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Chilling privation during dormancy period and carbohydrate mobilization in Japanese pear trees

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ABSTRACT: The flower bud abortion is one of the main problems that limit commercial pear (*Pyrus pyrifolia*) production in the southern region of Brazil. Insufficient chilling during the dormancy period is known as the main factor of this problem. One of the hypotheses to explain this problem is that the starch mobilization and carbohydrate fluxes to the buds are impeded when mild temperatures occurred during winter. This study compared the total soluble sugars (TSS) and reducing sugars (RS) concentrations, the cell wall acid invertase (CWAi - EC 3.2.1.26) and sucrose-phosphate synthase (SPS - EC 2.4.1.14) activities in wood of branches and floral buds of Japanese pear trees cv. Housui, grafted on *Pyrus calleryana* and submitted to chilling conditions during the dormancy period. Treatments were: (i) natural conditions; (ii) continuous artificial chilling; (iii) alternating temperatures, and (iv) total chilling privation. TSS and RS contents, as well as CWAi and SPS activities in tissues of branches that received insufficient chilling were lower than those that received sufficient chilling during winter. The starch concentration was superior in wood tissues of branches kept under chilling privation. The chilling privation disturbs carbohydrate mobilization in pear trees, reducing the sucrose synthesis capacity in wood tissues (source) and sucrose importation by the floral buds (sink).

Key words: *Pyrus pyrifolia*, carbohydrates, floral bud abortion, cell wall acid invertase, sucrose-phosphate synthase

Introduction

The southern region of Brazil has developed the cultivation of temperate fruit trees, especially apple (*Malus domestica* Borkh.), grape (*Vitis vinifera* L.), peach (*Prunus persica* (L.) Batsch) and plum (*Prunus salicina* Lindl.) cultures, but the pear (*Pyrus* sp.) culture did not show the same development, due to irregularity of the production and low yield of the orchards. Among the main limiting factors are: indefiniteness of the rootstocks, low rates of floral differentiation, high levels of flower bud abortion (variable with the cultivar, location and weather conditions) and lower percentage of fruit set (Nakasu et al., 1995).

In temperate-zone deciduous fruit trees, the most important factor in release dormancy is the accumulation of a certain amount of chilling (Lang, 1996). During dormancy, chilling temperature are associated with changes in carbohydrate contents and other substances, such as nucleic acids, proteins, polyamines, amino acids, organic acids and in the respiration rate, that may be related with bud break and the time of bloom (Wang and Faust, 1987).

Lack of chilling, associated with mild winter conditions, results in abnormal patterns of budbreak and development in temperate fruit trees (Mauget and Rageau, 1988) and is known as the main factor of pear flower bud abortion in Brazil (Petri et al., 2002; Petri and Herter, 2002) and New

Zealand (Klinac and Geddes, 1995; Do Oh and Klinac, 2003). Rakngan et al. (1996) observed that Japanese pear trees under enough chilling break dormancy earlier than plants with low chilling accumulation. The occurrence of intermittent hot days during dormancy period, with temperatures higher than 27°C, delayed the release dormancy more than mild temperature fluctuations (2 to 3°C).

Carbohydrates are the main source of energy for the metabolic changes that occurred during the dormant release period. Carbohydrate availability is presumably of major relevance to the control of bud growth and development during dormancy and dormancy release (Sherson et al., 2003), and might be related to the bud necrosis (Cottignies, 1986). Sugar comes from starch accumulated in reserve tissues during the preceding summer is converted to sucrose during winter and the enzymes involved in this conversion are induced by the low temperatures. Chilling effect on the changes in starch and sugar concentrations may be explained because amylase activity is induced by cold temperature, increasing starch hydrolysis and, consequently, sugar concentration (Elle and Sauter, 2000).

During winter, starch degraded by amylases is used to the sucrose synthesis by the sucrose-6-phosphate synthase (SPS) in response to decreasing temperature. The sucrose produced in reserve tissue is transported by the xylem pathway to the bud and hydrolyzed to glucose and fructose to supply energy and carbonic precursors (Yoshioka et al., 1988).

The SPS enzyme participates in the conversion of UDP-glucose plus fructose into sucrose-6-P and plays an important role in the mobilization of glucidic reserves in plant tissues. A close connection was observed between the sucrose synthesis and the SPS activity in photosynthetic and non-photosynthetic tissues of several fruit trees (Hubbard et al., 1991).

