interact with other hormones to regulate plant growth through crosstalk mechanisms (Nemhauser et al. 2006; Liu et al. 2014; Yang et al. 2014) (Figs. 8, 9, 10, 11). Furthermore, the hormonal imbalance caused by TE in eucalyptus seems to be an organ-specific control. For example, the concentration of leaf GA_1 was severely increased in eucalyptus leaves (+ 5-fold), obtaining, however, a 70% reduction in the SAB (Fig. 9b). Our results suggest that the hormonal control that modulates plant growth in response to TE is a complex signaling network (Wang et al. 2015; Duan et al. 2019). Thus, due to the dynamics of changing feedback and hormonal crosstalk (Figs. 8, 9, 10, 11), and in view of the limitations of the research methods, the understanding of the interaction between these molecules is still limited (Duan et al. 2019), especially when more than three hormones are evaluated.

Trinexapac-Ethyl Dose–Response Effect on *Eucalyptus* Growth and Physiology

Regarding TE effect on gas exchange, no positive response was observed for these characteristics in both water conditions until 15 DAP (Figs. 3, 4). In contrast, Bacha et al. (2019) reported a 19% increase in the net CO_2 assimilation rate at 40 DAP, for the dose of 60 g a.i. ha^{-1}, compared to the control. However, there were no deleterious effects on the photosynthetic characteristics of eucalyptus. This hypothesis was proposed by Pires et al. (2013) in that TE is not harmful to the photosynthetic processes of plants (Figs. 3, 4).

We verified that 150 g of TE increased eucalyptus total dry matter at 90 DAP by 49%, as compared to the non-treated control (Fig. 7). Similarly Bacha et al. (2017) reported gains of up to 30% in this same characteristic of *E. urophylla* (clone I-144) when treated with 30 g of TE. For clone GG-100 from *E. urograndis*, Pires et al. (2019) obtained a 76% increase in shoot biomass at 35 DAP with

15 g of TE. Under phosphorus-limiting conditions, Bacha et al. (2018) noted a 13% increase in eucalyptus total dry matter with 30 g of TE. As previously described, research has verified a positive effect of TE on different eucalyptus clones. However, there is no consensus regarding the dose that causes the greatest stimulus to plant growth. Data here indicated that 202 g TE provided the greatest stimulatory effect on eucalyptus growth (Fig. 7a). These results can support future research aiming to evaluate TE effect under field conditions (also considering sequential applications), focusing on maintaining such positive effect until harvest, especially considering short-rotation eucalypt areas.

Treating with 60 g TE, Pires et al. (2019) reported an increase in eucalyptus height (clone GG100), leaf area and total dry mass (clone GG100). For our research, this dose did not provide a significant effect on eucalyptus growth at 90 DAP ($p > 0.05$), despite an increase of almost 25% in total dry mass (Fig. 7b) and even with a higher concentration of GA_1 (Figs. 9b, 10b). The difference between these results is probably due to genotype-related response, in addition to the fact that Pires et al. (2019) only conducted their experiment out to 35 DAP. Despite this, it is important to emphasize that none of the doses were toxic to eucalyptus and all showed a tendency to increase the mass (Fig. 7), possibly due to the hormonal changes reported here (Figs. 8, 9, 10).

As no constant differences were detected in the eucalyptus photosynthetic characteristics in the first 15 days after TE application (Figs. 2, 3), it is likely that an increase in the plant's photosynthetic metabolism may have occurred later (after the hormonal homeostasis has been reestablished).

Since only WW plants exhibited the stimulatory effect, our results indicate that water was a limiting factor for 40-FC plants not obtaining the extra growth provided by TE (Fig. 6 and Table S4). These results are in agreement with those reported by Bacha et al. (2019), who also found no increase in *E. urophylla* (Clone I-144) growth treated with 30 and 60 g of TE, and cultivated under severe drought stress (20% of field capacity).

The reduction in gas exchange characteristics for treatments under 40-FC (Fig. 3), considering the average TE doses, is in in accordance with several studies with eucalyptus (Susiluoto and Berninger 2007; Granda et al. 2011; Correia et al. 2014, 2018). These results can be justified by the fact that water plays an important role in maintaining cellular metabolism (Warren et al. 2011), since its properties directly influence cell constituents, such as the structures of proteins, nucleic acids and membranes, among others (Chaves et al. 2003). Under limited water availability, stomatal closure is one of the first responses observed for plants under these conditions (Correia et al. 2014; Utkhao and Yingjajaval 2015). This process restricts water loss through evapotranspiration and helps maintain the leaf water balance (Jesus et al. 2015; Correia et al. 2018). This response is the result of a complex signaling network in which abscisic acid (ABA) plays a primary role (Zhang et al. 2006; Jiang and Hartung 2008; Martins et al. 2018), including being reported in eucalyptus plants under water-restricted conditions (Granda et al. 2011; Correia et al. 2014, 2018; Martins et al. 2018).

