



Cytokinins improve quality of 'Maxi Gala' apples produced in southern Brazil

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ABSTRACT: Cytokinins constitute a group of plant growth regulators responsible for a wide range of actions on plant growth and development. This study evaluated the effects of different concentrations and application timings of the cytokinins thidiazuron (TDZ) and benzyladenine (6BA) on fruit cell features, agronomic performance, and the quality of 'Maxi Gala' apples produced in southern Brazil over two growing seasons. The experiment comprised seven spray protocols of TDZ and/or 6BA applied at five different phenological stages. TDZ application during the dormant bud stage resulted in yield reduction in the subsequent growing season. TDZ and 6BA influenced fruit cell division and size, respectively, affecting intercellular spaces when applied at the pink bud (PB) and full bloom (FB) stages. The application of 20 mg L⁻¹ of TDZ at the PB and FB stages induced greater fruit cell division with smaller cells size. Application of 10 mg L⁻¹ of 6BA at PB and FB stages increased fruit cell size but reduced intercellular spaces. The application of TDZ or 6BA at PB and FB stages resulted in the lowest losses of fruit pulp firmness during refrigerated storage due to the reduced intercellular space. Among treatments with similar intercellular space, it was observed that a lower number of cells corresponded to a greater reduction in flesh firmness after storage. Treatments with TDZ or 6BA at PB and FB stages; 6BA at PB, FB, fruit 8-12 mm, and fruit 15-20 mm stages; and 6BA at fruit 8-12 mm and fruit 15-20 mm stages exhibited larger cell sizes and quantities, leading to larger fruit sizes but negatively affecting pulp firmness and productivity. Although, the application of TDZ or 6BA at PB and FB stages similarly affected fruit firmness and size. The application of 6BA at PB and FB stages resulted in higher productivity in the 'Maxi Gala' cultivar.

Key words: *Malus domestica* Borkh, Cell division, fruit caliper and quality, thidiazuron, 6-Benzyladenine.

Citocininas melhoram a qualidade de maçãs 'Maxi Gala' produzidas no sul do Brasil

RESUMO: As citocininas constituem um grupo de reguladores de crescimento vegetal, responsáveis por diversas ações no crescimento e desenvolvimento das plantas. Este estudo teve como objetivo avaliar o efeito de diferentes concentrações e épocas de aplicação das citocininas thidiazuron (TDZ) e benziladenina (6BA) nas características das células dos frutos, no desempenho agrônômico e na qualidade das maçãs 'Maxi Gala' produzidas no sul do Brasil durante duas safras. O experimento compreendeu sete protocolos de pulverização de TDZ e/ou 6BA aplicados em cinco diferentes estádios fenológicos. A aplicação do TDZ durante o estágio de gema dormente resultou na redução da produtividade na safra subsequente. TDZ e 6BA influenciaram, respectivamente, a divisão e o tamanho celular dos frutos, afetando os espaços intercelulares quando aplicados nos estágios de botão rosa (BR) e plena floração (PF). A aplicação de 20 mg L⁻¹ de TDZ nos estádios BR e PF induziu maior divisão celular nos frutos com células de menor tamanho. A aplicação de 10 mg L⁻¹ de 6BA nos estádios BR e PF aumentou o tamanho das células dos frutos, mas reduziu os espaços intercelulares. A aplicação de TDZ ou 6BA nos estágios BR e PF resultou em menores perdas de firmeza da polpa dos frutos durante o armazenamento refrigerado, devido a redução dos espaços intercelulares. Nos tratamentos com espaço intercelular similar, observou-se que um menor número de células correspondia a uma maior redução na firmeza da polpa após o armazenamento. Os tratamentos com TDZ ou 6BA nos estágios PB e FB, 6BA nos estágios de PB, de FB, de fruto de 8-12 mm, e de fruto de 15-20 mm, e 6BA nos estágios de fruto de 8-12 mm e de fruto de 15-20 mm exibiram células de maior tamanho e quantidade, resultando em frutos de maior tamanho, mas afetaram a firmeza da polpa e a produtividade. Embora a aplicação de TDZ ou 6BA nos estágios PB e FB tenha afetado de maneira semelhante a firmeza e o tamanho dos frutos, a aplicação de 6BA nos estágios PB e FB apresentou maior produtividade na cultivar 'Maxi Gala'.

Palavra-chave: *Malus domestica* Borkh, divisão celular, qualidade de fruto, thidiazuron, 6-Benzyladenine, calibre de fruto.

INTRODUCTION

The apple (*Malus domestica* Borkh) is one of the three most commercially significant fruit species in Latin America and southern Brazil (KIST

et al., 2019), accounting for approximately 98% of the country's production. According to the Brazilian Institute of Geography and Statistics, Brazil annually produces 1.2 million tons of apples across an area of 35.4 thousand hectares (IBGE, 2021).

Apple production is directly influenced by the fruit size, with larger fruit being a crucial factor from both consumer and commercial perspectives (PETRI et al., 2020). Fruit growth can be enhanced through management practices such as flower and/or fruit thinning, which reduce competition for carbohydrates and allow the remaining fruit to achieve a larger size (PETRI et al., 2020). Additionally, it is possible to increase fruit size using plant growth regulators. According to GUILLAMÓN et al. (2022), two crucial stages in fruit growth and development that determine apple fruit size are cell division and cell elongation. Auxins and cytokinins promote cell division and differentiation by regulating the activity of specific enzymes, while gibberellins stimulate cell elongation and play a role in the expression of essential genes involved in the cell division (GUILLAMÓN et al., 2022). The early phase, particularly the first few weeks after flowering, is especially vital for the exogenous application of plant growth regulators, especially cytokinins. Benzyladenine (6BA), in particular, enhances fruit size through its thinning effect and by promoting cell division (PETRI et al., 2013). Thidiazuron (TDZ), identified as a phenylurea with activity similar to cytokinin (GUO et al., 2011), stimulates an increase in fruit set at low concentrations (FAGUNDES et al., 2017) and promote fruit growth (PETRI et al., 2020).

Numerous treatments involving agrochemicals, plant growth regulators, and/or biostimulants have been conducted in warm regions to shorten endodormancy, advance flowering time, and increase fruit set and size (GUILLAMÓN et al., 2022). While these practices offer significant benefits, there remains a lack of knowledge regarding the effectiveness of these treatments, as well as the optimal concentrations and application times for different apple cultivars, environmental conditions, and regions. Therefore, the use of cytokinin-based plant growth regulators, when applied at specific concentrations, times, and phenological stages, emerges as a promising management strategy for increasing fruit size.

This study evaluated the effects of different concentrations and application timings of the cytokinins thidiazuron (TDZ) and benzyladenine (6BA) on fruit cell features, agronomic performance, and the quality of 'Maxi Gala' apples produced in southern Brazil over two growing seasons.

MATERIALS AND METHODS

The experiments were conducted in a 'Maxi Gala' apple orchard located in the municipality

of Vacaria, State of Rio Grande do Sul, southern Brazil (28° 30' S, 50° 56' W), at an average altitude of 970 m above sea level. The climate of the region is classified as humid mesothermic (Cfb) according to the Köppen classification. Daily data on maximum and minimum temperature, rainfall, and solar radiation were recorded by the A880 automatic station of the National Institute of Meteorology (INMET, 2022). The soil is a typical dystrophic brunoxisol (SANTOS et al., 2018), with high clay content (430 g kg⁻¹) and organic matter (95 g kg⁻¹). The orchard, established in 2008, consists of 'Maxi Gala' apple trees grafted onto 'M.9' rootstock, trained as slender spindle trees with a spacing of 4.5 m × 1.2 m, equivalent to 1,778 trees per hectare, all under black anti-hail net (12% shading).

