Chilling and forcing requirement of five international fig cultivars grown in Southeastern Brazil

Laís Naiara Honorato Monteiro[2]*, *Sarita Leonel*[3], *Jackson Mirellys Azevedo Souza*[4], *Rafael Bibiano Ferreira*[5], *Marcelo de Souza Silva*[6], *Emerson Loli Garcia*[7]

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ABSTRACT

Chill hours availability influence break dormancy, sprouting and production of temperate fruits trees in different regions. However, there are few reports on the subject for fig tree. This study aimed to evaluate the effects of the accumulated chilling hours on the physiology and dormancy release of fig cultivars. Stem cuttings of five fig cultivars were collected at the end of winter over two crop cycles and exposed to 0, 40, 80, 120 and 160 accumulated chilling hours (CH) in a cold chamber (8±0.5 ºC). Physiology of fig buds was then evaluated with regards to antioxidant enzymes activity, carbohydrate and nitrogen contents. The exposure of fig stem cuttings with two buds to different accumulated CH presented physiological changes for antioxidant enzymes activity, carbohydrates and nitrogen contents and confirming that the cultivars responded differently to the climatic conditions of each crop cycle. Results detected that the lowest accumulated CH in field in 2018 (2.7 CH) enabled a greater effect of the artificial CH when compared to 2017 (19.5 CH). Each fig cultivar had a critical accumulation point. The cultivars Roxo de Valinhos and Pingo de Mel require less CH to break dormancy, while Troyano requires more CH to finish this vegetative rest period.

Keywords: *Ficus carica* L.; antioxidant enzymes; biodiversity; carbohydrates; chilling requirements; gene pool.

INTRODUCTION

The fig tree (*Ficus carica* L.) has great importance worldwide, and in Brazil its success has lasted for several years, with the country in 11th place in the ranking of world producers. In an area of 2,208 ha in the year 2019 in Brazil, 22,526 tons were produced, and among temperate climate fruits, the Brazilian export of figs is second only to grapes and apples, which guarantees the country the first place as the largest exporter and producer in the Southern Hemisphere, an advantage acquired by the fact that the national production occurs in the off-season of the foreign market (Oliveira Junior *et al.*, 2018; Faostat, 2021).

The Brazilian fig orchards are mainly based on a single cultivar known as Roxo de Valinhos or Purple fig. The fig tree varieties were dispersed throughout the world and given local names, which makes varietal classification difficult. With the introduction of varieties outside the center of the fig tree origin, new local names were given, which created the varietal confusion (Pio & Chagas, 2011). The characterization and varietal diversification of fig trees enables the use of several cultivars and the expansion of productive areas.

The floral induction occurs in many plants when specific environmental conditions are satisfied and most plants bloom and bear fruit during the same season each year (Muñoz-Fambena *et al.*, 2011). In fig, by contrast, the time interval during which inflorescence differentiation occurs corresponds to the shoot elongation period. Fig trees thus differ from many species in their reproductive growth characteristics (Ikegami *et al.*, 2013). The flowers

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[2] Centro Universitário de Votuporanga, Votuporanga, São Paulo, Brazil. laismonteiiro@gmail.com
[3] Universidade Estadual Paulista, Faculdade de Ciências Agronômicas, Botucatu, São Paulo, Brazil. sarita.leonel@unesp.br
[4] Universidade Federal de Viçosa, Departamento de Agronomia, Viçosa, Minas Gerais, Brazil. jackson.m.souza@ufv.br
[5] Universidade Estadual Paulista, Faculdade de Ciências Agronômicas, Programa de Pós-graduação em Agronomia/Horticultura, Botucatu, São Paulo, Brazil. aprigio_bibiano@hotmail.com
[6] Faculdade de Educação Superior e Ensino Integral, Garça, São Paulo, Brazil. mace-lo-souza@hotmail.com
[7] Universidade Estadual Paulista, Centro de Raízes e Amidos Tropicais, Botucatu, São Paulo, Brazil. emerson.cerat@gmail.com
*Corresponding author: laismonteiiro@gmail.com

of the fig tree can be male, long-stemmed female, and short-stemmed female (Leonel & Sampaio, 2011). The fig fruit plant is of value mainly for its edible, particularly that of the sexual species females (Ikegami *et al.*, 2013). The long-stemmed female flowers (pomological type Cachopo) produce the fig infructescence by parthenocarpy, with no need for pollination. The cultivars with potential to diversify the Brazilian fig orchards are included in the Cachopo group (Leonel & Sampaio, 2011).

Fig tree as a low chill fruit species has his growth and development highly dependent on the environmental factors, which mainly affects vegetative rest period and consequently, resulting in low yields (Rodrigues *et al.*, 2019). Fig trees can grow in cold and dry climates, since they are native to the Mediterranean area, where prevails an essentially frost-free moderate temperate climate. They have a low or no chilling hour requirement during dormancy release to achieve satisfactory production (Oukabli & Mekaoui, 2012) and require less than 150 chilling hours (Vossen & Silver, 2000). In a study about thermal requirement of Roxo de Valinhos fig cultivar, Souza *et al.* (2009) reported the minimum (8 ºC) and maximum (36 ºC) basal temperatures after evaluating them for five years.

The budburst of any fig cultivar takes place once its chill and heat requirements have been met; however, winter temperatures are affected by climate change, which impacts the distribution of the species because, in general, warmer winters will result in reduced chilling hours leading to phenological changes and possible erratic phenological patterns, including the loss of synchronization in bud break at both the individual plant and population level. The need related to the biological mechanisms controlling thermal regulation is not well understood, as through its effect on enzyme activities and biochemical reactions, temperature directly affects plant physiology, and indirectly influences multiple signaling pathways (Bonhomme *et al.*, 2010; Tixier *et al.*, 2019).