Stomatal closure has also been described as an important consequence of the decrease in CO_2 in the photosynthetic parenchyma in long-term responses (Chaves et al. 2003; Vassileva et al. 2011), causing limited carbon assimilation. Here, limited soil moisture resulted in greater amounts of internal carbon (Fig. 3d), likely due to lower stomatal conductance (Fig. 3c). Despite this, plants in this treatment showed lower instantaneous carboxylation efficiency values (Fig. 3f), which suggests that carbon molecules were not being used in the chemical stage of photosynthesis due to the decrease in photosynthetic metabolism resulting from water limitation, which is important in the RuBP regeneration process and Rubisco activity (Lawlor 2002; Parry et al. 2002). Eucalyptus plants under water restriction tend to show limited gas exchange activity (Chaves et al. 2003; Susiluoto and Berninger 2007; Correia et al. 2014; Utkhao and Yingjajaval 2015) as observed in Fig. 3. These recurring results are justified due to the fact that water directly participates in the photochemical stage of photosynthesis, by acting as an electron donor for the electron transport chain. Furthermore, they are also essential in the production of ATP, because after the water photolysis process, H^+ protons are released inside the thylakoid for later use by the ATP-synthase pump (Lawlor 2002; Parry et al. 2002).

Regarding total chlorophyll content, from 20 DAP onward (Fig. 4b), the amount of this pigment in leaves under 40-FC was greater than under WW. Correia et al. (2014) found similar results in *E. globulus*, as well as a higher concentration of carotenoids, and attributed this response to lower leaf expansion. The authors emphasize that high amounts of carotenoids can play an important factor in protecting chlorophylls against photodegradation, thus maintaining the photosynthetic capacity of plants. Conversely, another hypothesis for the maintenance of high amounts of these pigments in eucalyptus plants was raised by Susiluoto and Beringer (2007). The authors emphasize that under conditions of water restriction, there is a greater root/shoot ratio, also observed by Li and Wang (2003), therefore, enhancing the plant's ability to exploit soil resources and absorb water and nutrients, particularly nitrogen.

For F_v/F_m, higher values ($p < 0.05$) for WW plants were found at 11 and 13 DAP. However, despite this difference, all values noted are within the range of those of healthy plants (0.75–0.85; Schreiber et al. 1994). Susiluoto and Berninger (2007) and Correia et al. (2014) found different results for *E. microtheca* and *E. globulus*, respectively. The

authors reported that stressed plants exhibited higher values for F_v/F_m, relating these results to the higher chlorophyll concentration in the leaves. Thus, a possible justification for the difference between the results found here and the previously cited works is due to the fact that F_v/F_m assessments occurred until 15 DAP (Fig. 4a), when the chlorophyll concentration in WW plants was higher than those from 40-FC (Fig. 4b).

The physiological processes that cause the stimulatory effect from TE application have not yet been completely elucidated. Thus, more research focusing on studying the effects of this PGR on eucalyptus crop must be carried out, once the understandment of this process can lead to increases in productivity in the near future.

We concluded that TE caused stimulatory effect on *E. urograndis* (Clone 1407) growth under WW condition, but not under 40% of field capacity. The estimated dose for the greatest stimulatory effect on WW eucalypt plants is 202 g a.i. ha^{-1}.

TE did not cause an increase in the plant's photosynthetic characteristics up to 15 DAA, suggesting that an increase in the plant's primary metabolism must occur after this period. TE caused a 5-fold increase in leaf GA_1 as short-term effect (5 DAA), but significantly decreased SAB-GA_1 concentration. In the leaf, the concentrations of IAA, SA, ABA and iP also increased. TE caused changes in both 13-hydroxylated (GA_{20}, GA_1 and GA_8) and non-13-hydroxylated (GA_9, GA_4 and GA_{34}) GA metabolic pathways in an organ-specific manner.

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Declarations

Conflict of interest None declared.

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