Experimental design

The experiment followed a randomized block design, consisting of eight treatments and four replications. Each replication comprised eight trees per plot. The treatments included seven spraying protocols that varied in concentration, frequency, and application timings of two plant growth regulators: the cytokinins thidiazuron (TDZ) and benzyladenine (6BA). Additionally, untreated control trees were included for comparison (Table 1). For sprays conducted during the dormant bud stage with TDZ, mineral oil was used at a rate of 35 ml L⁻¹. During flowering and fruit development, a silicone non-ionic adhesive spreader was added to the solution at a concentration of 1 ml L⁻¹. The treatments were applied using an electric backpack sprayer with constant pressure and flow, delivering a volume equivalent to 1,000 L ha⁻¹.

Fruit cellular features

Eight central fruits originating from the king flower, located in the middle part of the tree with an approximately diameter of 20 mm, were collected seven days after the last spray protocol. Fresh mass, equatorial diameter, and height of the fruit were measured by precision scale and digital caliper, respectively. For the evaluation of cellular structures, fruit tissue was fixed in a preservative solution composed of formaldehyde, glacial acetic acid, and alcohol (FAA_{70%}), following the methodology adapted from MANN et al. (2005). The fixing solution consisted of 50 mL of formalin (formaldehyde 37% -Dinâmica®), 50 mL of 70% ethanol (ethyl alcohol -Dinâmica®), and 900 mL of glacial acetic acid.

Median slices of the preserved fruit tissue were removed and placed in a FAA_{70%} solution. The material was placed in a desiccator and connected to

Table 1 - Description of cytokinins benzyladenine (6BA) and thidiazuron (TDZ) spray protocols at the 'Maxi Gala' apple orchard in the municipality of Vacaria/RS, southern Brazil during 2018/19 and 2019/20 growing seasons.

Treatments	Sprays timing at different phenological stage-----				
	1	2	3	4	5
	Dormant bud BBCH*01	Pink bud BBCH 57	Full bloom BBCH 65	Fruit 8-12 mm BBCH 71	Fruit 15-20 mm BBCH 72
T1	TDZ 300 mg L ⁻¹	TDZ 20 mg L ⁻¹	TDZ 20 mg L ⁻¹	-	-
T2	-	TDZ 20 mg L ⁻¹	TDZ 20 mg L ⁻¹	-	-
T3	-	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹	-	-
T4	-	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹
T5	-	-	-	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹
T6	TDZ 600 mg L ⁻¹	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹	-	-
T7	TDZ 600 mg L ⁻¹	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹	6BA 10 mg L ⁻¹
T8 Control	-	-	-	-	-

*BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemical industry) phenological growth stages of all mono- and dicotyledonous plant species scale, according to MEIER et al. (1994).

a vacuum pump at a negative pressure of 600 mmHg for a period of 48 hours. Following the fixation process, the material was gradually dehydrated in ethyl alcohol solutions (50%, 60%, 70%, 80%, 90%, and 95%), under vacuum, with each solution applied for 90 minutes. Subsequently, the alcohol was replaced by a solvent forming a basic resin matrix, composed of 2-hydroxyethyl methacrylate and an activator solution, dibenzoyl peroxide, all from Leica Biosystems®.

After drying the historesin blocks containing the sampled fruit, histological sections were cut at a thickness of 8µm using a Leica SM2010 R sliding microtome. Distilled water was applied to the surface of the histological slides, which were then positioned on a magnetic stirrer with heating set between 35-40 °C. The sliced samples were placed over the warm water to stretch the material. Subsequently, a drop of basic fuchsin stain was applied for approximately 30 seconds. With the tissue properly stained and stretched, a clear stained-glass varnish was applied, and a coverslip was placed atop section. The images of the histological sections were captured using a Leica Microsystems® microscope, processed, and analyzed using the Leica Application Suite (LAS) software platform version 4.8.0.

Fruit cellular analyses were performed manually, involving the quantification of cell numbers and measurement of the longitudinal and transverse lengths of each cell in the microscopic images sized at 1,311 × 983 µm. Averages were subsequently calculated from the individual values. To determine cell area, the formula ($A = \pi \times R1 \times R2$) was

employed. The resulting value was then multiplied by the number of cells and subtracted from the area of the analyzed image ($1,311 \times 983 \mu\text{m} = 1,288.713 \mu\text{m}$) to yield the area of the intercellular space.

Agronomic performance

During the phenological stage from the beginning of flowering (10% in open flowers) to the end of flowering (90% of buds in 'G' stage or 100% in the F2 stage), spanning from early to late October in the 2019/2020 and 2020/2021 growing seasons, one hundred reproductive structures (comprising terminal buds and spur buds) per plot were sampled to determine the number of reproductive and vegetative structures. This allowed for the calculation of the return bloom based on the percentage of floral buds. The estimated yield in tons per hectare was determined by harvesting and weighing all the fruit from each tree. The production per tree was then multiplied by the planting density to obtain the overall yield.

Fruit quality

The harvest of 'Maxi Gala' apple fruit began around February. The average fruit weight was determined by dividing the yield of each tree by the number of fruits. Additional variables were obtained using a sample of 40 fruits per plot, with 20 fruits evaluated immediately after harvest and the remaining 20 fruits after 30 days of storage in a refrigerated atmosphere at $3 \text{ °C} \pm 1$ and $90\% \pm 2$ relative humidity. The samples were stored in an open, ventilated plastic crate during cold storage. This refrigerated storage condition was used to simulate

one of the primary commercial storage conditions for apples in southern Brazil, but with a temperature that is ± 3 °C higher than typically used for ‘Gala’ cultivar. This adjustment allows for a higher ripening rate, facilitating the assessment of treatment influence within a shorter storage period.

The average diameter and height of the fruit were measured using an L-shaped wooden gutter graduated in millimeters. The fruit were arranged side by side, allowing for the simultaneous measurement of 20 fruits. The obtained value was then divided by the number of fruits to determine the average diameter and height in millimeters. Flesh firmness was assessed using a digital texture analyzer TA.XT express/TA.XT2icon, equipped with an 11 mm tip. Two readings were taken per fruit, positioned on opposite sides of the equatorial region. Soluble solids content was determined by extracting juice from the sampled fruit and measuring it with a digital refractometer ITREFD-45. To assess fruit peel color, a digital colorimeter Konica Minolta CR-400 was used, with two readings taken on opposite sides of the fruit, focusing on areas with the highest and the lowest red color intensity.