Signals or cycles of oxidation and reduction are crucial for dormancy release, activation of the cell cycle and expression of genes related to growth and development (Hernandez *et al.* 2021). Reactive oxygen species (ROS) are involved in these processes, at response to biotic and abiotic environmental stimuli, and in addition may act as signal transducers (Caverzan *et al.*, 2016). Sub-lethal oxidative stress may be involved in bud dormancy release, and increased hydrogen peroxide (H_2O_2) levels may activate budburst acting as a signaling molecule triggering this process. (Prassinos *et al.*, 2011; Sudawan *et al.*, 2016; Beauvieux *et al.*, 2018). Moreover, the transcription of genes encoding H_2O_2 enzymes, and/or the corresponding enzyme activities, were induced after dormancy release in order to prevent the accumulation of lethal levels of H_2O_2 (Hernandez *et al.* 2021).

There is evidence that carbohydrate dynamics in buds are closely related to temperature, cold accumulation and water flow that occurs during dormancy and dormancy release (Kaufmann & Blanke, 2017). According to the same authors, when the temperature drops in late autumn, deciduous plants enter the para-dormancy stage, wherein the hexoses content (glucose + fructose) are slightly higher than the starch content in yolks. Furthermore, with the accumulation of cold hours during winter, the plants enter the endo-dormancy stage, when the hexose contents increase significantly. At the end of winter (eco-dormancy), the carbohydrate contents remain stable, but there is a significant increase in the relative water content in the buds, which decreased during the previous phases. When plants are subjected to low temperatures, amylases and phosphorylases activities increase and convert starch into soluble sugars, conferring chilling tolerance (Oliveira *et al.*, 2012).

Vegetative rest is triggered by a great number of enzymes and biochemical reactions, besides being directly dependent on the endogenous plant growth substances, such as reserves and nutrients. Moreover, some contrasting changes occur in nutrient concentrations in plant tissue; for instance, nitrogen is stored in the shoots, but migrates to meristematic regions during sprouting. The shoots are responsible for managing the relationship between nutrient supply and bud requirement, acting as a sink and source, as carbon and nitrogen skeletons (Gupta & Kaur, 2005).

Overall, the response of plants towards the biotic and abiotic stresses causes oscillation in enzymes activities, carbohydrates reallocation and nutritional profile. Thus, understanding the physiological principles and environmental factors during dormancy release period is necessary to select an appropriate fig cultivar according to the growing area. Moreover, these factors are indispensable for the employment of management practices that aim to decrease problems caused by insufficient chilling hours, and the selection and zoning of fig cultivars can be considered an alternative to Roxo de Valinhos cv. (Martinazzo *et al.*, 2013).

Therefore, the present study aims to evaluate the effects of the accumulated chilling hours on the physiology and dormancy release of fig cultivars and propose a better understanding of the dormancy and chilling accumulation in a low chill specie requirement.

MATERIAL AND METHODS

Site description

The experiment was carried out at the Lageado Experimental Farm in the city of Botucatu, of the School of Agriculture (FCA UNESP), where stem cuttings were collected from fig trees, located at 22°51’55"S, 48°26’22"W at an altitude of 810 m above sea level. According to the Köppen classification, the climate of the area is the *Cfa* type, that is, a hot temperate climate (mesothermic), with concentrated rains from October to March and an average annual rainfall of 1376.7 mm; the mean temperature of the hottest month exceeds 22 °C (Cunha & Martins, 2009).

The fig trees (*Ficus carica* L.) were four years old in 2017 and were planted at 3 m spacing between rows and 2 m between plants. Regarding treatments, stem cuttings were collected over two crop cycles (July 2017 and 2018). Crop management and fertilization were performed based on soil analysis and crop recommendations.

The five fig cultivars were therefore selected as follows: Roxo de Valinhos, which has its likely origin in Southern Italy and was brought to Brazil by Italian immigrants being also known as the Purple fig (Pio & Chagas, 2011). This cultivar is well-known by Brazilian growers with fruits intended for fresh consumption (in natura) or food industry (green figs); Brown Turkey, which is commonly produced in the United States (California); Pingo de Mel, which is also called Kadota or Dottado (in Italy, Adriatic, Verdone), Mission (in California) and Fraga or Sepe (in Spain); Troyano, an Italian cultivar that is popularly grown in the United States and White Genova, which has a Italian background and was selected at the Campinas Agronomic Institute (IAC).

Accumulated chilling hours until fig cuttings collection

The accumulated chilling hours (CH) until fig cuttings collection were computed in each crop cycle. The lower basal temperature used in the model for fig tree was determined by Souza *et al.* (2009), that is, the daily thermal sum when the temperature remained equal or below 8 °C. This was considered the chill accumulation occurred in field.

The air temperature was obtained through thermo hygrometer (HMP45C, Vaisala, Fl) with a Model 41002 Gill Multi-Plate Radiation Shield (YOUNG, OH), installed 2 m high, and connected to a CR23X MICROLOGGER (data storage output every 10 minutes). The cuttings were sampled during the traditional winter pruning at the end of July 2017 and 2018. The plants had accumulated 19.5 and 2.7 CH when harvested in each crop cycle.

Cuttings collection and artificial chilling application

The 250 fig cuttings were collected per cultivar in two crop cycles. The cuttings were standardized to the 20 cm length and an average diameter of 7 mm, with at least two buds. The cuttings of each cultivar were separated into five lots (50 cuttings each), which were wrapped in moistened paper towel and packed horizontally. Afterwards, they were submitted to 0, 40, 80, 120 and 160 accumulated chilling hours (CH) in a cold chamber (8 ± 0.5 ºC) (Gariglio *et al.*, 2006).

Antioxidant enzymes activities

Fig cuttings with vegetative buds in the apical region were selected for enzymatic activity quantification. Only two buds were sectioned and macerated in a cryogenic grinder (6770 SamplePrep Freezer/Mill, SPEX™). The evaluation of antioxidant enzymes peroxidase (PODs, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1) followed the methods proposed by Teisseire & Guy (2000) and Giannopolitis & Reis (1977), respectively.

