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Study of the Consequences of Global Warming in Water Dynamics During Dormancy Phase in Temperate Zone Fruit Crops

Robson Ryu Yamamoto, Paulo Celso de Mello-Farias,
Fabiano Simões and Flavio Gilberto Herter

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1. Introduction

For a normal development of temperate zone fruit trees, a very complex phase of their life-cycle called dormancy is regulated by environmental conditions during autumn-winter season. This phase allows those species to survive under adverse environmental conditions, and one of the most important factors which induce and release dormancy stage is temperature. It was adopted a terminology suggested by Lang et al. (1987) for the different stages of dormancy: para-, endo-, and ecodormancy.

From the end of the 19th century, cultivation of temperate zone fruit crops were set up in areas warmer than those traditionally cultivated. As the cultivars grown were those used traditionally, the chilling requirements were not adequately fulfilled. These difficulties led to identification and study problems of growing temperate zone fruit crops in warm areas, which was approached in two different ways. On the one hand, traditional cultivars were selected and bred to obtain new cultivars with commercial quality and low chilling requirement, which is a heritable character. On the other hand, new cultural practices, such as applications of rest-breaking agents, were developed to avoid or reduce the negative consequences of an insufficient chilling accumulation. The most useful option for temperate zone fruit crops in warm areas has frequently been a combination of low-chill cultivars and the adoption of cultural practices to break or avoid dormancy. Although different species of the *Rosaceae* family have been bred for low-chill cultivars, the furthest advances have been made in peaches and apples.

During dormancy, several events occur simultaneously, which modifies water and carbohydrate dynamics, hormonal balance, among others. Water is important for bud and plant development, such in solute translocation, enzymatic reactions, and osmotic regulated events, reason why many studies are focused on its dynamics. Recent studies in water dynamics during dormancy phase, such as the embolism (loss of hydraulic conductivity) in xylem of plants and water status (free or bound water) in dormant buds determined by magnetic resonance (MR) techniques, showed the importance of water on dormancy release process. And under stressed conditions this importance seems to be strongly accentuated.

1.1. Physiology of dormancy

Dormancy is an adaptive behavior of temperate zone tree species that allows the plant to survive under unfavorable conditions during winter. From the appearance of the bud only, it is difficult to ascertain what kind of biochemical and physiological changes are happening during this period (Faust et al., 1997; Yooyongwech et al., 2008a).

Winter dormancy is an important adaptive mechanism for plant survival in temperate and cold climates. It is essential that the dormant condition is established within the plant well in advance of the cold season. This requires the timely sensing and physiological processing of a regular and reliable environmental signal (Heide & Prestrud, 2005).

Lang et al. (1987) classified the different phases of dormancy as ecodormancy, which is found in late winter and spring and imposed by unfavorable conditions to growth; paradormancy, which is equivalent to correlative inhibition or apical dominance; and endodormancy, also called deep or winter dormancy. The last is the genuine dormancy that characterizes woody plants in temperate zones and has been subject of many studies that have shown the enormous complexity of this phenomenon.

Lack of plant environmental synchrony is considered to be the primary cause of abiotic stress injury. Plant synchrony requires timely responses to environmental cues to minimize risk from abiotic stresses. The timing of growth cessation and dormancy, and subsequent cold acclimation, deacclimation and the depth of cold hardiness are all critical components of winter survival in temperate climates. The degree to which temperature mediates this response is important in order to determine the impact of future temperature change in a global warming context on timing of growth cessation and cold acclimation in woody plants (Tanino et al., 2010).

In temperate zone deciduous fruit trees, the most important factor in dormancy release process 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 might be related with bud break and the time of bloom (Wang et al., 1987).

Most of the temperature effects on plants are mediated by their effects on plant biochemistry. Carbohydrates are the main source of energy for the metabolic changes that

occurred during the period of dormancy release. Carbohydrate availability is presumably of major relevance to the control of bud growth and development during dormancy induction and its release (Sherson et al., 2003), and might be related to the bud abortion (Cottignies, 1986). Starch, which is accumulated in reserve tissues during the preceding summer, is converted to sucrose and other soluble sugars during winter. Effects of chilling accumulation on changes in both starch and sugar concentrations may be explained because amylase activity is induced by cold temperature, which result in increasing of starch hydrolysis and, consequently, sugar concentration (Elle & Sauter, 2000).

During winter, dynamics of carbohydrates in the different tissues reflects the inter-conversion between starch and soluble sugars as described previously by Améglio et al. (2001), Lacoïnte et al. (1993), and Sauter (1980). Just before bud break, a strong increase in hexoses in apical buds seems closely linked to the decrease in sucrose and starch. The sucrose decrease revealed high activity of enzymes involved in sucrose catabolism (Bonhomme et al., 2009). Starch, which is degraded by amylases, and 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 (Marafon et al., 2011; Yoshioka et al., 1988).

Researchers have investigated the relationships among carbohydrates (Fahmi, 1958; Monselise & Goldschmidt, 1981; Sarmiento et al., 1976; Stutte & Martin, 1986), hormones (Chen, 1987; Lavee et al., 1983; Mullins & Rajasekaran, 1981; Ramirez & Hoad, 1981; Stephan et al., 1999), mineral nutrients (Golomp & Goldschmidt, 1981; Hartmann et al., 1966; Priestly, 1977), and flower bud formation in different fruit species.

Ulger et al. (2004) observed that the abscisic acid (ABA), indole-3-acetic acid (IAA), and gibberellic acid (GA₄) levels in leaves, nodes and fruits of olive (*Olea europea* L.) during the induction, initiation, and differentiation periods in the on year were lower than those in the off year. Similarly, Pal & Ram (1978) in mango (*Mangifera indica* L.), Ulger et al. (1999) in olive, and Chen (1990) in litchi (*Litchi chinensis* Sonn.), demonstrated higher GA₃ level in the on year. The fact that GA₃ decreased and GA₄ levels increased during the induction and initiation periods in the off year suggest that they affect flower bud formation. Looney et al. (1985) determined that exogenous GA₄ application on apple (*Malus domestica* Borkh.) trees promoted flowering and yield, and Stephan et al. (1999) also noted that lack of GA₄ induced a biennial bearing habit.