Statistical analysis

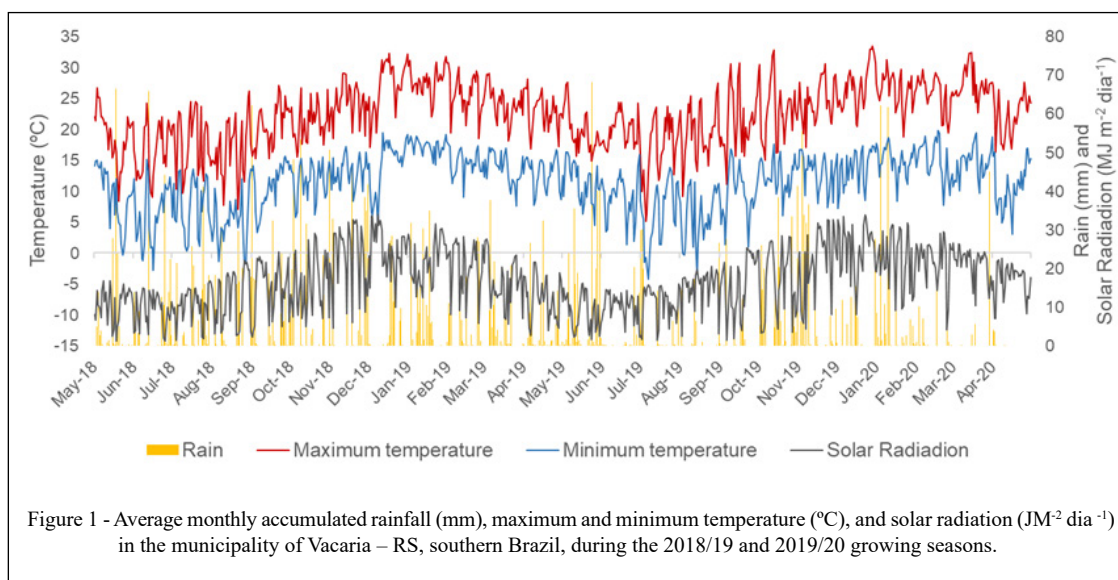
The multivariate data were subjected to Principal Component Analysis (PCA) using a correlation matrix, performed in Minitab software. Additionally, univariate analysis of variance (ANOVA) was performed as a complementary analysis to PCA. The univariate data underwent the Shapiro-Wilk test ($\alpha = 0.05$) to verify the adherence to a normal

distribution, and Bartlett’s test was used to assess homogeneity of variances. When differences were identified at a 5% error probability level, the Scott-Knott test ($\alpha = 0.05$) was used to separate the means, utilizing the R software.

RESULTS

During the evaluation period from May 2018 to April 2020, the average maximum and minimum temperatures, total rainfall, solar radiation, and chilling hours were approximately 23 °C, 8 °C, 1,878 mm, 26 $\text{JM}^{-2} \text{dia}^{-1}$, and 593 h, respectively (Figure 1). The average minimum and maximum temperatures during the key phenological stages of beginning of sprouting (August), fruit growth and development (December to January), and harvest (February to March), were 8 °C to 14 °C, 19 °C to 21 °C, and 18 °C to 22 °C, respectively (Figure 1). However, the average maximum temperature from the beginning (February) to the end of the harvest (March) was approximately 29 °C (Figure 1).

In the 2018 growing season, there were 116 days with rain, totaling 2,015 mm. However, rainfall was poorly distributed at times, with the most critical period in January, just before the harvest. The 2018/19 growing season was preceded by a winter with temperatures favorable for chilling accumulation, resulting in 706 h below 7.2 °C. In contrast, the 2019 growing season experienced a lower volume of rain (1,707 mm), which; although still high, was spread over 112 days. Rainfall distribution was irregular, with few rainy days and low volumes in November



and December 2019 and January and February 2020, when apple trees were in the ripening stage. The winter preceding the 2019/20 growing season had low chilling accumulation, totaling only 480 hours below 7.2 °C.

Fruit cellular features

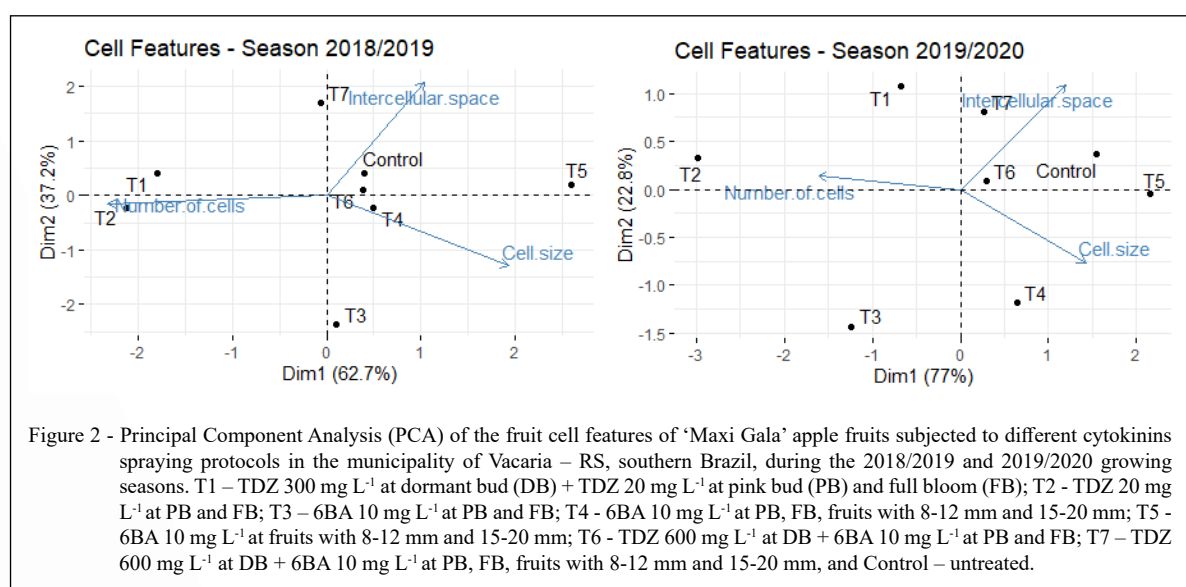
All cytokinin growth regulators treatments are displayed in the table 1. The treatments T1 [300 mg L⁻¹ of TDZ at dormant bud (DB) and 20 mg L⁻¹ of TDZ at pink bud (PB) and full bloom (FB) stages] and T2 (20 mg L⁻¹ of TDZ at PB and FB stages) resulted in a high number of fruit tissue cells, with small cell size and reduced intercellular space compared to T4 (10 mg L⁻¹ of 6BA at PB, FB, fruit 8-12 mm, and fruit 15-20 mm stages), T5 (10 mg L⁻¹ of 6BA at fruit 8-12 mm, and fruit 15-20 mm stages), T6 (600 mg L⁻¹ of TDZ at DB stage and 10 mg L⁻¹ of 6BA at PB and FB stages), and T7 (600 mg L⁻¹ of TDZ at DB stage and 10 mg L⁻¹ of 6BA at PB, FB, fruit 8-12 mm, and fruit 15-20 mm stages) during the 2018/2019 growing season (Figures 2A, 3A). The only difference between T1 and T2 was the additional application of 300 mg L⁻¹ of TDZ at dormant bud in T1 (Table 1). The trees treated with T4, T5, and T6 produced fruit tissue with fewer, larger cells, and greater intercellular space (Figures 2A, 3A). T7 exhibited fruit tissue with fewer and smaller cells along with greater intercellular space (Figures 2A, 3A).

The fruit from T5-treated trees displayed the poorest cell features, even worse than untreated fruit (control), indicating that the application of 10

mg L⁻¹ of 6BA only at the fruit sizes of 8-12 and 15-20 mm is not effective in increasing fruit cell number and reducing intercellular space in 'Maxi Gala' apples. Fruit from T3-treated trees (10 mg L⁻¹ of 6BA at PB and FB stages) showed distinct cellular features from all other treatments, with a significant increase in number of cells, although less pronounced than other treatments that applied TDZ in the same PB (BBCH-57) and FB (BBCH-65) stages. However, fruit from T3-treated trees had the smallest intercellular space, attributed to the larger cell size (Figures 2A, 3A). In the first evaluated 2018/19 growing season, 6BA had a more pronounced effect on cell size, while TDZ had a greater effect on cell division (Figures 2A, 3A).