For POD, the reaction system was composed of 30 µL of diluted enzymatic extract (1:10 in extraction buffer); potassium phosphate buffer 50 mmolL^{-1}; pH 6.5; pyrogallol (1,2,3-benzenotriol) 20 mmolL^{-1} and hydrogen peroxide (H_2O_2) 5 mmolL^{-1}, with a total volume of 1 ml. The reaction was conducted at 25 °C (room temperature) for 5 minutes. The formation of purpurogallin was measured in a UV-visible spectrophotometer at wavelength of 430 nm; and the molar extinction coefficient (2.5 mmol$L^{-1}cm^{-1}$) calculated the specific enzyme activity. The POD activity was expressed in ì mol of purpurogallin $^{-1}$ mg^{-1} protein (µmol min^{-1}mg^{-1}prot).

For SOD, the activity was determined considering the inhibition in photochemical reduction of the Nitro-blue tetrazolium test (NBT) that was measured in spectrophotometer at wavelengths of 560 nm. The enzyme unit was defined as the amount of SOD needed to produce 50% inhibition of NBT reduction. The activity was determined by adding 50 ì L of fig tree extract to reaction system, which contained 13 mM methionine; 75 ì L of NBT; 100 nM EDTA and 2 ì M riboflavin in 3.0 mL of 50 mM potassium phosphate buffer; pH 7.8. The reactions were carried out at 25 °C (room temperature), initially by exposing the tube to light in a chamber composed of fluorescent lamps (15 watt). To calculate the specific enzyme activity in the sample, it was considered the percentage of inhibition obtained, the volume of the sample and the concentration of soluble protein (U mg^{-1}prot).

Carbohydrates and nitrogen compounds contents

The fig cuttings were sectioned into 1 cm pieces and packed in kraft paper bags, then dried in a forced air circulation at 65 °C until obtaining constant mass. Subsequently, samples were crushed and homogenized in a knife mill (Willye® type).

The carbohydrate contents were evaluated according to Somogy-Nelson method (Nelson, 1944) by the quantification of starch content (SC), total sugars (TS), reducing sugars (RS, glucose + fructose) and non-reducing sugars (NRS, sucrose). Regarding SC, 1.5 g of homogenized sample were diluted in 50 ml of distilled water and 6 ml of 0.1 N HCl, and then transferred to the autoclave (1 atm for 15 min). After cooling, samples were neutralized with sodium carbonate and filtered; 1.5 ml of the filtered volume was then used for analysis. For TS and RS, 1 g of homogenized sample was diluted in 100 ml of distilled water and filtered; 7 and 10 ml of the filtered volume were used for analyses. For TS, the diluted samples were heated in a water bath at 100 °C for 10 min, with 3.0 ml of sulfuric acid (100%) and neutralized with sodium carbonate (30% w/v) and filtered. After sample preparation, readings were performed on a UV-Vis Bel SPECTRO SP-2000 spectrophotometer. For non-reducing sugars, results were expressed as a percentage (%), where considered:

$$NRS\ (\%) = (TS - RS) * 0,95$$

The total nitrogen content was determined according to the Association of Official Analytic Chemists method 967.21 (AOAC, 2019). The first stage involved the digestion of 150 mg of homogenized sample at 350 °C with 7 ml of concentrated sulfuric acid. For quantification, 10 ml of the boric acid solution was pipetted to 125 ml Erlenmeyer. For the first extract tube, it was placed in the nitrogen distiller, added 15 ml of 50% sodium hydroxide. About 75 ml of distillate was obtained by placing the Erlenmeyer flask at the end of distiller; sample was titrated with 0.1 N sulphuric acid. For the calculation of the total nitrogen content, the volume spent on titration was considered.

Experimental design and statistical analysis

The split-plot setup organized in a randomized complete block design was used with plots represented by fig cultivars and subplots by chill hours, with four replicates and twenty cuttings per parcel. The crop cycles were analysed separately. Data were submitted to the hypothesis of normality by the Shapiro-Wilk test; the F test was used in the analysis of variance. When a significant difference was found in the sources of variation, the Scott-Knott test (plots) was used at 1% significance, and polynomial regression (subplots). All analyses used the System for Analysis of Variance program (Sisvar, version 5.6) (Ferreira, 2019).

RESULTS AND DISCUSSION

There was a significant interaction ($P < 0.01$) in both crop cycles between cultivars and accumulated chilling hours for SOD and POD activities. With regards to SOD activity, Pingo de Mel, Troyano and White Genova cultivars were significantly affected by artificial chilling exposure in 2017 (Figure 1A). Pingo de Mel and Troyano cultivars presented high SOD activity in the absence of artificial chilling hours, while there was a linear reduction in their averages by increasing the chilling hours up to 160, when they were equal to the others. White Genova cv. presented lower averages between 0 and 80 CH, the quadratic increase in SOD activity after that period allowed it to be equal to the others by exposing them to 120 and 160 CH.

For SOD activity, the artificial chilling exposure varied among Troyano, Roxo de Valinhos and Pingo de Mel cultivars in 2018 (Figure 1B). The Troyano cv. behaved like the previous cycle, whereas the averages of Pingo de Mel cv. presented a linear increase, but also an increased SOD activity was observed up to 160 CH. Furthermore, the exposure to artificial chilling provided a significant reduction in the averages of Roxo de Valinhos cv., since high SOD activity was reported in the absence of chilling exposure.

Most plants, especially those adapted to the temperate climate zones, can overcome moderate or temporary temperature decreases by cold sensing and subsequent adjustment of metabolism, morphology and growth to the new conditions (Hernandez *et al.*, 2021). Signals or cycles of oxidation and reduction are essential for dormancy release, activation of the cell cycle and expression of genes related to growth and differentiation (Hernandez *et al.*, 2021). The regulation of redox and oxygen metabolism is critical for organ development, and the release of dormancy depends on the action of reactive oxygen species (ROS) and the metabolism of antioxidant enzymes (Meitha *et al.*, 2015; Beauvieux *et al.*, 2018).