An increase in zeatin levels during the induction period in the off year suggests that had possibly a positive effect on floral formation (Chen, 1987, 1990; Mullins & Rajasekaran, 1981; Ramirez & Hoad, 1981; Ulger et al., 2004). Many experiments confirmed the direct involvement of different growth regulators in promoting or inhibiting flower bud induction and differentiation. However, all these studies were related to the effect of a single regulator or its quantitative changes before, during or after flower bud induction (Lavee, 1989). On contrary to plant growth regulators, Ulger et al. (2004) suggested that carbohydrates and mineral nutrients might not have a direct effect to induce flower initiation.

1.2. Global warming and dormancy

Global climate change can alter significantly plant phenology because temperature influences the timing of development, both alone and through interactions with other cues, such as photoperiod (Bernier, 1988; Partanen et al., 1998). Temperature records showed that, over the past 30 years, global average surface temperatures increased by 0.28°C per decade (Cleland et al., 2007).

Fruit and vegetable growth and development are influenced by different environmental factors. Studies conducted by Moretti et al. (2012) showed that warmer temperatures affect photosynthesis directly, causing alterations in carbohydrates, organic acids, and flavonoid contents, firmness and antioxidant activities; carbon dioxide (CO₂) accumulation in the atmosphere had direct effects on postharvest quality causing tuber malformation, occurrence of common scab, and changes in reducing sugars contents on potatoes; high concentrations of atmospheric ozone can potentially caused reduction in the photosynthetic process, growth, and biomass accumulation. Understanding how climate changes will impact fruit crop production in the next decades is extremely important for survival.

All economically important fruit and nut tree species that originated from temperate and cold subtropical regions have chilling requirements that need to be fulfilled each winter to ensure homogeneous flowering and fruit set, and generate economically sufficient yields (Luedeling et al., 2009). Elevated concentration of atmospheric CO₂, warmer temperatures in general, and changing precipitation regimes will affect the exchange of energy, carbon, water and nutrients between forests and the environment, leading to changes in forest growth, survival and structure. Interactions with biotic and abiotic disturbance agents will also shape future forests (Chmura et al., 2011).

Lack of chilling, associated with mild winter conditions, results in abnormal patterns of bud break and development in temperate zone fruit trees (Mauget & Rageau, 1988) and is known as the main factor of pear flower bud abortion in Brazil (Petri et al., 2002; Petri & Herter, 2002) and New Zealand (Do Oh & Klinac, 2003; Klinac & Geddes, 1995). Rakngan et al. (1996) observed that the Japanese pear trees under enough chilling released from dormancy earlier than plants with insufficient chilling accumulation. The occurrence of intermittent warm days during dormancy period, with temperatures higher than 27 C, delayed the dormancy release more than mild temperature fluctuations (2-3 C) (Marafon et al., 2011).

1.3. Apple and pear production under warm winter conditions of Brazil

Apple orchards in Brazil has been highlighted by growth in harvested area of 34%, 30% in production, 948% in exported quantity and 1328% in exportation value in the last 10 years. These results reinforce the trend that has been happening since the beginning of pomiculture in the country and reveal the great exportation potential of Brazil. The responsible factors for this great development of the apple orchards is due to the development of technologies used in crops, the logistics in place, the definition of cultivars (Gala 58% and Fuji 36%) and clones capable to meet consumer demands (Fachinello et al., 2011).

In addition to government incentives to apple crop on 1970's decade, which resulted in improvement of plant breeding, nutrition and phytotechnical management, post-harvest technology, made by research centers and extension programs, enabled to change from an importer country to export in a few years. One decade after starting the apple crop production on a commercial scale, Brazilian apple importations began to decline, and actually is considered self-sufficient (Boneti et al., 2002; Fachinello et al., 2011).

On contrary, pear crop production is not considered a fruit of great expression despite the large domestic market for pear fruit, moreover, it has the lowest expression in terms of production, crop area and production value (Fioravanço, 2007). In the beginning of 1960's decade, the pear crop in Brazil was economically more important than apple. According to FAOSTAT (2012), in 1961, the harvested area, production, and yield of pear crop were 70%, 22%, and 34% higher than apple, but in 2010 it was 25 times, 78 times, and 3 times lower, respectively. Such situation was changed at the end of 1970's, and nowadays Brazil imports around 90% of fresh pear fruit (140,000 tones), corresponding to USD 130 million in 2009.

It is therefore possible to note that pear crop represents an important market opportunity. Isolated initiatives of pear (European and Japanese cultivars) crop in highlands of Southern Brazil have confirmed the viability of its commercial production. A great demand of this fruit in local market occurs in Brazil, but it is observed a technological lack available for growers, such as development of cultivars of both rootstock and scion adapted to mild winter conditions, crop management adapted to such environmental conditions, appropriate logistic systems, among other factors (Faoro, 2001). Low rates of fruit set (Petri, 2008) and high incidence of physiological disorder called locally "flower bud abortion" (Marafon et al., 2011; Petri & Herter, 2002; Petri et al., 2001; Trevisan et al., 2005) are cited as factors of low yield in pear crops. In mild winter conditions of Southern Brazil, over 60% of "flower bud abortion" was found in 2001 (Petri et al., 2002) and more than 90% in 1999, resulting in low numbers of opened flowers at the bud break and consequently low production (Veríssimo et al., 2002). Another consequence of low chilling accumulation during dormancy stage is a prolonged and poorly synchronized flowering, which results in poor uniformity of fruit size at harvest (Yamamoto, 2010).

Nishimoto et al. (1995) cited the value of 750 chilling hours as the requirement in 'Housui' buds, but the amount of 600 hours below 7.2°C (80% of requirement) brought about the release from endodormancy stage (Yamamoto et al., 2010a). According to Yamamoto (2010), however, abnormalities on floral primordia (partial or total necrosis) and eventual development of new inflorescences, during dormancy progression (Fig. 1) and at flowering, were observed in all treatments in lateral buds of Japanese pear shoots (Fig. 2). These symptoms became severe with prolonged cold deprivation before chilling accumulation (simulation of delayed mild winter, treatment 3) and after consecutive seasons (simulation of permanent global warming situation) (Yamamoto et al., 2010a, b).

2. Past works carried out in water dynamics by using magnetic resonance techniques

Water is one of the basic determining factors of bud development because it is quantitatively the major component of plant tissues. Besides, it is the essential medium for many metabolic

processes, such as transpiration, CO₂ uptake, and photosynthesis, which regulates plant production, yield, and reproduction. Dormancy, an important phase which allow temperate zone fruit crops to overwinter, is considered to be closely related to changes in water movement (Welling & Palva, 2006).