The cell wall acid invertase (CWI) is associated with the sugars absorption and its activity determines the sink strength of a tissue, an organ or plant tissue. Hexoses (glucose and fructose) released by CWI associated with the H⁺-hexose cotransporter of the transfer cells of the xylem, results in a regulatory mechanism capable of attracting more sucrose from the source tissue to the sink tissue (Godt and Roitsch, 1997).

One of the hypothesis to explain the pear bud necrosis problem is that the reserve mobilization and the carbohydrate fluxes to the buds are blocked when mild temperatures occurred during winter. The objective of this study was to compare the carbohydrate concentrations, the cell wall acid invertase and sucrose-phosphate synthase activities in wood of branches and floral buds of Japanese pear trees (*Pyrus pyrifolia*) cv. Housui, under different chilling conditions during the dormancy period.

Materials and Methods

This experiment was conducted in the winter period of 2007, under controlled conditions in Pelotas, state of Rio Grande do Sul, Brasil (31°52' S, 52°21' W, average altitude

of 224 m). The climatic classification of Pelotas is of type 'Cfa' (according to Köppen), temperate humid climate with hot summer. Annual averages are 1,582 mm of rainfall, 18.4°C of temperature, 78% of relative humidity and around 350 h ($\leq 7.2^{\circ}\text{C}$) of natural chilling accumulation during the winter period.

The experimental material consisted of branches of Japanese pear trees cultivar Housui, collected from 16-year-old plants at the experimental orchard. The chilling requirement of 'Housui' is around 720 chilling hours ($\leq 7.2^{\circ}\text{C}$) and has shown high rates of floral bud abortion (close to 100% in most years) in southern Brazil (Faoro, 2001).

The collected branches were grafted on rootstocks of *Pyrus calleryana* in May 2007, kept in a greenhouse (around 25°C) for 40 days until the start of treatment in June. Branches underwent the following treatments: T1) natural conditions: 235 hours ($\leq 7.2^{\circ}\text{C}$); T2) continuous artificial chilling: 792 hours (under 5°C) in a cold room; T3) alternating temperatures: 264 h (5°C) in a cold room + 264 h (15°C) in a growth chamber + 264 h (5°C) in a cold room and T4) lack of chilling: 792 h (15°C) in a growth chamber (Figure 1). Upon completion of treatments, branches were transferred to the greenhouse ($\pm 25^{\circ}\text{C}$) to induce the budbreak. After three days, samples (wood and floral buds) were collected, frozen in liquid nitrogen, freeze dried and kept at -70°C until assayed. Evaluations were performed on three-sample groups from 20 stems for each treatment.

A total of 200 mg of dry matter for the quantification of carbohydrates was used. After grinding, the samples were