Fruit tissue from trees that received only TDZ applications (T1 and T2) had a higher number of cell than the fruit tissue from trees that received only 6BA applications. However, the greatest reduction in intercellular space was primarily due to the increase in cell size (Figures 2A, 3A). Moreover, when 6BA was applied in combination with TDZ to induce budburst (600 mg L⁻¹), there was little or no increase in the cell number, as observed in fruit from T6 and T7-treated trees. The cell number remained low, and the intercellular space was larger, similar to that fruit of untreated trees.

The T1, T2, and T3 treatments, exhibited the best fruit cellular features in the 2018/2019 growing season, with T2 displaying the greater number of fruit cells among them and surpassing all other treatments (Figures 2A, 3A). The fruit tissue from T2-treated trees showed 20 % and 15 % higher



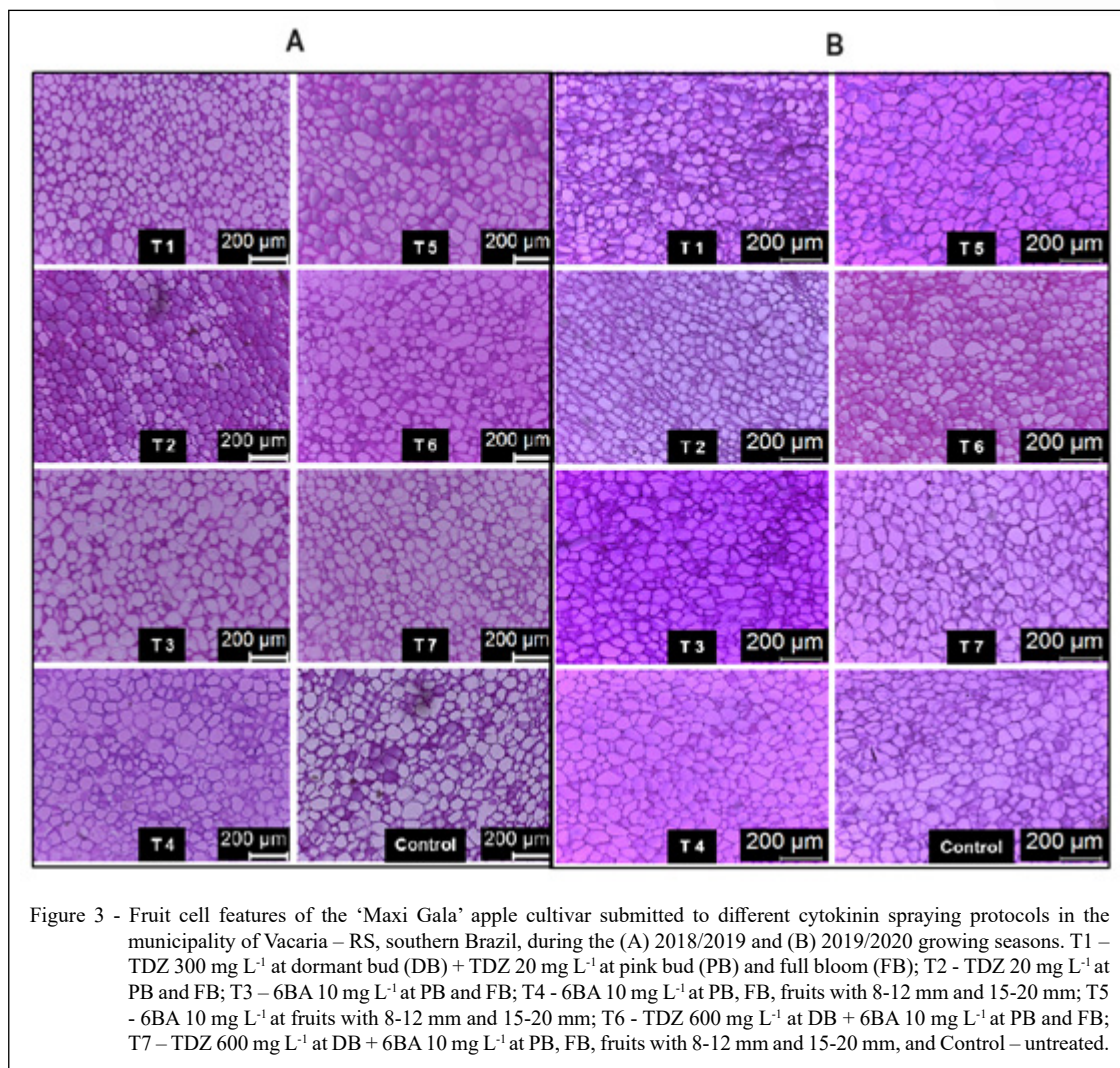


Figure 3 - Fruit cell features of the 'Maxi Gala' apple cultivar submitted to different cytokinin spraying protocols in the municipality of Vacaria – RS, southern Brazil, during the (A) 2018/2019 and (B) 2019/2020 growing seasons. T1 – TDZ 300 mg L⁻¹ at dormant bud (DB) + TDZ 20 mg L⁻¹ at pink bud (PB) and full bloom (FB); T2 - TDZ 20 mg L⁻¹ at PB and FB; T3 – 6BA 10 mg L⁻¹ at PB and FB; T4 - 6BA 10 mg L⁻¹ at PB, FB, fruits with 8-12 mm and 15-20 mm; T5 - 6BA 10 mg L⁻¹ at fruits with 8-12 mm and 15-20 mm; T6 - TDZ 600 mg L⁻¹ at DB + 6BA 10 mg L⁻¹ at PB and FB; T7 – TDZ 600 mg L⁻¹ at DB + 6BA 10 mg L⁻¹ at PB, FB, fruits with 8-12 mm and 15-20 mm, and Control – untreated.

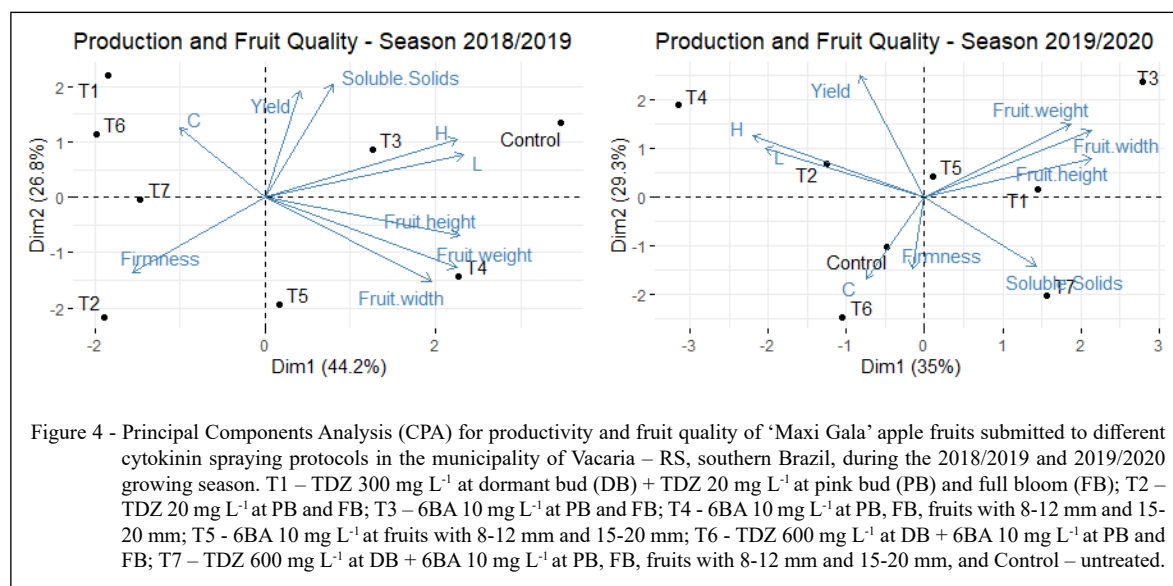
number of cells compared to fruit from untreated control and T3, respectively. However, fruit tissues treated with T3 exhibited cells that were 22% larger than those treated with T2, resulting in 13% less intercellular space. In the 2019/2020 growing season, T2 and T3 maintained the characteristics observed in the 2018/2019 growing season and can be considered the best results in terms of intercellular space, either due to the greater number of cells (T2) or the larger cell size (T3) (Figures 2B, 3B).