Winter temperatures can impair the hydraulic functions of trees because water uptake from soil water reservoirs is very limited when upper soil layers are cool or frozen during winter months or early spring (Mellander et al., 2006; Peguero-Pina et al., 2011).

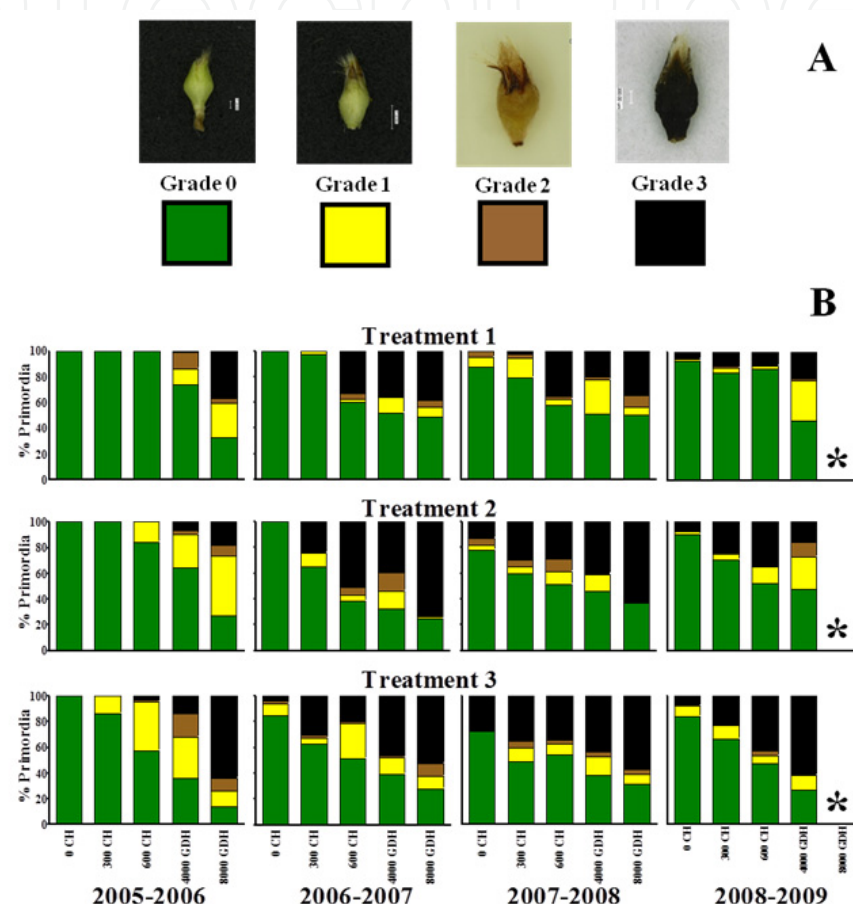


Figure 1. Scale adopted to evaluate the severity of floral primordia necrosis: grade 0, normal primordia (green); grade 1, yellow primordia; grade 2, primordia with partial necrosis; grade 3, completely necrosed primordia (A). Incidence and severity of floral primordia necrosis in buds of 'Housui' Japanese pear grown under mild (600 CH) winter conditions during four consecutive seasons (from 2005-2006 to 2008-2009), expressed as percentages of primordia (B). ($n = 10$). CH, chilling hours; GDH, growing degree hours; Treatment 1, normal chilling treatment; Treatment 2, one month of cold deprivation before exposure to chill; Treatment 3, two months of cold deprivation; Asterisks, not analyzed (Yamamoto, 2010).

2.1. Hydraulic conductivity in plants

In temperate zone woody plants, hydraulic conductivity describes resistance of xylem against embolism formation (Mayr et al., 2003) and can be impaired by xylem embolism, which is mainly caused by water stress or frost (Cochard et al., 2001; Cochard & Tyree, 1990; Sperry & Sullivan, 1992). Frost-induced embolism can occur as a consequence of alternating

frost-thaw events (Améglio et al., 1995; Cochard & Tyree, 1990; Just & Sauter, 1991; Pockman & Sperry, 1997). As the sap freezes, previously dissolved gases escape because of their low solubility in ice (Sperry & Sullivan, 1992). On subsequent thawing, these bubbles can either dissolve back into the xylem sap or grow to obstruct the entire xylem conduit (Cruiziat et al., 2002, 2003; Yang & Tyree, 1992), resulting in an embolized conduit that is unable to transport water (Améglio et al., 2002).

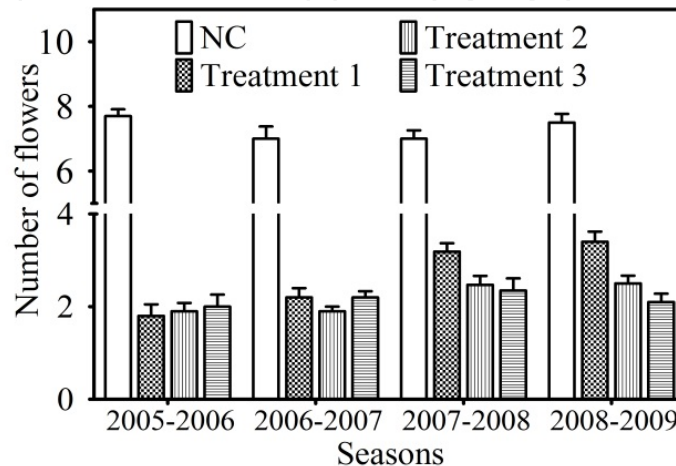


Figure 2. Average number of opened flowers per bud, from 2005-2006 to 2008-2009 seasons in treatments 1 (normal chilling treatment), 2 (one month of cold deprivation before exposure to chill), 3 (two months of cold deprivation), and natural conditions (NC). The vertical bars are mean \pm SD (n = 10) (Yamamoto, 2010).

It was demonstrated loss of hydraulic conductivity in xylem vessels during winter on peach trees conditioned to temperatures between 4-19 C after several cycles of freeze-thaw (Améglio et al., 2002). In deciduous fruit species, an increase in sap tension caused by dehydration of the tissue can affect the resumption of growth and production, moreover, in the xylem pathway between buds and twigs, affecting translocation of nutrients to the developing bud (Lauri & Cochard, 2008).