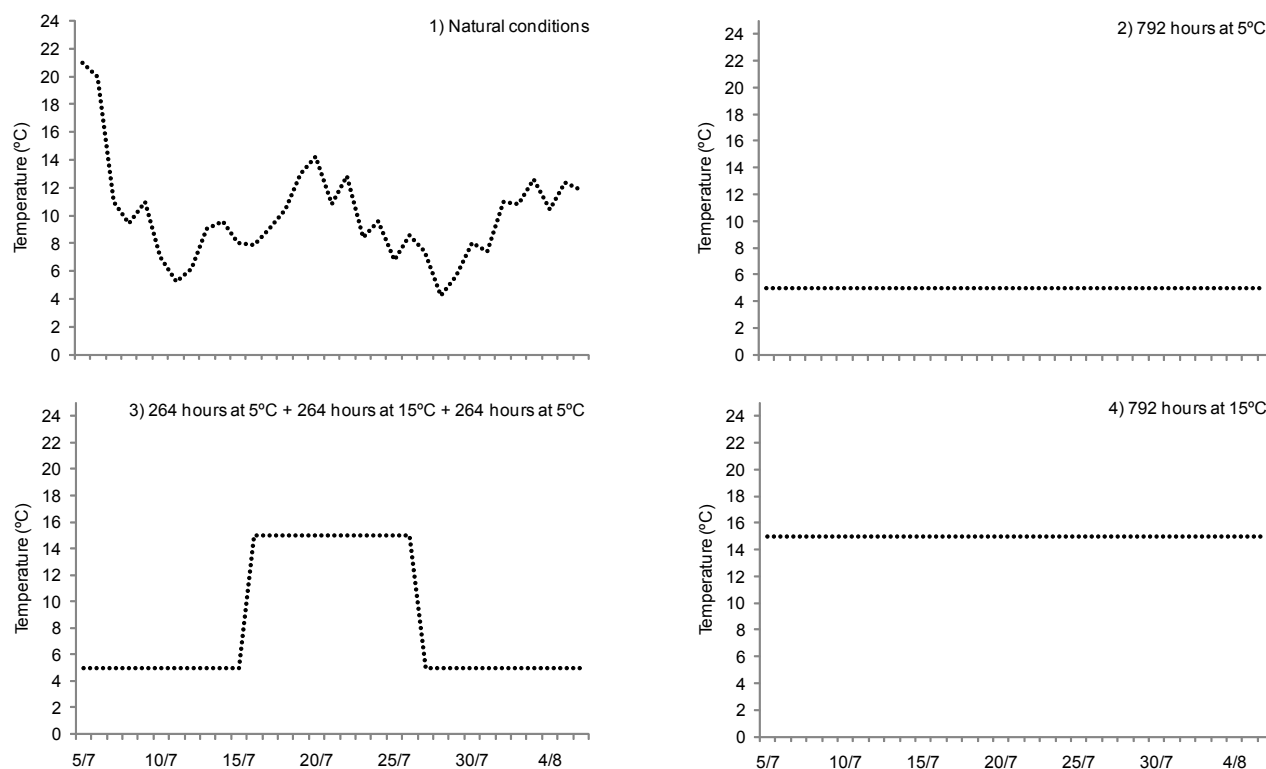


Figure 1 – Chilling treatments applied in grafted branches of Japanese pear cv. Housui.

placed in test tubes, 5 mL of ethanol at 80% pre-heated (80°C) were added, stirred and incubated at 85°C for 5 min in water bath. After centrifugation at $3,000 \times g$ (6,000 rpm) for 10 min, the supernatant was collected into volumetric flask of 50 mL. The pellet was resuspended in 5 mL of ethanol at 80%, pre-heated and centrifuged again, collecting the supernatant and add to the flask, completing the volume of the flask to 50 mL with distilled water. From this sample, an aliquot of 200 μL of supernatant was used for quantification of total soluble sugars (TSS) by the anthrone method (Dische, 1962) in a spectrophotometer at 620 nm of wavelength (λ). The quantification of reducing sugars (RS) was performed with aliquots of 750 μL of supernatant by the dinitrosalicylic acid method (Miller, 1959) at 540 nm of wavelength (λ). The TSS and RS concentrations were expressed in mg g^{-1} DW (dry weight) for both determinations.

For the starch quantification, the dry pellet was re-suspended in 3.33 mL of perchloric acid at 30% and stirred for 30 min. Next, 6.67 mL of distilled water were added and was centrifuged at $3,000 \times g$ (6,000 rpm) for 10 min. The supernatant was collected into a volumetric flask of 50 mL, repeating the same procedure again. Both supernatants were mixed and the volume of the flask was complete to 50 mL with distilled water. The starch quantification was carried out using 200 μL of supernatant in 1 mL of distilled water. Then, it received 2 mL of anthrone (0.1%) diluted in sulfuric acid p.a., and the test tubes were kept in ice bath (Dische, 1962). After, tubes were boiled for 3 min at 100°C and then cooled again in ice bath. The quantification was made at 620 nm (λ), and the starch concentration expressed in mg g^{-1} DW.

The enzyme extraction was performed by maceration of 150 mg of tissue in liquid nitrogen. After grinding, 1200 μL of 50 mmol L^{-1} HEPES/KOH buffer, pH 7.0, with 10 mM DTT, 5 mM MgCl_2 , and 1 mM EDTA were added into Eppendorf tubes.