SOD in the cells represents the first line of defence against ROS, this group of enzymes catalyse the dismutation of superoxide radicals (O_2^-), which has a short life and low oxidative action, to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). However, H_2O_2 has a great damage potential, POD and Catalase (CAT) activities are then needed in preventing lipid peroxidation (Marafon *et al.*, 2009). Therefore, the joint action of these enzymes becomes more important than their isolated effects on the regulation of plant metabolism during vegetative rest. According to a study on Chinese pear (*Pyrus bretschneideri* Rehd.), Shao & Ma (2004) reported that ROS metabolism is strongly associated with buds in vegetative rest period during winter.

Regardless of chilling hours, Troyano, Pingo de Mel and White Genova cultivars presented great POD activity in both cycles, while Roxo de Valinhos and Brown Turkey cultivars presented the lowest of all (Figure 1C e 1D). Furthermore, there was POD activity (Figure 1C) without any exposure to artificial chilling (0 CH). In field, the accumulated chilling hours was of 19.5 from January to July 2017. Therefore, it was enough to promote stress and trigger POD activity in fig cuttings, especially in Pingo de Mel cv., since quadratic reduction occurred when exposed to 97.4 CH. Also, an adjusted equation did not fit for the other cultivars in this cycle.

There was less accumulated chilling hours (2.7 CH) in field in 2018, but there was a great effect of artificial chilling hours (Figure 1D). POD activity in Pingo de Mel cv. increased in a quadratic model up to 82 CH, which may indicate that this cultivar was already in the final stage of vegetative rest. Before dormancy release, there was an increase in H_2O_2 content and POD activity; in this case, it was preceded by a decrease. Nevertheless, a linear increase was observed in Roxo de Valinhos and Troyano cultivars. Regarding to Brown Turkey cv., there was a quadratic reduction in POD activity up to 97 CH. Finally, CH did not significantly affect the averages in White Genova cv., probably because this cultivar was originated in the Northern Italy, with high chill but was selected by breeding programs in Brazil, aiming at a low chill requirement (Ferraz *et al.*, 2020).

The artificial chilling exposure presented significant effects, since the accumulation of chilling hours in field dropped down in 2018 (i.e., 2.7 CH) (Figure 1D). In Pingo de Mel cv. a quadratic growth occurred in POD activity for 82 CH, while Roxo de Valinhos and Troyano cultivars presented a linear increase. Regarding to Brown Turkey cv., a quadratic decrease was observed in POD activity up to 97 CH. The chilling hours did not affect the averages of White Genova cv.

With regards to the linear growth, the POD activity did not reach the peak level in Roxo de Valinhos and Troyano cultivars, unlike Pingo de Mel cv. (Figure 1D). In peach trees, the increased activity of antioxidant enzymes occurs until the peak level during winter rest completion, with a subsequent reduction after (Gonçalves *et al.*, 2015). The Roxo de Valinhos and Troyano cultivars presented great demand for chilling hours when compared to Pingo de Mel. According to Ferraz *et al.* (2020), Troyano cv. has a longer and later harvesting than White Genova, Roxo de Valinhos and PI-189 cultivars in the city of Botucatu, São Paulo state, Brazil.