The fruit tissue from the T4-treated trees in the second 2019/2020 growing season exhibited greater cell size than observed in the first 2018/2019 growing season, equating to the cell size observed in the T3 treatment. The distinction between the effects of T3 and T4 was in the number of cells, with a 10 % higher count in the fruit of trees that received 10 mg L⁻¹

6BA only at the PB (BBCH-57) and FB (BBCH-65) stages (T3) (Figures 2B, 3B). T1 showed a reduction in the number of cells in the 2019/2020 growing season, resulting in an increase in intercellular space.

Agronomic performance

The agronomic performance of fruit production, fruit quality, yield, and weight of the 'Maxi Gala' cultivar was influenced by TDZ and 6BA spraying protocols during the 2018/19 and 2019/20 growing seasons (Figure 4). T1 and T3-treated trees exhibited similar yields of around 85 t ha⁻¹, which were higher than those of the other treatments during 2018/2019 growing season (Figure 4A). Untreated trees also showed high productivity, around 70 t ha⁻¹. In contrast, there was a reduction in yield for trees that received treatments T2, T4, and T5, with yields



ranging between 53 and 54 t ha⁻¹. The lowest yield was obtained for trees that received treatment T7 in both 2018/2019 (41 t ha⁻¹) and 2019/2020 growing seasons (30 t ha⁻¹), which involved the highest number of applications and the highest concentration of cytokinins, resulting in a thinning effect.

The fruit yield in the second 2019/2020 growing season was lower than in the previous year due to alternate bearing, with untreated trees (control) producing only 35.9 t ha⁻¹ in the 2019/2020 growing season. This facilitated an increase in production with the use of certain cytokinin based growth regulator treatments. T3 and T4-treated trees stood out in term of yield compared to the other treatments, being around 20 % more productive than the untreated trees (control).

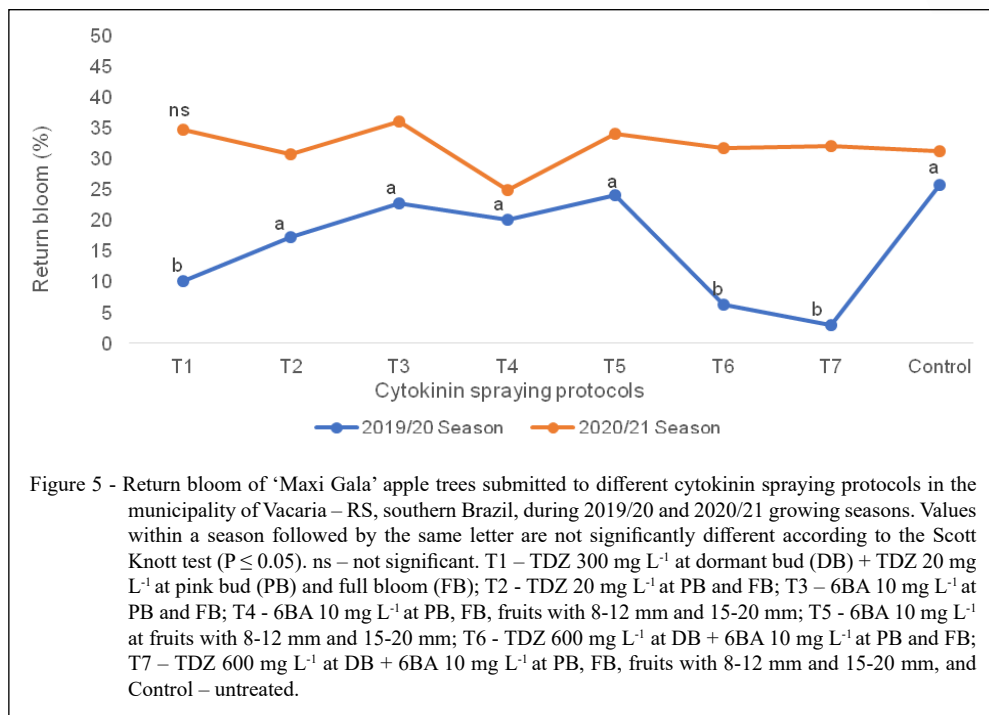
The Principal Components Analysis (CPA) of T3 and T4 treatments showed a similar positioning on the graph concerning yield, but in different quadrants due to distinct responses to variables corresponding to epiderm color at 2019/2020 growing season (Figure 4B). The fruit from T3-treated trees were heavier and larger than those from T4-treated trees. There was a reduction in the return bloom in trees that received different doses of 300 or 600 mg L⁻¹ of TDZ applied in the DB stage (T1, T6 and T7) in the 2019/2020 growing season compared to the 2020/2021 growing season (Figure 5). The greatest reduction in return bloom was observed in trees that received the highest concentration of 600 mg L⁻¹ of TDZ in DB stage (T6 and T7), practically canceling the floral formation of these trees (Figure 5). However, the return bloom of 2020/2021 growing season was

not affected by cytokinin applied in the previous 2019/2020 growing season. The trees influenced only of 6BA (T2, T3 and T4) remained more stable in relation to the return bloom compared to the others (Figure 5). The application of TDZ at the BD stage also caused early bud burst and abnormal growth pattern in the 2019/20 growing season, forming short-growing structures similar to a hyperhydricity (Figure 6).

Fruit quality at harvest and after refrigerated storage

The results of the effects of the cytokinin growth regulators TDZ and 6BA on fruit quality are shown in the figure 4. T3, T4, and T5 exhibited fruit with largest cell sizes, while T2 had the highest fruit cell quantity in the 2019/2020 and 2020/2021 seasons. These treatments resulted in larger fruit sizes; however, there was no significant difference compared to the untreated control. In the 2019/2020 growing season, the difference between these treatments were qualitative in terms of pulp firmness and soluble solids content, and quantitative in terms of productivity. T2 produced fruit with greater pulp firmness but lower soluble solids content and yield. Conversely, T3 resulted in lower firmness but higher soluble solids content and productivity.

It is important to note that there was a water deficit during the fruit growth and ripening stage in the 2019/2020 growing season, which significantly limited fruit growth. In the 2020/2021 growing season, pulp firmness did not significantly contribute to the first two principal components and was therefore excluded from the evaluation of differences



between T2, T3, T4, and T5. The main differences between these treatments in the 2020/2021 season were related to soluble solids content and yield, with T3 showing the best values for both.

The greatest reduction in flesh firmness after 30 days of refrigerated storage was observed in fruit from T5-treated trees, which were characterized by the poorest fruit cellular features. This highlighted a strong relationship between fruit cellular features and the potential for fruit refrigerated storage (Figure 7). In contrast, fruit from T1, T2 and T3-treated trees showed the highest flesh firmness after refrigerated storage and displayed the smallest reduction in flesh firmness during the storage period, with reductions of 2.7 %, 2.4 % and 3.0 % respectively, in the 2019/2020 growing season (Figure 7). Fruit from untreated trees (control) showed the lowest flesh firmness (77 N) and the second largest reduction in pulp firmness (6.7 %) during the 30 days of refrigerated storage. When examining treatments with similar intercellular space, it was observed that a lower number of cells corresponded to a greater reduction in flesh firmness after refrigerated storage.