After well mixed, samples were centrifuged at $13,000 \times g$ (18,900 rpm) for 15 min at 4°C, and the supernatant was recovered to determine the activity of SPS (EC 2.4.1.14) according to the method of Schrader and Sauter (2002) and Hauch and Magel (1998). SPS activity was determined in non-limiting substratum conditions, in an incubation medium composed of 100 mM HEPES/NaOH pH 7.5, 25 mM MgCl_2 , 25 mM glucose-6-phosphate, 5 mM fructose-6-phosphate, and 20 mM uridine diphosphate glucose (UDP-glucose). UDPUDP is the product formed during the synthesis of sucrose and is measured indirectly by the nicotinamide adenine dinucleotide in reduced form (NADH) consumed by a multi-enzymatic reaction catalyzed by a complex of Pyruvate Kinase/Lactate Dehydrogenase (PK 450 U/LDH 450 U), using a of 100 mM TRIS/HCl buffer, pH 7.5, with 10 mM MgCl_2 , 0.8 mM phosphoenolpyruvate (PEP), and 0.3 mM NADH. The reaction was developed for 40 min at 30°C, being paralyzed by boiling at 100°C for 4 min. Control samples were first boiled at 100°C for 4 min. The absorbance was made in quartz cuvette at 340 nm (λ). The enzyme activity was expressed as nanomoles of NADH per minute per gram of fresh weight ($\text{nmol NADH min}^{-1} \text{g}^{-1}$ FW).

The CWAI extraction was performed by acid dialysis of the pellet resulting from the SPS enzyme extraction. This pellet was washed with 800 μL of extraction buffer and centrifuged at $13,000 \times g$ (18,900 rpm) for 5 min, repeating the same procedure again. After this, it was resuspended in 500 μL of 70 mM K_2HPO_4 / 40 mM Citrate buffer with 1M NaCl pH 5.0, remaining in the eppendorf tubes for 18h under refrigeration. Then, samples were centrifuged at $13,000 \times g$ (18,900 rpm) at 4°C for 15 min, recovering the supernatant to determine the CWAI activity (EC 3.2.1.26) according to the method of Roitsch et al. (1995). The CWAI incubation medium was composed of a 70 mM K_2HPO_4 / 40 mM Citrate 40 buffer, pH 5.0 and 50 mM sucrose. The reaction was developed during 30 min at 30°C and was stopped by boiling at 100°C for 5 min. The control samples were first boiled for 5 min at 100°C. Then, it was followed by the estimation of reducing sugars according to the methodology of Nelson (1944). The absorbance was performed at 520 nm (λ) and the enzyme activity expressed in nanomoles of glucose produced per minute per gram of fresh weight ($\text{nmol min}^{-1} \text{g}^{-1}$ FW).

The experimental design was completely randomized with three replications in a double factorial scheme (2×4), with two levels of tissue factor (bud and wood) and four levels of chilling treatments factor (T1, T2, T3 and T4). Analysis of variance (ANOVA) was calculated and averages were grouped by Tukey test ($p \leq 0.05$) using WinStat Program Software version 2.11 (Machado and Conceição, 2003).

Results and discussion

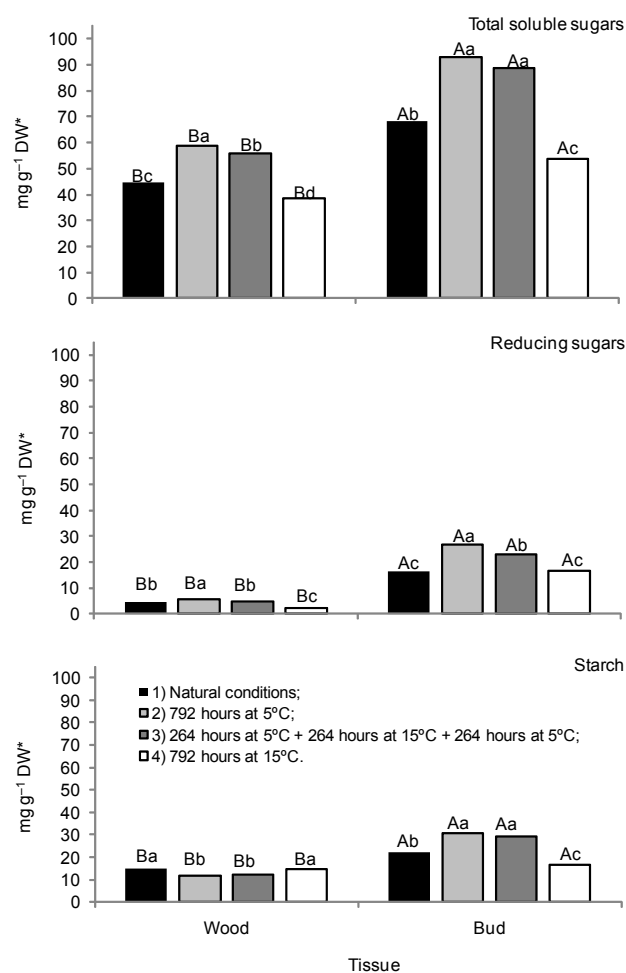
Interactions ($p \leq 0.05$) between factors occurred to the total soluble sugars (TSS), reducing sugars (RS) and starch (mg g^{-1} DW) contents and to cell wall acid invertase (CWAI) and sucrose-phosphate synthase (SPS) activities ($\text{nmol min}^{-1} \text{g}^{-1}$ FW) (Table 1). Concentrations of TSS, RS and starch were higher in the buds concerning the continuous (T2) and alternate artificial chilling (T3) compared to natural conditions (T1) and lack of chilling (T4) treatments. The reducing sugars were higher in both tissues of T2 treatment than in other treatments. The total soluble sugars concentrations were higher in wood tissues of T2 treatment and in bud tissues of T2 and T3 treatments. The accumulation of soluble sugars in the branches submitted to the cold occurred possibly due to the effect of low temperatures during the dormancy period, leading to a great accumulation of sugars in the buds exposed to chilling during the dormancy release phase (Figure 2).