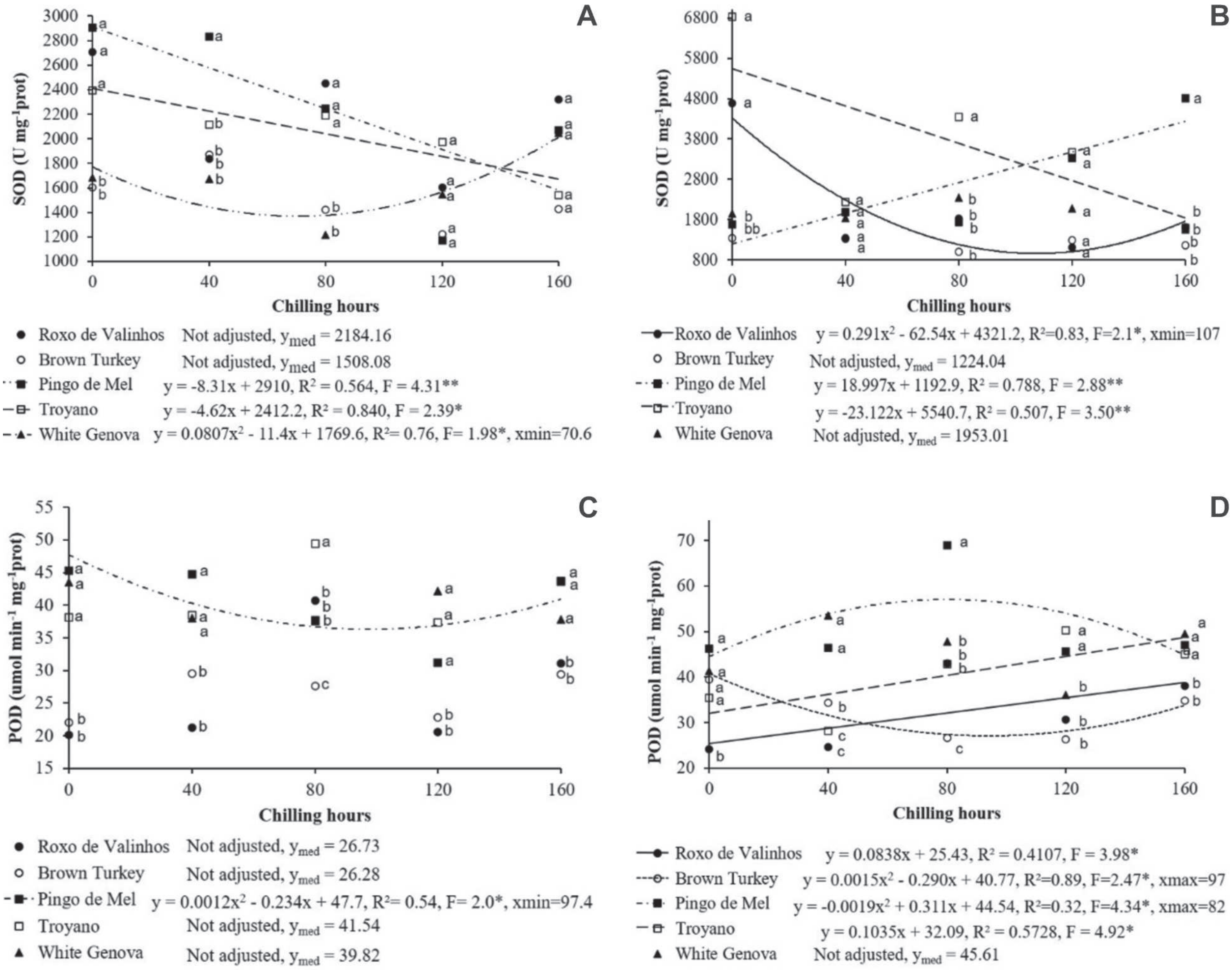

Figure 1: Superoxide dismutase activity (U mg^{-1}prot) (A and B) and peroxidase activity (μmol min^{-1}mg^{-1}prot) (C and D) in fig stem cuttings subjected to chilling hours in the agricultural cycles of 2017 and 2018. The same letter between cultivars indicates that the results do not differ by Scott-Knott test at 1% probability.

POD activity increases under stressful situations and the exposure to low temperatures promotes a sudden increase in the production of ROS, that is, H_2O_2 is highly prejudicial to cells, leading to oxidative damage by inducing protein denaturation, lipid peroxidation and DNA mutations (Barbosa *et al.*, 2010). Up-regulation or accumulation of antioxidant enzymes involved in ROS removal after or at the dormancy release stage indicates the need to eliminate ROS so that toxicity does not occur (Takemura *et al.*, 2015; Sudawan *et al.*, 2016; Hernandez *et al.*, 2021). The exposure to artificial chilling hours had a lesser effect in the first cycle, since chilling hours were probably enough until stem cuttings collection in order to trigger and anticipate the enzyme activity. Throughout the second cycle, the accumulated chilling hours was lower in field, which allowed the greatest effect of chilling hours exposures for POD activity.

The lowest SOD activity is often preceded by the highest POD activity, which is clearly seen in Troyano cv. The increase in H_2O_2 levels, as a result of SOD activity in buds exposed to chilling hours, acts as a signaling molecule and can interfere with signal transmission pathways such as hormones (gibberellins and cytokinins); which consequently triggers the dormant bud break process (Shao & Ma, 2004; Prassinos *et al.*, 2011). Gholizadeh *et al.* (2017) observed that SOD progressively increased, reaching its highest values at dormancy release of lateral buds from low chill walnut trees (*Juglans regia* L.). Redox interactions can trigg overcome vegetative rest, suggesting that this period requires an oxidative signaling networking, the cultivars with lower chilling requirements being able to develop and react to this oxidative signalling earlier that cultivars with higher chilling needs (Gholizadeh *et al.*, 2017). Therefore, H_2O_2-accumulation in plant tissues, stimulated by chilling exposure, trigger the oxidative signaling required for overcome vegetative rest in Troyano cv.

In addition to the activity of antioxidant enzymes, the entry and exit of vegetative rest may be related to the dynamics of carbohydrates and reserves translocation at a short distance, which varied according to temperature level and water flow (Bonhomme *et al.*, 2010; Kaufmann & Blanke, 2017). For starch content, there was no significant interaction between cultivars and chilling hours in 2017. However, a significant effect was observed in starch, total sugars, reducing sugars and non-reducing sugars contents in 2018 ($P < 0.01$); besides these factors individually affected the starch content in 2017 ($P < 0.01$).

In relation to the artificial chilling hours, the levels of starch decreased linearly with the increase in chilling hours in 2017, with the highest value (10.08%) obtained at 0 CH (Figure 2A). A similar effect was reported by Marafon *et al.* (2011) in a study on the bud dormancy of Japanese pear (*Pyrus pyrifolia* cv. Housui), who also observed that the lower the accumulated chilling hours, the higher the starch contents. The authors attributed this outcome to the reduced action of starch enzymes in conditions of lesser accumulated chilling hours, which directly affects the synthesis and import of sucrose, implying the accumulation of starch.

Among cultivars, Troyano cv. had the highest average for starch (Table 1). Moreover, starch showed higher percentages by comparing to other carbohydrates. This outcome corroborates the findings of Oliveira *et al.* (2012), who reported higher levels of starch in blueberry branches (*Vaccinium ashei* Reade) when compared to the total soluble sugar content. In plants, the accumulated chilling hours indicates various metabolic processes such as the end of dormancy; therefore, explaining the reduction in starch contents, since they were converted into sugars.

Regarding to artificial chilling, there was a significant interaction effect between cultivars and chilling hours in 2018, since starch values decreased linearly in Pingo de Mel cv.; while White Genova cv. had a quadratic growth up to 84 CH and then subsequent reduction (Figure 2B). The reduction in the contents of Pingo de Mel cv. may indicate the end of vegetative rest, as it corroborates studies by Gholizadeh *et al.* (2017) and Ito *et al.* (2012), who observed in walnut and Japanese pear (*Pyrus pyrifolia*, 'Kosui'), respectively, higher levels of starch at the beginning of dormancy followed by a progressive decline until the release of this process.