DISCUSSION

Fruit cellular features

The application of cytokinins TDZ and 6BA in the 'Maxi Gala' apple cultivar alters the

cellular features of fruit, primarily impacting fruit cell size and quality, depending on the cytokinin type, concentration, and timing of application. The influence of TDZ and 6BA application on fruit cell division in the 'Maxi Gala' apple cultivar is more pronounced when applied at the pink bud - PB (BBCH-57) and full bloom - FB (BBCH-65) stages. Research conducted by JING & MALLADI (2022) on the cellular features of 'Gala' apples in USA described a steady increase in cell number in the fruit cortex from 8 days after full bloom (DAFB) until 18 DAFB, followed by a gradual increase until around 43 DAFB. The relative cell production rate significantly increased between 3 and 8 DAFB, peaked around 11 DAFB, declined from 14 DAFB, and remained low from around 24 DAFB. The application of cytokinin in PB and FB stages may extend the period of cell multiplication or increase the rate of multiplication. This effect is not observed with the application of studied concentrations at the beginning of fruit development, possibly because the cytokinins are applied at the final stage of cell division.

The use of 6BA in different apple cultivars usually involves higher concentrations (MILIC et al., 2017; GUILLAMÓN et al., 2022) that those used in this study, as well as application on young fruits up to 10-12 mm for fruit thinning, with contradictory results on cellular anatomy following 6BA application

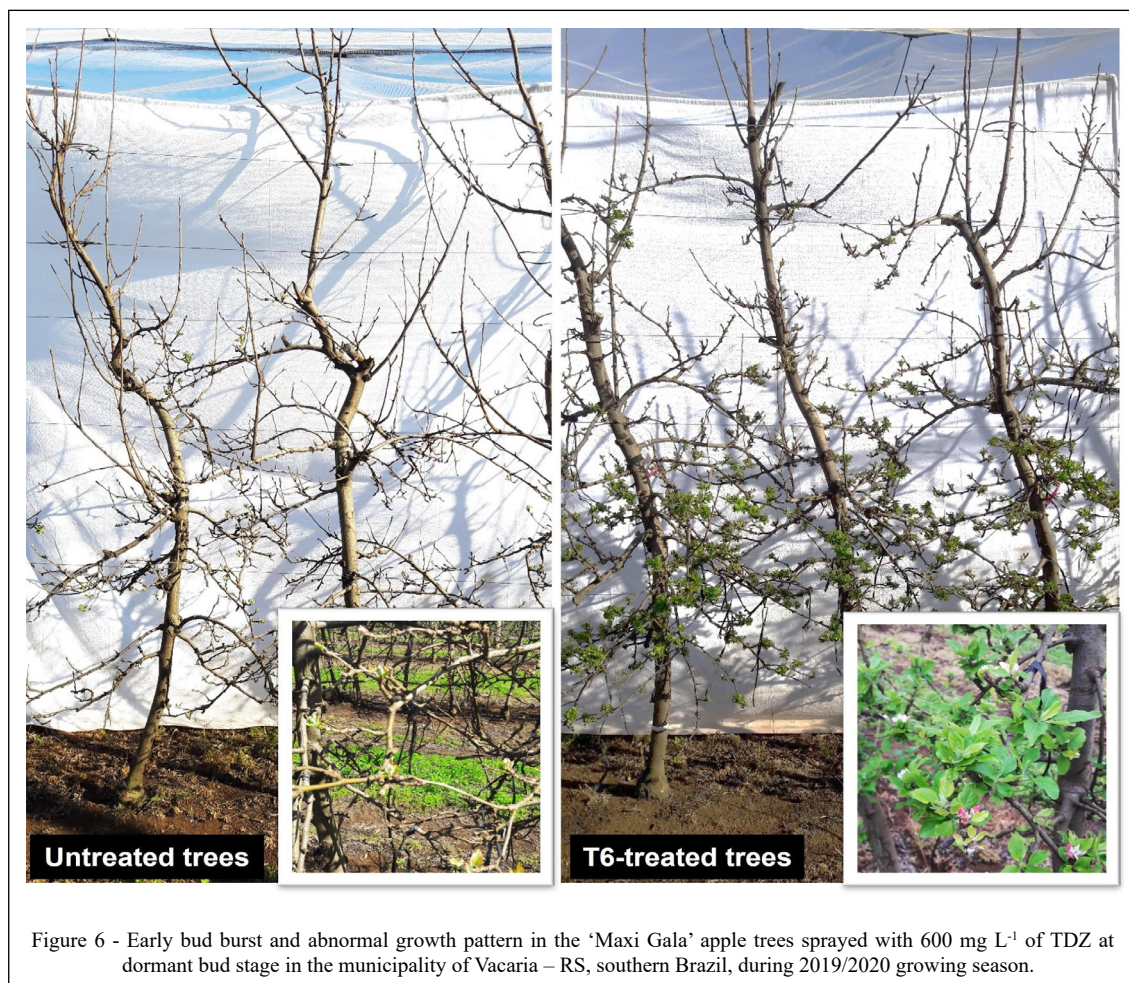
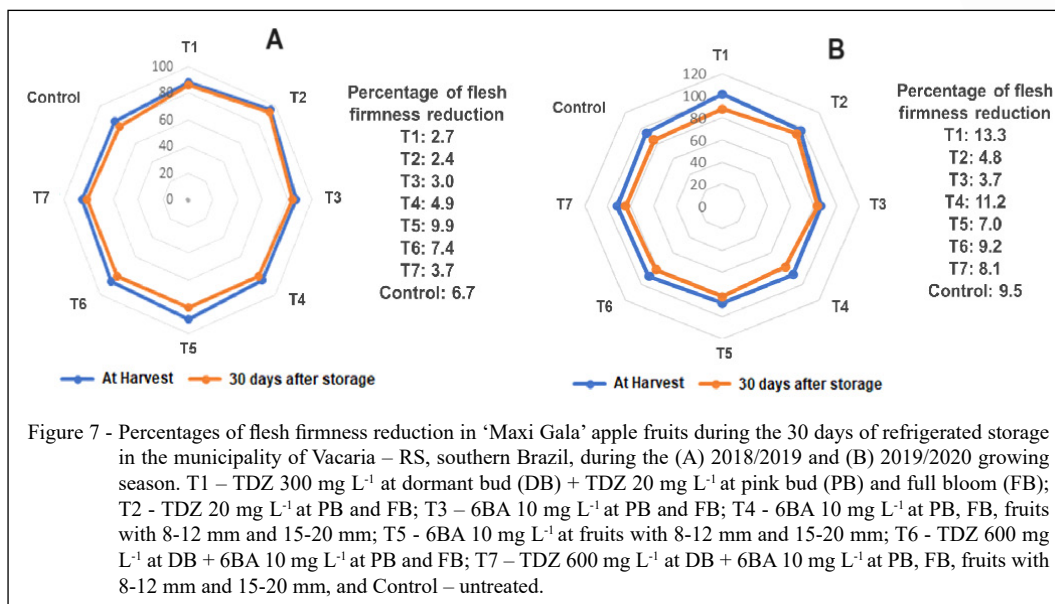


Figure 6 - Early bud burst and abnormal growth pattern in the 'Maxi Gala' apple trees sprayed with 600 mg L⁻¹ of TDZ at dormant bud stage in the municipality of Vacaria – RS, southern Brazil, during 2019/2020 growing season.