2.2. Water status determined by MRI techniques in buds during dormancy

Nuclear magnetic resonance (NMR) possibly the determination of water properties in biological systems by measuring proton spin density and relaxation time by spectroscopy (Chudek & Hunter, 1997). Magnetic resonance imaging (MRI) technique, a spatially resolved NMR in essence, is cited as an important tool for providing detailed and quantitative information on both water transport and status in intact plants (Van As et al., 2009). In addition, it is a non-destructive technique, allowing a continuous developmental analysis that provides morphological and molecular structure information, measurements of biophysical parameters, including diffusion, viscosity, and solute status (Van As et al., 2009; Van der Toorn et al., 2000).

The long-distance transport of water (sap) plays an important and crucial role in the exchange of nutrients and plant hormones between different organs. MR techniques have

been used to study the sap flow in xylem (Johnson et al., 1987; Köckenberger et al., 1997; Scheenen et al., 2007; Van As, 2007; Wistuba et al., 2000) as well as in its embolism occurrence (Clearwater & Clark, 2003; Fukuda et al., 2007; Holbrook et al., 2001; Umebayashi et al., 2011; Utsuzawa et al., 2005). In contrast to MRI, images obtained by synchrotron X-ray has higher spatial resolution, with possibility to obtain in real time. Such technique was used to study the mechanism of water-refilling of embolized xylem vessels, and provided a dynamic data on water transport (Lee & Kim, 2008). Brodersen et al. (2010) presented the in vivo 3D visualization and quantification of the vessel refilling process in grapevine (*Vitis vinifera*) by using a high-resolution X-ray computed tomography.

Portable MRI systems were developed recently (Rokitta et al., 2000; Van As et al., 1994), but limitations on magnetic field or MRI probes, equipments weight, external electromagnetic noise, temperature drift, among others, were cited. Umebayashi et al. (2011) monitored the developmental process of xylem embolism in Pine wilt disease by using a compact magnetic resonance imaging system with a U-shaped probe coil. Kimura et al. (2011) adapted a probe with a local electromagnetic shielding, and a flexible rotation and translation mechanism in an electric wagon system. These authors did a whole-day outdoor MRI measurements in not detached Japanese pear shoots, and a correlation between water flow and solar radiation were observed. Fukuda et al. (2007) found that cavitation and embolism events in water-stressed Japanese black pine (*Pinus thunbergii* Parl.) seedlings were detected by acoustic emission coupled with magnetic resonance microscopy, respectively.

NMR and MRI has been used to provide details during cold acclimatization and/or dormancy stage control in organs and tissues of several species, such as in dogwoods (Burke et al., 1974), Norway spruces (de Fay et al., 2000), tulip bulbs (Kamenetsky et al., 2003; Van der Toorn et al., 2000b), hybrid poplars (Kalcsits et al., 2009), blueberries (Parmentier et al., 1998; Rowland et al., 1992), grapes (Fennell & Line, 2001; Gardea et al., 1994), peaches (Erez et al., 1998; Sugiura et al., 1995; Yooyongwech et al., 2008b), Japanese pears (Yamamoto et al., 2010a), apples (Faust et al., 1991; Liu et al., 1993; Millard et al., 1993; Snaar & Van As, 1992), among others.

Differences in MRI signal strength between endo- and ecodormant buds is attributed to water content and its mobility (Faust et al., 1991). MRI primarily detects differences in proton relaxation time of water, spin-lattice or longitudinal T_1 , and spin-spin or transverse T_2 . A short relaxation time is interpreted as an indicator of water associated or bounded to macromolecules and a longer relaxation time is indicative of freer water (Parmentier et al., 1998).

A mobility restricted form or bounded water become freer with progression of endodormancy or chilling accumulation in apple (Faust et al., 1991) and blueberry buds (Rowland et al., 1992), among other fruit species. At endodormancy stage, freer water appeared but as a slower response to forcing conditions, indicating that changes on water status is more correlated to growth resumption in peach buds (Erez et al., 1998). Apple buds in terminal portions of shoot had both higher water content and proportionally more free water that did buds in the lower portions, which demonstrate a gradient in dormancy (or

resumption of growth ability) along the shoot (Liu et al., 1993). The relaxation time measured in flower buds is an indicator of the physiological development of ecodormancy because of water absorption (Sugiura et al., 1995). Peach cultivars with lower chilling requirement increased both T_2 and PD after release of dormancy, which occurred earlier compared to high-chill cultivars (Yooyongwech et al., 2008b). Water transport in twigs of *Picea* was firstly slow during winter season and then increased relatively fast in early spring (de Fay et al., 2000).

In addition to these two parameters, apparent coefficient of self-diffusion (ADC) behavior of molecules can also contribute to the image contrast (Chen et al., 1978), by providing information on water diffusion at the cellular or tissue level (de Fay et al., 2000). Proton density (PD) predominantly represents water molecular mobility at cellular level and water membrane permeability (Van der Toorn et al., 2000).

Under water deficit conditions, membrane permeability seems to have two opposite actions: increase permeability to facilitate water transport to maintain cell expansion and rehydration of tissues, or induces a decrease permeability to preserve cellular water within the tissues (Barrieu et al., 1999; Smart et al., 2001; Van der Weerd et al., 2002). The discovery of aquaporins in the beginning of 1990's popularized the concept of membrane water channels (Connolly et al., 1998; Engel et al., 2000; Preston & Agre, 1991), and their roles were studied on membrane permeability (Johansson et al., 2000; Maurel, 2007; Sakr et al., 2003; Sarda et al., 1999; Van der Weerd et al., 2002). The most immediate decrease in ADC under dormancy inducing conditions occurred in the axillary buds of poplar followed by the vascular bud trace region (de Fay et al., 2000). Yooyongwech et al. (2008), by comparing two cultivars of peaches, found increase of such proteins in bud portions after the endodormancy release irrespective of their chilling requirements. From the same experiment, they suggested that an increase in ADC can be used as a indicator of ecodormancy release in peach buds.

de Fay et al. (2000) used diffusion-weighted images to demonstrate water movement and activity during budburst of spruce (*Picea abies* (L.) Karst.) after fulfillment of chilling requirement. Similar methodology was used by Van der Toorn et al. (2000) to assess water mobility, which is proportional to ADC value. Past investigations had been limited by low resolution of the images, but recently the ADC measurements correlated better with dormancy development than did the vascular tissue measurements (Kalcsits et al., 2009).