Furthermore, Troyano cv. had the highest starch contents in both cycles (Figure 2B), which maybe explains the greatest vegetative vigour and the largest branch diameter (Ferraz *et al.*, 2020). Fig cultivars might have high starch content whether they were well-adapted to the region, since they will show more vigour and resistance to diseases, since accumulated starch in reserve tissues will be required during dormancy period, as well as to begin a new growth cycle.

Troyano cv. presented the highest starch average of all, even higher than Roxo de Valinhos cv., which is highly known by Brazilian growers (Table 1 and Figure 2B). Troyano cv. has a great potential to be introduced, since

Table 1: Starch and total nitrogen content in fig stem cuttings during dormancy stage. Botucatu, SP, 2017

Cultivars	**Starch (%)**	**Total Nitrogen ($g\ kg^{-1}$)**
Roxo de Valinhos	5.19 b*	7.85 b*
Brown Turkey	7.75 b	8.60 a
Pingo de Mel	7.90 b	7.95 b
Troyano	14.52 a	8.67 a
White Genova	7.29 b	8.46 a

* The same letter between cultivars indicates that the results do not differ by Scott-Knott test at 5% probability.

this cultivar is a feasible alternative to incorporate diversity into the Brazilian fig orchards (Ferraz *et al.*, 2020). This cultivar presents longer harvest interval when compared to Roxo de Valinhos cv., since fruits accumulate carbohydrates during their development, which allow harvest marketable fruits after the end of traditional figs production in the subtropical regions. The characterization of Troyano, as a late cultivar, has enabled the diversification of figs cultivars; whose have different ripening stages (Ferraz *et al.*, 2020).

Overall, fig cultivars presented higher total sugars contents in relation to starch contents in 2017, except of Troyano cv. (Figure 2C and Table 1). Plants were therefore in full dormancy, since starch is converted into total soluble carbohydrates throughout this stage (Waldie *et al.*, 2010). Plants reached full vegetative rest in 2017 by considering both accumulated chilling hours in 2018 and 2017 (19.5 CH), because the sum of environmental and artificial chilling introduced them into the vegetative rest process.

However, Troyano cv. had higher starch content than total sugars, which required more chilling hours to finish their vegetative rest; besides the increase in artificial chilling promoted a linear increase in values of total sugar for Troyano cv. in 2017, as this cultivar presented higher averages than the others. According to Kaufmann & Blanke (2017), in a study with cherry cultivars, the beginning of the dormancy period (para-dormancy) of plants is marked by a 2:1 ratio between hexoses (fructose and glucose) and starch, whereas during end-dormancy this ratio is up to 20:1 and at the end, in eco-dormancy, it is 10:1. This evidences that the cultivar Troyano, due to the predominance of starch, was in the initial dormancy stage, and that it demands a greater accumulation of cold hours. The White Genova cv. only achieved the total sugars peak level at 71.7 CH and, subsequent reduction, the sugars in this cultivar may have activated the metabolism and signal to leave the vegetative rest (Figure 2C).

Cultivars behaved differently in 2018, since total sugars contents were lower than starch contents (Figure 2B and Figure 2D). Thereat, the high starch values in relation to those of total sugars demonstrated that stem cuttings had just begin their vegetative rest period, when carbohydrates are stored in plants roots and cuttings (Gupta & Kaur, 2005). Therefrom, the field chilling hours (2.7 CH) were insufficient in advancing the vegetative rest in 2018, unlike 2017. Rosa *et al.* (2014) attributed the lowest number of accumulated chilling hours to the highest content of total soluble carbohydrates in ‘Cabernet Sauvigon’ grapevine cultivar (*Vitis vinifera* L.). Furthermore, the starch reserves are vital for plants to protect them against low temperatures and, consequently, for plants to sprout and develop flowers and fruits (Gupta & Kaur, 2005).

The chilling hours in Pingo de Mel cv. increased in 2018 and promoted a linear increase in total sugars, confirming its lower requirement; but these values decreased in Brown Turkey cv. in 2018; while the others did not show any significant variation for artificial chilling exposure (Figure 2D). In 2018, the total sugar values decreased in Brown Turkey cv., while the artificial CH did not promote any significant variations in the other cultivars. The increase in CH in this cycle promoted a linear increase in sugars for Pingo de Mel cv. (Figure 2D), reaffirming its lower requirement to cold weather that can be explained by his origin in regions with low chill from Northern Algarve, Portugal (Pio & Chagas, 2011). Similar behaviour was observed by Marafon *et al.* (2011) in Japanese pear trees (*P. pyrifolia* cv. Housui), as the authors reported that the accumulation of soluble sugars in branches exposed to chilling hours occurred because of low temperatures during the dormancy period.

Regarding to reducing sugars (glucose and fructose), a quadratic growth was observed in Roxo de Valinhos and White Genova cultivar with increasing chilling hours in both cycles (Figures 2E e 2F). On the contrary, the CH in Troyano cv. presented a linear increase in reducing sugar content in 2017, but a decrease in 2018, showing that this cultivar was more sensitive to accumulated chilling hours in field, probably by his origin from Northern Italy, a region with high chill accumulation. In the first cycle, the linear growth allowed the highest reducing sugar average for Troyano cv. at 160 CH. The variation of reducing sugars in Brown Turkey cv. was adjusted to the linear decreasing model in 2018, while Pingo de Mel cv. did not adjust to any tested models for this variable in any cycle (Figures 2E and 2F).

It is worth noting that in the absence of chilling hours in a cold chamber, regardless of the accumulation obtained in field, there were no differences among cultivars for reducing sugars in both cycles (Figures 2E and 2F). In this condition of less accumulated chilling hours in field in 2018, the fig cultivars showed higher levels of starch when compared to reducing sugar levels (Figures 2B and 2F), which was also observed by Marafon *et al.* (2011) in Japanese pear branches (*Pyrus pyrifolia* cv. Housui) that were not subjected to low temperature exposures.