(MILIC et al., 2017). The application of 10 mg L⁻¹ of 6BA only on fruit measuring between 8-12 mm and 15-20 mm proved ineffective in increasing cell number. Instead, it enhanced cell size and the intercellular space, contradicting the results obtained by MILIC et al. (2017). In their study, the application of 200 mg L⁻¹ of 6BA in 11 mm fruits of 'Golden Delicious' apple led to an increase in cell number without changing cell size, with no thinning effect. Differences in concentration and varietal characteristics could explain the distinct cell features resulting from 6BA application on young fruit. The same authors found no effect of applying 200 mg L⁻¹ of 6BA on 11 mm fruit of 'Red Delicious'. It appears that, during the cell division phase, lower concentrations of 6BA are efficient in altering cell number and size during the flowering stage. In contrast, higher concentrations are necessary to achieve similar effects in young fruit. However, varietal characteristics play a crucial role in determining cell response to 6BA application.

The fruit tissue from T1 (300 mg L⁻¹ of TDZ at DB stage and 20 mg L⁻¹ of TDZ at PB and FB stages) displayed a lower number of cells than T2 (20 mg L⁻¹ of TDZ at PB and FB stages), possibly associated with the application of TDZ at the DB stage intended to promote bud burst. According to GUO et al. (2011), TDZ induces a series of physiological and chemical effects in plants, modifying endogenous plant growth regulators. Consequently, this process directly or indirectly triggers reactions in cells or tissues that are necessary for their division or regeneration. The effect of high cell division and the consequent suppression of dormancy caused by the application of TDZ in DB stage by T1, T6 (600 mg L⁻¹ of TDZ at DB stage and 10 mg L⁻¹ of 6BA at PB and FB), and T7 (600 mg L⁻¹ of TDZ at DB stage and 10 mg L⁻¹ of 6BA at PB, FB, fruit 8-12 mm, and fruit 15-20 mm stages), may have influenced competition for carbohydrates between leaves and flowers during the 2019/20 growing season. This is



evident in the abnormal bud burst pattern observed in trees that received TDZ in DB stage (Figure 6). During the flowering period, the tree needs to utilize its accumulated reserves, and at this stage, vegetative and reproductive growth compete for carbohydrates. In trees experiencing excessive vegetative growth or vigorous bud sprouting, vegetative growth tends to dominate carbohydrate partitioning, thereby reducing contributions to the reproductive organs. This dominance may have limited the initial cell division of flowers and fruit at the beginning of development (GUO et al., 2011; VERJANS et al., 2018a).

Regarding the two types of cytokinins used, TDZ exhibited a more pronounced effect on cell division in fruit, while 6BA primarily influenced the increase in cell size and consequent reduction of intercellular spaces. According to GREENE (2019) and CARRA et al. (2021), 6BA enhances protein synthesis in apple cells from preexisting RNA without modifying its synthesis, likely due to an increase in the rate of monosomes converting to polysomes. The authors also note an elevation in metabolic activity with the application of 6BA, which can favor both cell division and cell growth. However, the greater proliferation of protoplasts caused by the application of TDZ (ROSA et al., 2018) and the increase in endogenous auxin, ethylene, and abscisic acid in response to TDZ treatment (FAGUNDES et al., 2017) may be the determining factors in the enhanced cell multiplication obtained with TDZ in this study.

The 'Maxi Gala' trees subjected to the T3 treatment (10 mg L⁻¹ of 6BA at PB and FB stages)

consistently exhibited the most favorable results in both 2018/2019 and 2019/2020 growing seasons, demonstrating positive outcomes in cellular features, yield, and fruit quality. Fruit from T2-treated trees displayed optimal cellular features and fruit quality, resulting in minimal loss of pulp firmness during refrigerated storage in both years. However, yield was influenced differently in the two studied seasons. Regardless of the cytokinins used, the best results in terms of fruit cellular features and quality when observed when the application was carried out during flowering. Exploring the impact of alternative applications of TDZ and 6BA during flowering could be valuable, given TDZ's superior capacity for cell division and 6BA's ability to provide consistent yields at the studied concentrations.

Agronomic performance

The primary use of 6BA and TDZ in apple orchards is for chemical thinning purposes and to increase fruit set, respectively (ARUME et al., 2020; CARRA et al., 2021). GREENE et al. (2016) described the development of 6BA as a chemical thinner in apple trees, noting that various studies have found that 6BA promotes fruit abscission and increases both fruit weight and fruit size. In a seven-year study, PETRI et al. (2013) described the effect of TDZ on the fruit set of different temperate climate fruit trees, finding that on 'Gala' apple trees, TDZ increased fruit set and fruit weight without affecting the return bloom.

Despite significant differences in yield, particularly in relation to fruit weight, the present

data did not corroborate the findings of most studies involving 6BA and TDZ in apple trees. The present data indicated a tendency for larger fruit in treatments T2, T3, T4 (10 mg L⁻¹ of 6BA at PB, FB, fruit 8-12 mm, and fruit 15-20 mm stages) T5 (10 mg L⁻¹ of 6BA at fruit 8-12 mm, and fruit 15-20 mm stages), which increased fruit cell number or cell size. However, the increase in fruit size in these treatments was not significantly different from the untreated control. In some studies, involving 6BA and TDZ in apple trees, an increase in fruit size or weight is often reported and attributed to factors such as reduced competition for carbohydrates and water due to the thinning effect of 6BA or the growth stimulus of TDZ. (GREENE et al., 2016; VERJANS et al., 2018a). However, our results align with ROSA et al. (2018), who used 150 mg L⁻¹ of 6BA on 'Royal Gala', 'Cripps Pink' and 'Red Delicious' at 9-10 mm fruit, and VERJANS et al. (2018b), who used 150 mg L⁻¹ of 6BA on 'Nicoter' at 9-10 mm fruit. In these studies, no significant change in fruit size or weight was observed.

The 'Maxi Gala' trees subjected to T2, T4, and T5 treatments exhibited a significant yield reduction, ranging between 53 and 54 t ha⁻¹, compared to untreated trees (control), which displayed a high yield of around 70 t ha⁻¹. 6BA is recognized for its thinning activity in apple trees, typically at concentrations between 150 and 300 mg L⁻¹ when applied on young fruit up to 10 mm (VERJANS et al., 2018b; GREENE et al., 2016). Additionally, TDZ applied at concentrations between 15 and 30 mg L⁻¹ during flowering is known to increase fruit set in apple trees (PETRI et al., 2020; FAGUNDES et al., 2017). However, there are also reports of a thinning effect of TDZ on fruit trees, with concentrations ranging from 10 to 30 mg L⁻¹ applied at FB stage of 'Rocha' pear (CARRA et al., 2021). The lowest yield was obtained from trees that received treatment T7 in both 2018/2019 and 2019/2020 growing seasons (41 t ha⁻¹ and 30 t ha⁻¹, respectively), which involved the highest number of applications and the highest concentration of cytokinins, resulting in a strong thinning effect with no benefits on fruit quality.