3. Present work: Determination of water status by MRI in flower buds under mild winter conditions

The following experiment was conducted in order to verify the effect of delayed mild winter conditions and consecutive seasons on water dynamics by using the MRI techniques, and to analyze a possible correlation with floral primordia necrosis.

Plants of Japanese pear (*Pyrus pyrifolia* (Burm. f.) Nakai) var. culta 'Housui' grafted on *P. pyrifolia* var. *pyrifolia* ('Yamanashi') rootstock were obtained from a commercial nursery, transplanted into a 30 cm diameter plastic pots in April 2005 and grown at the Agricultural

and Forestry Research Centre, University of Tsukuba, Japan (36°2' N, 140°4' E, 25 m above sea level). One-year old shoots were reoriented to horizontal position in order to promote flower bud formation (Ito et al., 1999).

Plants were divided into three groups, in order to simulate different conditions of chilling accumulation: plants were kept under natural conditions, with chilling accumulation superior that than required for this cultivar (NC); two groups of plants were submitted to two months (November and December) of cold deprivation in a heated greenhouse before exposure to chilling amount of 600 hours below 7.2°C (CH), in order to simulate a delayed mild winter condition of subtropical climates. These two groups of plants were exposure to such conditions for either a single season of 2008-2009 (treatment SS) and during four consecutive seasons, from 2005-2006 until 2008-2009 (treatment CS). The later condition was set as a tentative to simulate conditions of permanent warm winter. After chilling accumulation, plants were again moved to a heated greenhouse, with minimum temperature set at 16°C, to force bud break (Fig. 3).

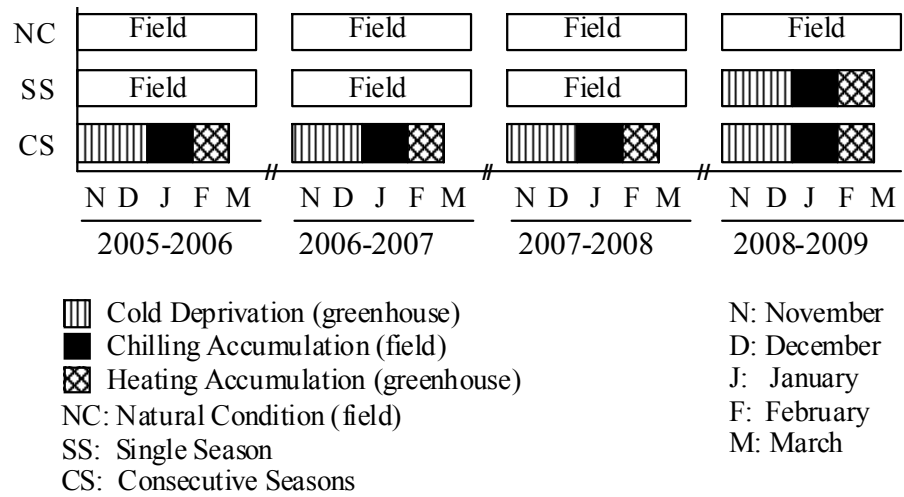


Figure 3. Experimental design for MRI measurements, from November (N) to March (M). Plants were kept under natural conditions (NC) and cold deprivation during two months before exposure to 600 hours of chilling accumulation below 7.2°C under two regimes: a single season of 2008-2009 (SS), and four consecutive seasons from 2005-2006 to 2008-2009 (CS) (Yamamoto et al., 2010a).

Both field and greenhouse air temperature was recorded by a data logger (TR-51A, T and D Co., Matsumoto, Japan). Chilling calculation started when two consecutive hours below 7.2°C were detected in field. In the heated greenhouse, maximum temperature was not controlled and plants were submitted do natural photoperiod.

All MRI measurements were made in 2008-2009 season. Three lateral buds were collected randomly from one-year old shoots. Samples were taken from SS and CS after one month of cold deprivation (CD1), at the end of cold deprivation and just before the chilling treatment (0 CH, SS only), at the end of chilling treatment (600 CH), and before bud break under forced conditions (4000 GDH). Under NC, samples were taken in the middle of endodormancy (December), during transition from endo- to ecodormancy (January), middle (February) and end of ecodormancy stage (March).

MRI measurements were performed using an NMR spectrometer (DRX 300WB, Bruker, Karlsruhe, Germany) equipped with a microimaging accessory at a magnetic field of 7.1 Tesla at $\approx 21^\circ\text{C}$ (Fig. 4A). Magnetic resonance images were acquired and reconstructed with ParaVision imaging software (ver. 3.0.2 Bruker). The sample was placed on a homemade plastic holder and putted in a 15 mm NMR coil (Fig. 4B). Morphological images and 32 sequential echo images in longitudinal sections of flower buds were obtained by a multi-slice multi-echo MRI pulse program. For morphological images the repetition time was set to 1 s with the echo time of 5.524 ms, the matrix size of 256×256 , the field of view of $15 \times 15 \text{ mm}^2$ or $18 \times 15 \text{ mm}^2$, and the slice thickness of 0.5 mm. The sequential echo images were obtained with the repetition time of 5 s, the echo time of 3.069 to 115.5 ms with a constant interval of 3.069 ms, the matrix size of 128×128 , and same field of view and slice thickness with morphological images. T_2 (spin-spin relaxation time) and relative proton density (PD) maps were calculated from 32 sequential images using the image sequential tool in ParaVision. Three regions of interest (ROIs) of grouped floral primordia, bud base, and whole bud were determined manually in a longitudinal section at the highest bud base portion of flower bud (Fig. 4C) and T_2 values of each ROI was also calculated. There were three replications of each analysis.