Carbohydrate mobilization varies throughout artificial chilling exposure on fig cuttings, when starch content reached a peak level and followed degradation (Figures 2A and 2B). Subsequently, there was an increase in reducing sugar content to then decrease (Figure 2E and 2F). Since cuttings remained under low temperature, the starch hydrolysis and sucrose synthesis happen through enzymes activities (á-amylase and sucrose phosphate

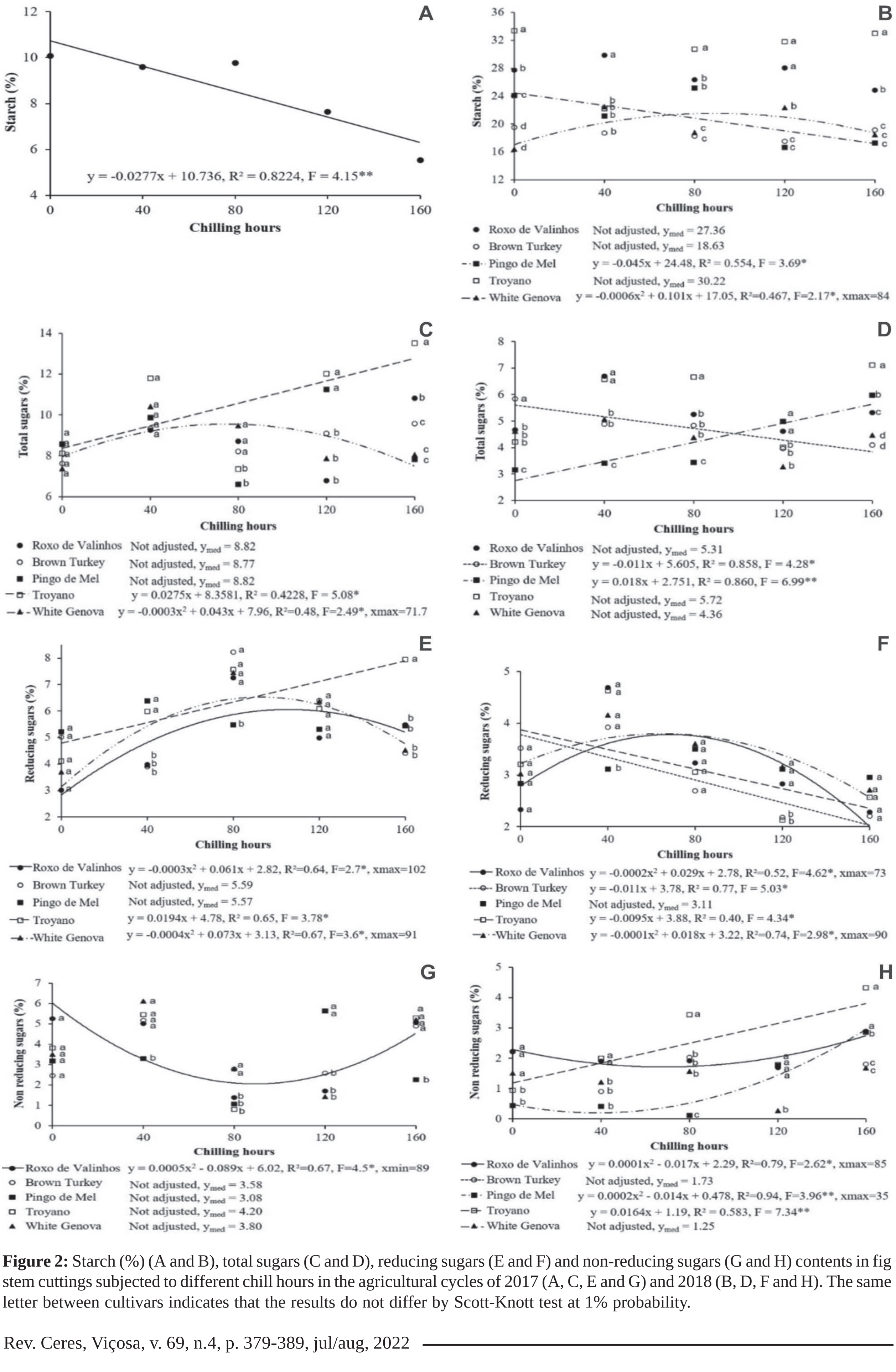

Figure 2: Starch (%) (A and B), total sugars (C and D), reducing sugars (E and F) and non-reducing sugars (G and H) contents in fig stem cuttings subjected to different chill hours in the agricultural cycles of 2017 (A, C, E and G) and 2018 (B, D, F and H). The same letter between cultivars indicates that the results do not differ by Scott-Knott test at 1% probability.

synthase, respectively) to protect plant organs against the cold (Gupta & Kaur, 2005); consequently, the relation between starch conversion and increased content of glucose and fructose were disproportionate among cultivars.

When starch contents are lower than reducing sugars, it is believed to be the end of dormancy period, since the increase in reducing sugars contents may indicate starch hydrolysis, converting it into soluble sugars (Oliveira *et al.*, 2012). Therefore, explaining the biggest gap between starch and reducing sugars contents in 2018, as the number of chilling hours dropped in 2018. In the first cycle, fig cuttings presented high accumulated chilling hours, the starch hydrolysis process had already intensified. Soluble sugars are also called non-reducing sugars and are less reactive than reducing sugars, bearing in mind that sucrose is the most common sugar translocated (Gupta & Kaur, 2005).

The non-reducing sugars (sucrose) tended to behave unlikely reducing sugars, especially in 2018. In 2017, cuttings were exposed to 80 artificial chilling hours, they presented less non-reducing sugars than reducing sugars contents (Figure 2E and 2G), besides cultivars behaved differently among them. In 2018, the same behaviour was observed in cuttings exposed to 0, 40 and 80 CH (Figure 2F and 2H). Thus, non-reducing sugars are easily transported to the growth organs (i.e., buds and roots), where they will again be converted into starch and then stored (Gonçalves *et al.*, 2015). Corroborating with Alburquerque *et al.* (2008) who assessed chilling hour requirement in cherry (*Prunus avium* L.) and pistachio (*Pistacia vera* L.), this study noted that the evaluated fig cultivars generally exhibited different outcomes with regards to accumulation of chilling hours and, consequently, in the carbohydrates dynamics.