The elevated RS (glucose and fructose) content in the tissues which receive more artificial chilling than others during dormancy enabled the 'resynthesis' of starch next to the floral buds tissues and provided more reserves to support the blossom and leafing processes. The starch mobilization from the reserve tissues (the stems and/or roots) to the growth areas (the meristems) of the woody plants, aims to increase the amount of soluble sugars potentially useful for cellular metabolism (Beck and Ziegler, 1989). Carbohydrates dynamics in tissues of temperate-zone deciduous fruit trees reflects the inter-conversion between starch and soluble sugars, since

Table 1 – Summary of variance analysis for total soluble sugars (TSS), reducing sugars (RS), starch, cell wall acid invertase (CWI) and sucrose-phosphate synthase (SPS) activities in 'Housui' pear tissues exposed to chilling treatments.

Source of variation	Degree of freedom	Mean Square				
		TSS	RS	Starch	CWI	SPS
Tissue (T)	1	4220.60*	1586.18*	758.58*	92851.53*	97207.28*
Chilling treatment (C)	3	1144.29*	59.75*	36.58*	60054.05*	21396.11*
T × C	3	120.73*	24.26*	94.38*	31079.48*	5352.53*
Error	16	2.99	0.08	0.44	230.53	26.81
CV (%)		2.76	2.27	3.49	8.92	4.64
General mean		62.69	12.49	19.06	170.17	111.40

*5% probability level.

Figure 2 – Total soluble sugars, reducing sugars and starch in tissues of 'Housui' pears at budbreak induction after chilling treatments. Means with the same lowercase in the same tissue and means with the capital letters in the same chilling treatment do not differ (Tukey, $p \leq 0.05$).

The increase in RS content in the buds that received cold may be associated with the decreased in starch content in reserve tissues to support the dormancy release processes (Maurel et al., 2004b; Bonhomme et al., 2005). According to these authors, the necrosis occurrence would be primarily a

that starch hydrolyzed during dormancy period is temporally resynthesized before being used during dormancy release (Ameglio et al., 2004).

Starch concentration in the wood was higher under insufficient (T1) and deprived chilling (T4) than under chilled branches (T2 and T3). Probably, the starch concentration was lower in the wood tissue of chilled branches due to the induction of starchy enzymes by the low temperatures as observed in poplar trees (*Populus* sp.) by Sauter (1988). Unlike, the highest starch content in the wood of the branches under insufficient and deprived chilling could reflect the lack of induction of these starchy enzymes during the dormancy period, as also observed by Citadin et al. (2009) in wood tissues of walnut trees. Sauter et al. (1998) found a high correlation between the low temperature, the starch degradation and the increasing in the content of sugars in *Populus* stems. The nature of the sugars released after exposure of the shoots to cold showed that it was essentially the alpha-amylase enzyme responsible for starch hydrolysis at low temperatures. The contact of this enzyme with the starch granules increased strongly and linearly with decreasing temperature in the xylem parenchyma cells during the dormancy period.