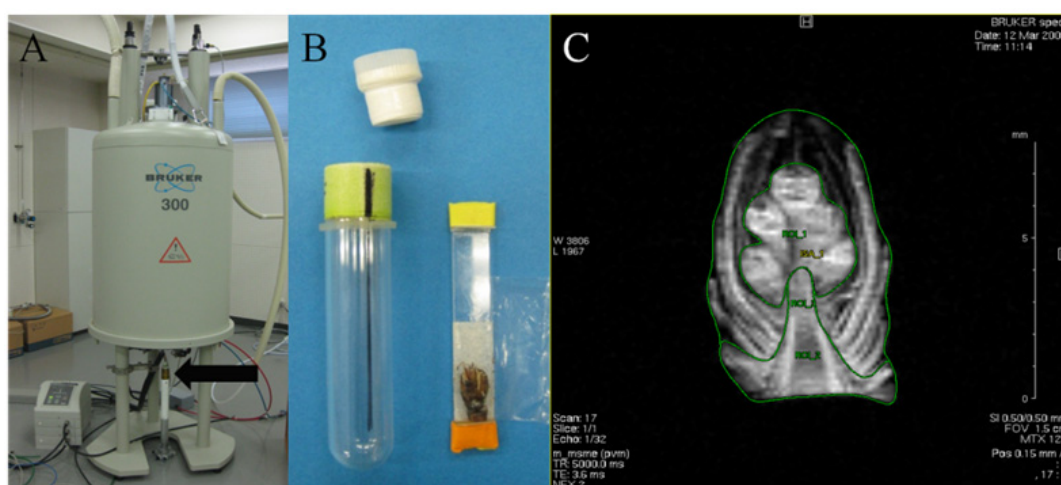


Figure 4. NMR spectrometer (Bruker DRX 300WB) equipped with a 7.1 Tesla magnetic field tank (A). The arrow indicates the NMR coil with the glass tube. A 15 mm glass tube and a homemade plastic holder with sampled bud of Japanese pear (B). MR morphological image of Japanese pear bud with regions of interest (ROI) determined manually (C) The scale bars represent 8mm (adapted from Yamamoto, 2010).

3.1. Results

T_2 values of floral primordia increased considerably at the transition from endo- to ecodormancy stage (January) in buds grown under normal winter conditions (NC) (Fig. 5A). Under warm and delayed winter conditions (SS and CS), however, T_2 values of floral primordia increased only after the end of chilling accumulation (600 CH to 4000 GDH). The bud base of plants under NC showed a gradual increase in T_2 values until the end of chilling accumulation, but under SS were lower than those of NC until after submitting plants to

heating accumulation (Fig. 5B). After several season under warm winter conditions (CS) buds showed constant T_2 values until submitting plants to heating accumulation, when increased abruptly. The average T_2 values in whole bud increased gradually in plants kept under NC, whereas treated plants (SS and CS) had low values at the end of the chilling treatment (600 CH) (Fig. 5C).

T_2 and proton density (PD) maps (Fig. 6) were almost entirely calculated in detected parts of the morphological images (Fig. 7). Water mobility, as determined by T_2 (Fig. 6A), showed intermediate values (16 to 24 ms) in the floral primordia, bud base, and bud scales of plants kept under fulfillment of chilling requirement (NC) on March. In contrast, at the end of chilling treatment (600 CH), buds of plants kept just a single season under warm winter conditions (SS) showed high T_2 values (over than 24 ms) in the lower portions of the bud scales, while values were similar to NC in the bud base. Compared to SS and NC, T_2 values increased (to higher than 16 ms) after several seasons of mild and delayed winter conditions (CS) before flowering only in specific portions of bud base and scales near the bud base.

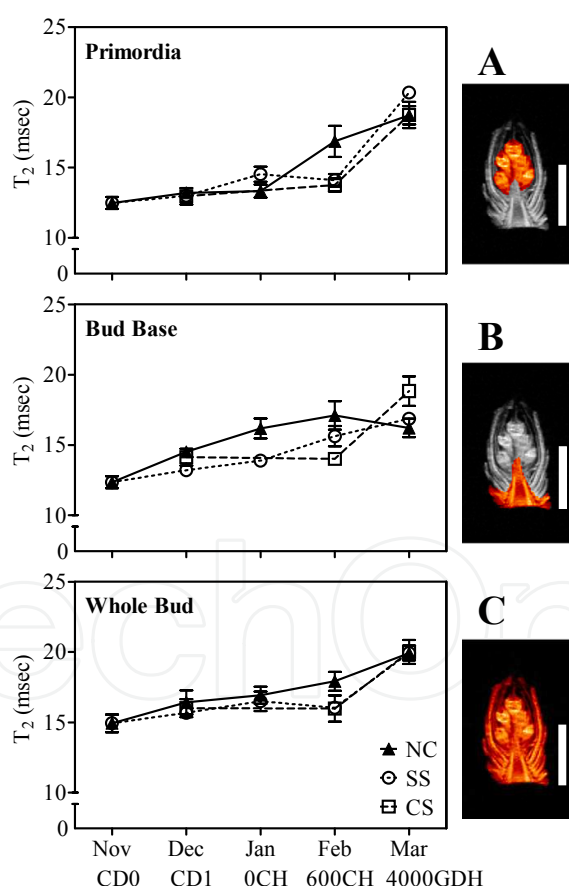


Figure 5. Averages of T_2 values (ms) of floral primordia (A), bud base (B), and whole bud (C), measured in mixed buds of 'Housui' Japanese pear grown under natural conditions (NC), one (SS) and four consecutive seasons of mild and delayed winter conditions. Means \pm SE ($n = 3$). CD0: start of cold deprivation; CD1: one month of cold deprivation. Scale bars in each ROI represent 5 mm (adapted from Yamamoto, 2010).

Relative amount of water, represented by PD maps (Fig. 6B), gradually increased in the floral primordia of buds grown under normal winter conditions (NC) during the transition from endo- to ecodormancy (January). Similar increase in PD was observed in primordia under SS. At the end of chilling accumulation (600 CH) in CS, the PD value was medium to