The 'Maxi Gala' fruit yield in the 2019/2020 growing season was lower (35.9 t ha⁻¹) than in the previous 2018/2019 growing season, primarily due to the alternate bearing, compared with untreated control plot. However, the application of 10 mg L⁻¹ of 6BA to T3 - and T4-treated trees resulted in increased fruit yield compared to the other treatments, with yields around 20% higher than the untreated trees. Although, T3 and T4 showed similar fruit yield, T4 reduces the refrigerated storage potential due to its impact on

cellular features, making it more suitable to apply the same concentration only in PB and FB (T3). This treatment not only increased yield but also improved refrigerated storage potential, as the fruit exhibited less loss of flesh firmness during refrigerated storage, attributed to a better cellular arrangement.

Applications of cytokinins in horticultural fruit crops increase fruit set, either by the forming parthenocarpic fruit or by extending the period of effective pollination through enhanced ovule viability. This increase is primarily controlled by ethylene, which is suppressed by the antagonistic action of cytokinins (AREMU et al., 2020). Conversely, the application of cytokinins can also have a thinning effect. This occurs as cytokinins induce stress at the beginning of fruit development, accentuating differences in the photosynthate required for all fruit to persist and the spur's ability to supply carbohydrates. This stress can lead to the abscission of the weakest fruit due to a carbohydrate deficit (GREENE, 2019). Therefore, the same cytokinin used worldwide to control crop load through its thinning action can be applied in smaller concentrations with the opposite objective.

Previous studies have demonstrated the significance of the cytokinin/gibberellin ratio in apple flower induction, with a higher ratio favoring floral initiation (GUILLAMÓN et al., 2022). Contrary to the results found by LI et al. (2019) using 300 mg L⁻¹ of 6BA applied 27 to 30 days after full bloom on 'Nagafu No.2' apple trees in China, our study did not show an increase in flowering with the use of TDZ and 6BA. However, our findings align with those of the latter authors, who observed no response in return bloom with a single application of 100 mg L⁻¹ of 6BA on 'Golden Delicious' apple trees from full bloom up to 25 mm fruit.

There was a reduction in return bloom due to the use of TDZ in dormant buds, which contradicts the results of other studies that used TDZ to break dormancy of apple trees (DE MARTIN et al., 2017). This contradiction is likely attributed to the use of lower concentrations in those studies and the accumulative effect of different applications. The lowest return bloom was observed in trees that received the highest concentration of TDZ in dormant buds and the highest number of cytokinin applications (T7-treated trees). FAGUNDES et al. (2017), during an investigation of different concentrations of TDZ at pink bud and full bloom on 'Royal Gala' apple trees in southern Brazil, found a reduction in flowering in the season following the application of TDZ. They attributed this reduction to alternate bearing, as there was an increase in fruit set and yield with the use of

TDZ. This differs from our study, where the floral reduction cannot be solely attributed to yield. In the same study, the authors also observed an increase in return bloom after using TDZ in another season. They related this to the control of vegetative growth through greater fruit production, favoring the partition of carbohydrates to the reproductive organs rather than shoot growth. However, it's important to note that a reduction in return bloom has been reported in other cultivars, such as 'McIntosh', 'Double Red Delicious' and 'Gala' apple trees by the application of TDZ during flowering and post-flowering at concentrations ranging from 5 to 50 mg L⁻¹.

TDZ has been studied as a stimulant bud burst of apple trees in regions that do not meet the chilling hours requirement in winter, particularly in regions dependent on the use of hydrogen cyanamide, such as Brazil. It has proven to be efficient for this purpose without causing abnormal bud burst patterns (DE MARTIN et al., 2017). While the beneficial effects of TDZ in increasing bud burst and fruit set in some apple cultivars are known, caution should be taken when using TDZ in apple trees, as it may negatively impact on return bloom. This includes avoiding high concentrations, especially when used for bud burst promotion. Additionally, factors such as the number of applications and the cumulative concentration applied during the season must be carefully considered.

Fruit quality at harvest and after refrigerated storage

Maintaining flesh firmness has been a major goal in improving the shelf-life performance of fruit (AREMU et al., 2020). The rate of reduction in apple flesh firmness during storage is influenced by factors such as fruit cell size, anatomy, and cell wall structure (MANN et al., 2005). The present data exhibited that for treatments with similar intercellular space, the smaller the number of cells, the greater the reduction in flesh firmness after refrigerated storage. This result is possibly linked to the fact that in fruit with fewer cells number, where intercellular space is equal and cell are larger, the dehydration process of the larger cells more intensively affects the reduction of flesh firmness compared to the dehydration of smaller cells. This confirmed the observation that a higher number of smaller cells may help maintain the qualitative attributes of the fruit for a longer time during storage, compared to fruit with larger cells, regardless of intercellular space. The reduction of the intercellular space, either by the increasing the number of cells or by increasing cell, caused by the applications of both cytokinins at PB and FB, positively impacts

storage potential and reduces the negative aspects related to a soft and drier perception of ripe apples during consumption (AREMU et al., 2020). The flesh firmness of the 'Maxi Gala' apples at harvest was not as influenced by the different treatments as it was after the refrigerated storage period. The treatments that promoted the best cellular arrangement in the fruit, where applications of TDZ or 6BA occurred during flowering (T1, T2 and T3), were also the treatments that provided the lowest loss of flesh firmness during refrigerated storage. This is because the fruit had smaller intercellular space, which corroborates the findings of MALLADI & JOHNSON (2011) who describe a strong influence of cellular arrangement on storage capacity. Fruit with a greater number of smaller cells may maintain equivalent or superior quality in storage compared to apples whose size is only a result of increased cell size.

Two likely causes for the observed effect due to the application of cytokinins are the larger cell layer and smaller cell size, which may serve as a stronger physical barrier to the loss of firmness. Another important factor is the composition of the cell membrane, which can be altered by the application of cytokinins. AN et al. (2024), demonstrated that, in many fruits, flesh firmness is closely related to the structure and composition of the cell wall, which includes components like pectin, cellulose, and hemicellulose. A specific study on strawberries showed that cell size distribution, influenced by nutritional factors such as calcium and nitrogen, directly impacts the fruit's mechanical properties. This study suggested that fruits with larger cells may have less dense cell walls, contributing to reduced tissue firmness. Additionally, another study on various fruits species revealed that tissue firmness is correlated with the composition of cell wall polysaccharides, implying that variations in cell size could directly influence fruit texture by altering cell wall content (LIU et al., 2024). According to MILIC et al. (2017), TDZ-stimulated enzymes associated with cell walls may cause changes in membranes and membrane fluidity. NG et al. (2013) described that cell wall structures related to tissue microstructure and cell adhesion, developed early during apple growth, lead to genotypic differences in softening rates well before the induction of the ripening process. It seems likely that the application of DTZ and 6BA during the flowering of 'Maxi Gala' apple trees led to changes in the structure of the cell membrane, as there is a relationship between how the wall is composed and assembled from early development. This plays an important role in affecting subsequent fruit firmness changes, as described by NG et al. (2015).

CONCLUSION

The cellular features of 'Maxi Gala' apple trees can be improved by the application of TDZ and 6BA, thereby enhancing fruit quality, particularly in terms of flesh firmness. This effect is most notable in fruit from trees that received 20 mg L⁻¹ of TDZ or 10 mg L⁻¹ of 6BA at the PB and FB stages. In this context, 6BA acted more intensely by increasing cell size and reducing intercellular space, while TDZ had a greater effect on increasing cell division, which reduced both cell size and intercellular space. The use of TDZ at concentrations of 300 or 600 mg L⁻¹, applied at dormant bud stage, along with the application of TDZ and 6BA during flowering and the beginning of fruit growth, is not recommended, as it does not improve the cellular and post-harvest features and may even reduce return bloom and storage potential by reducing the loss of flesh firmness during refrigerated storage.

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DECLARATION OF CONFLICT OF 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.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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