The uptake of nutrients by the meristematic zone could depend on the capacity of transportation from the reserve domain (wood and/or root) to the growth area (meristems). During dormancy, the bud exhibited low sugar absorption potential and increased the hexose concentration by starch hydrolysis. However, after dormancy release, the buds become high sink tissues, increasing the soluble sugars importation, by active transport, which are metabolized to provide energy and carbon storage (Marquat et al., 1999).

In our study, branches submitted to insufficient chilling had higher contents of starch in wood and low RS content in bud tissues. Leite et al. (2004) found that peach branches kept under natural conditions, with their complete chilling requirement satisfied, showed increases in the RS and starch contents in bud tissues. Unlike, in plants kept under chilling privation (insufficient chilling), the carbohydrates accumulation in the buds was not sufficient to allow their normal development. Lacointe et al. (1993) observed that the starch and soluble sugars concentrations follow a reverse pattern during dormancy, suggesting that a process of interconversion occurs between them.

consequence of the floral bud incapacity to metabolize the available sucrose and/or sorbitol to provide the required hexoses during the blossom and leafing phases. Bonhomme et al. (2010) measured the bud capacity of walnut (*Juglans regia*) to use the xylem sap carbohydrates during the rest period until bud break, observed that the bud uptake capacity varies according to the temperature. The flow rates between xylem vessels and buds increased 1 month before bud break, when buds were able to import high sugar quantities (around 2000 μg of sucrose $\text{h}^{-1} \text{g}^{-1} \text{DW}$). Bonhomme et al. (2010) also showed a close correlation between influx of soluble sugars and the concomitant DW increase in buds.

In chilled branches (T2 and T3) the activities of SPS in both tissues and CWAI in the bud tissue were higher than in the other treatments, which received insufficient (T1) or were deprived of chilling (T4). The increases in RS and starch contents in buds and in the TSS content in the wood of the chilled branches are probably related to the increases in the activities of CWAI in buds and SPS in the wood, respectively (Figure 3).

The continuous chilling treatment (T2) was more effective to convert starch into sucrose in wood and more effective in the hydrolysis of this sucrose into reducing sugars in

buds than all the other treatments. Activities of CWAI in the buds and of SPS in the wood tissues were higher in branches submitted to continuous cold than in other treatments and possibly has increased the amount of sucrose that reached the buds and was hydrolyzed in glucose and fructose. CWAI activity may be inversely correlated to the sucrose concentration and positively correlated with the glucose and fructose concentrations in the sink tissues (Ito et al., 2002). Maurel et al. (2004a) found an increase in CWAI activity in peach buds just before the leafing in natural conditions, but when branches were maintained under chilling privation this activity remained low.

During winter, starch degraded in the wood tissue, by the action of amylases induced by cold, is metabolized into sucrose by the enzyme SPS, which exports sugar to the buds, where it is hydrolyzed into glucose and fructose by CWAI or sucrose synthase (SuSy) for the supply of energy and carbonaceous precursors (Yoshioka et al., 1988). The low SPS activity observed in the wood tissues of insufficient (T1) and lack of chilling (T4) treatments demonstrates its limited ability to export sucrose under these circumstances. Likewise, in the same treatments, a low activity of CWAI in the buds was observed, which characterizes the low import capacity of sucrose. Thus, these low SPS and CWAI activities together characterizes a weak capacity to mobilize reserves from the source (wood) to the sink tissue (floral bud), causing a blockage in the bud growth and development vital processes and, consequently, the possible flower bud necrosis.

The high temperatures during dormancy nullify chilling accumulation in Japanese pear trees, which disturb carbohydrate mobilization from the reserve tissues and the absorption of sugars by the buds (Honjo et al., 2002). The intensification of flower bud abortion may be related to the lack of chilling and to the temperature fluctuations during the dormancy period. The necrosis symptoms became more intense during winter period, proportionally to the increasing temperatures, affecting most of plants at end of dormancy (Marafon et al., 2010).

In branches subject to sufficient chilling accumulation, the capacity of synthesis and exportation of sucrose from the apoplast becomes larger after dormancy release and coincides with the increase of starch enzyme activity. The starch stored in xylem parenchyma cells is converted rapidly to sugars, particularly sucrose, and is then secreted by contact xylem cells into the xylem vessels. Sucrose is transported along the xylem pathway together with the water, to the buds, where it is directly absorbed or further hydrolyzed in the apoplast, contributing to their import. Temperature may determine whether parenchyma secrete or absorb sugars, with secretion dominating at low temperatures (Ameglio et al., 2004). Schrader and Sauter (2002) found out that the SPS activity in the xylem parenchyma of the branches of *Populus*, *Salix*, *Acer* and *Fagus* increases in late autumn, is maximum in the winter (during the starch conversion into soluble sugars), and decreases in early spring, during the starch resynthesis in the adjacent tissues to the buds. These authors found that the SPS activity in branches kept at 0°C during dormancy was higher than those kept at 10°C.