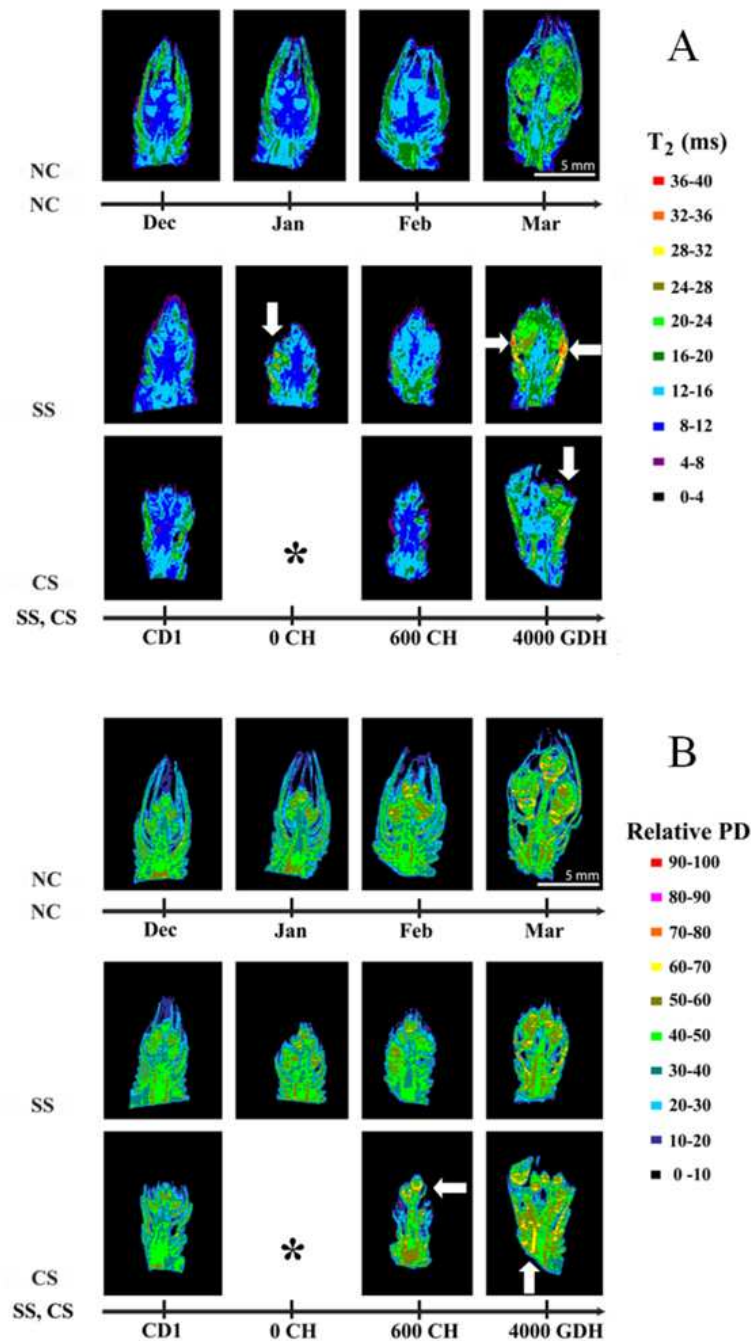


Figure 6. Transverse relaxation (T_2) time maps (A) and Proton density (PD) maps in longitudinal sections of flower buds of Japanese pear 'Housui', obtained by magnetic resonance imaging, determined after a single season of 2008-2009 (SS) and four consecutive seasons from 2005-2006 to 2008-2009 under partial chilling accumulation (CS), and from plants kept under natural conditions (NC). The arrows indicate significant changes on water dynamics. Asterisk: not analyzed. CD1: one month of cold deprivation (adapted from Yamamoto, 2010).

high (40-70 %) in the bud base, and high (until 80%) in the floral primordia. During the heating accumulation (4000 GDH), PD was higher in primordia and some specific portions of bud base after consecutive seasons of mild winter conditions (CS).

Structural changes during dormancy until flowering were observed in morphological MR images of longitudinal sections at the central portion of 'Housui' flower buds (Fig. 7). Plants grown under normal conditions of winter (NC) had a single inflorescence, with a high and uniform signal intensity of bud scales and floral primordia during all dormancy stage. However, bud scales and floral primordia configurations were remarkably different under mild winter conditions. Signals from upper portion of bud scales could not be detected at the end of cold deprivation (0 CH). Signals in floral primordia were not uniform from the end of chilling accumulation, and numbers of detected primordia and inflorescences were different. After four consecutive seasons of mild winters (CS), buds showed also variations on primordia size especially during heating accumulation (600 CH and 4000 GDH).

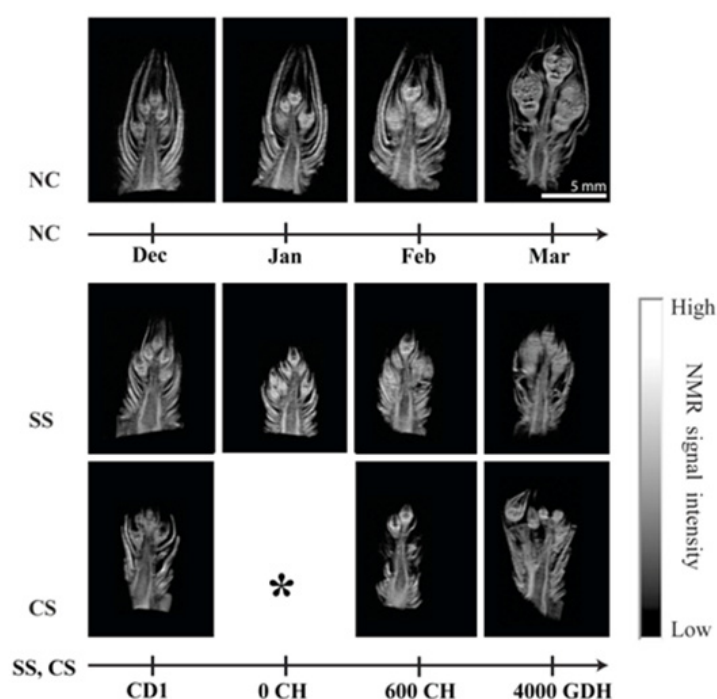


Figure 7. Morphological images in longitudinal sections of Japanese pear 'Housui' buds, obtained by magnetic resonance imaging, determined after a single season of 2008-2009 (SS) and four consecutive seasons from 2005-2006 to 2008-2009 under partial chilling accumulation, and from plants kept under natural conditions (NC). Asterisk: not analyzed. CD1: one month of cold deprivation (Yamamoto et al., 2010a).

Morphological comparison between buds of plants growing under natural (NC) and warm winter conditions for several seasons (CS) is showed in Fig. 8. Externally there was no morphological differences between then in photographs obtained from digital microscope (Figs. 8A, D), but under NC both floral primordia and bud scales remained green (Fig. 8B), whereas necrosed primordia was observed under CS (Fig. 8E). MR images showed such differences (Figs. 8C, F). Differences on coloration of bud scales between bud of plants grown under natural and warm winters could be observed also in photographs obtained from digital microscope (Fig. 9).

During November, there were no morphological differences observed in SEM images, but during cold deprivation (December), buds of plants grown under mild winter conditions (SS and CS) developed a second inflorescence (Fig. 10). Digital photographs obtained in February (after chilling treatment) showed a progression of floral primordia necrosis and development of new inflorescences in different levels. Reduced number of opened flowers, variations on length of pedicels, and differences on flowering stage were observed in buds of plants grown under mild winter conditions.