The cultivar factor individually affected the total nitrogen content in 2017 ($P < 0.01$). However, there was a significant effect of the interaction between cultivars and chilling hours in 2018 ($P < 0.01$). Such inconsistent results between agricultural cycles may be because of plants staying at the same place for longer periods, under climatic conditions that vary over the years, which provides different physiological aspects for each cycle. The stem cuttings of Roxo de Valinhos and Pingo de Mel cv. presented the lowest total nitrogen content of all (Table 1).

During autumn, nitrogen is accumulated in perennial organs in woody plants such as the fig tree, mainly in the sprouted buds. During spring, mobilized nitrogen is translocated and redistributed to the growing tissues in the subsequent cycle (Chao & Anderson, 2010). Besides the stored nitrogen responds to specific stimuli such as chill accumulation, presenting a physiological role of resistance to low temperatures; thus, the nitrogen level can influence the dormancy period of temperate plants.

The artificial chilling exposure promoted a linear increase of total nitrogen content in the averages of Roxo de Valinhos and Brown Turkey cv., results presented those peak values did not have any difference for these cultivars (Figure 3). When nitrogen content increases before sprouting, protein hydrolysis occurs concomitantly, which coincides with the end of dormancy. This may indicate that the sum of 2.7 CH in 2018 with the artificial chilling hours being insufficient to end the vegetative rest period. Seif Elyazal and Rady (2012) observed higher levels of total nitrogen in apple (*Malus sylvestris* cv. Anna) buds subsequent to dormancy release.

When the accumulated cold hours are insufficient to break dormancy, it is common to use sprouting inducers, such as hydrogen cyanamide (Dormex®) and Erger®, a product that presents organic nitrogen in its composition and presented a good performance anticipating the sprouting and promoted a higher number of buds overcome the dormancy in fig 'Roxo de Valinhos' (Souza *et al.*, 2021). During the process of induction and overcoming of dormancy, several changes occur in the chemical components of plant tissues, mainly in the buds (Seif Elyazal & Rady, 2012). Studies indicate that buds sprayed with sprouting inducers, present higher nitrogen contents, since they may play a role in inducing enzyme activity, promoting the retranslocation of stored reserves and increasing the uptake of nitrogen leading to bud break (Seif Elyazal & Rady, 2012).

There was no significant effect of the interaction between cultivars and nitrogen contents (Figure 3). These variations in nitrogen contents are due to the seasonal amplitudes that plants present, since it is shown on different occasions annual minimum and maximum levels of this nutrient (Chao & Anderson, 2010).

The reproductive buds of fig trees are involved in the newly formed, besides having endogenous factors such as carbohydrates and nitrogen compounds. Also, buds fertility is fundamental in fruit production, the buds cuttings of fig trees showed great fertility in the present study, due to the nutrient uptake, since the soil had a high content of organic matter in the experimental area; thus, releasing a high amount of mineral nitrogen to the soil, as a result it increased the levels in shoots.

Moreover, the cuttings of Troyano cv. performed the highest starch content when exposed to 120 CH (Figure 2B), also showed the highest total nitrogen value under the same condition (Figure 3). Thus, the inhibition of á-amylase enzyme activity by higher nitrogen

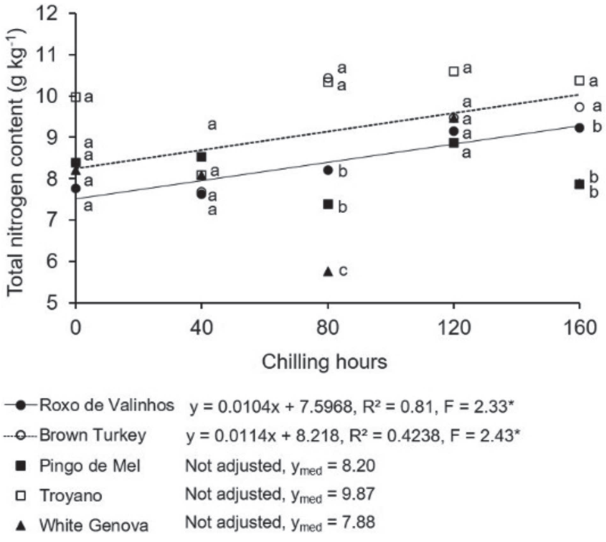

Figure 3: Total nitrogen content (g kg^{-1}) in fig stem cuttings subjected to the chill hours in 2018. The same letter between cultivars indicates that the results do not differ by Scott-Knott test at 1% probability.

concentrations (Shao & Ma, 2004) is evidenced in the low contents of total sugar (Figure 2F), due to the starch limitation in the starch hydrolysis process. Possibly, bud fertility is associated with carbohydrate reserves that are accumulated in these organs, especially starch; besides protein content such as nitrogen that promotes budding in the subsequent cycle (Chao & Anderson, 2010).

CONCLUSIONS

The effects of chilling hours on fig cuttings vary according to the cultivars and accumulated chilling hours (CH). The different accumulations of chilling hours (CH) impact the activity of antioxidant enzymes, as well as carbohydrates and nitrogen contents. The Roxo de Valinhos and Pingo de Mel cvs. requires less accumulation of chilling hours (CH) to break dormancy. However, Troyano cv. requires more, which explains its sprouting and late production in subtropical areas. This first report on the fig stem cuttings chilling requirement in Brazil will facilitate the breeding programs for low chilling requirement cultivars.

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DECLARATION OF CONFLICT INTEREST

The authors declare no conflict of interest.

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