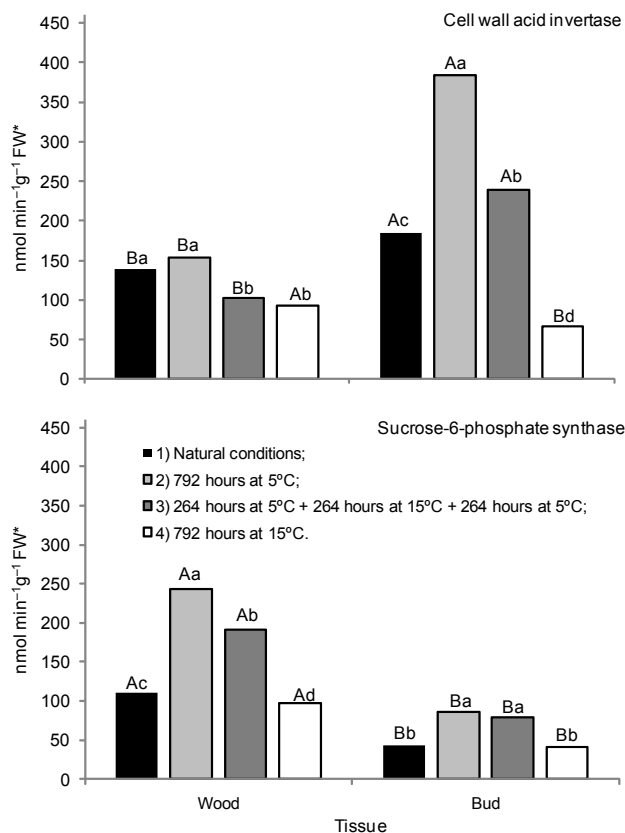


Figure 3 – Cell wall acid invertase and sucrose-6-phosphate synthase activities in tissues of 'Housui' pears at budbreak induction after chilling treatments. Pelotas, Rio Grande do Sul, Brazil, 2007. Means with same lowercase in the same tissue and means with the same capital letters in the same chilling treatment do not differ (Tukey, $p \leq 0.05$).

Hauch and Magel (1998) stated that the reduction in starch content in the cells of the xylem parenchyma, in *Robinia* branches, is associated with increased SPS activity during winter, indicating the conversion of starch into sucrose. When sucrose is translocated to non-photosynthetic tissues, it is rapidly converted into fructose and glucose by invertase enzymes. In these organs, fructose and glucose may be used in three ways: i) in the respiration, for Adenosine Tri-Phosphate (ATP) formation and other important compounds; ii) be stored in the vacuole for later use, and iii) glucose, that may be converted into starch serving as an energetic reserve for use in other circumstances. Therefore, the lower activity of SPS in the branches with insufficient or lack of chilling accumulation can be interpreted as a lower ability to export sucrose and the lower activity of CWAI can be a consequence of the low quantity of sucrose in the xylem parenchyma vessels of Japanese pears. This low capacity to import sugar, water and other nutrients of the bud tissues caused by high temperatures during winter, lead to the exhaustion of carbohydrate reserves and could be the cause of the flower bud abortion.

Conclusions

The exposure of branches at low temperature affected starch and soluble sugar contents and sucrose-metabolizing enzymes activities in wood and buds tissues of Japanese pears. While starch content decreases in wood, RS content increases in bud tissues of chilled branches. Unlike, starch content remained high in wood tissues and the RS content does not increase in those branches kept under chilling privation.

The chilling privation disturbs carbohydrate mobilization in pear trees, reducing the sucrose synthesis capacity in wood tissues and the sucrose importation by the floral buds. Therefore, the sufficient chilling supply during the dormancy period increases the cell wall acid invertase and sucrose-6-phosphate synthase activities providing increases in the reducing sugars and starch contents in bud tissues that are used to budburst and blooming in the spring.

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