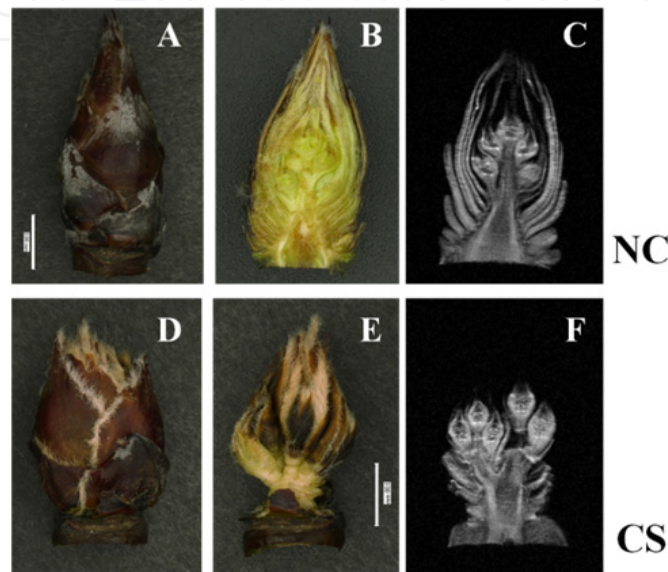


Figure 8. Differences between flower buds of 'Housui' Japanese pear under natural conditions (NC) and four consecutive seasons of plants submitted to warm and delayed mild winter conditions. Photographs obtained from digital microscope (A, D), longitudinal section (B), and after removing all twelve scales (E), and morphological images of longitudinal sections of same flower bud obtained by MRI (C, F). Scale bars represent 3 mm.



Figure 9. Photographs obtained from digital microscope of flower buds sampled on February from plants grown under natural conditions (A) and a single season of 2008-09 under artificial mild winter conditions (B). From left to right: normal bud; after removed 4, 8, all 12 scales, and inner scales. Scale bars represent 3 mm (Yamamoto, 2010).

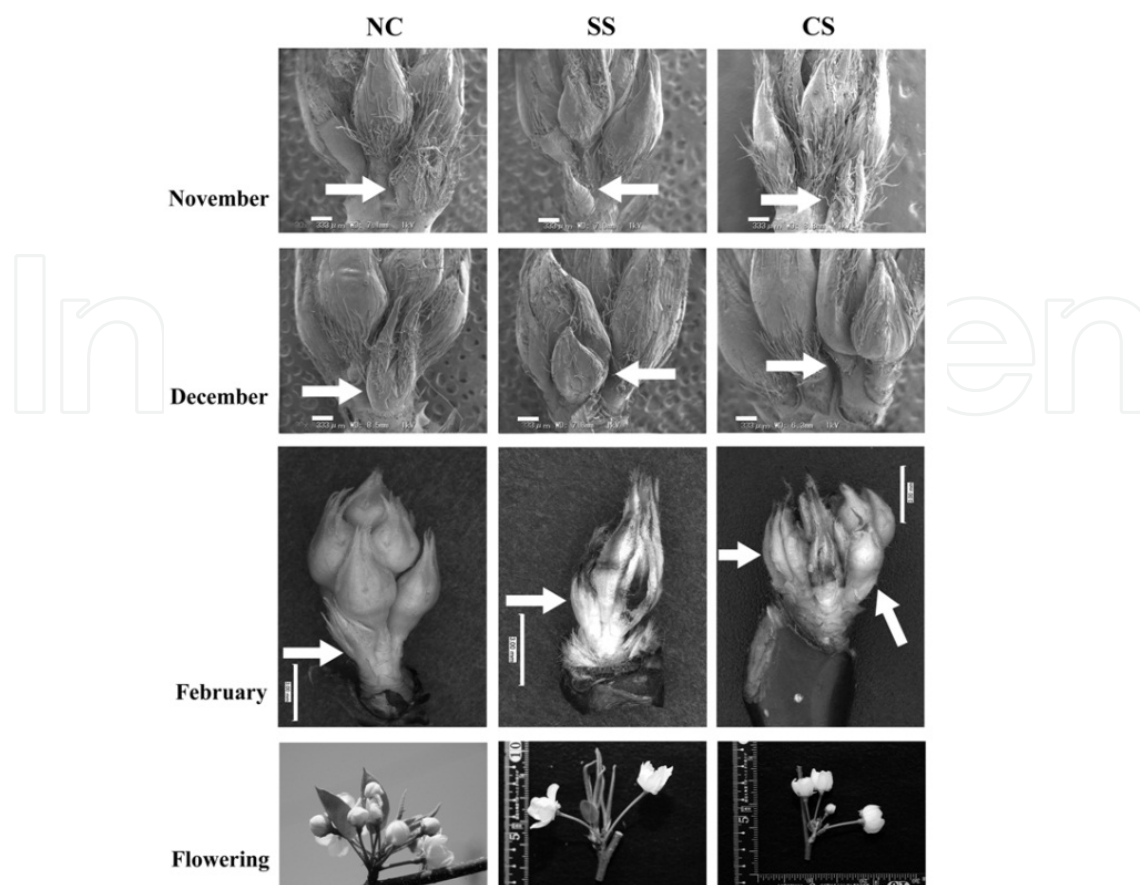


Figure 10. Micro-images of flower buds obtained from scanning electron microscope (SEM) before any chilling accumulation (November) and after one month of cold deprivation conditions (December). Photographs of buds during the transition from chilling to heating treatment (February) obtained from digital microscope. NC: natural conditions; a single (SS) and four consecutive seasons (CS) under mild and delayed winter conditions. The arrows indicate the leaf primordia or inflorescence duplication. The scale bars represent 333 μm in SEM and 3 mm in digital microscope images (Yamamoto et al., 2010a).

3.2. Discussion

The data presented suggest that MRI techniques can be an useful non-destructive and rapid analysis tool of internal anatomical details of tissues or organs, the spatial distribution of water, dynamics and mobility of water (Yamamoto et al., 2010a). Japanese pear plants grown under conditions of sufficient chilling accumulation showed a gradual increase in T_2 values in the whole bud, indicating a conversion from bound to free water (Fig. 6A). In other words, the endodormancy stage takes place to the ecodormant phase, which is correlated to free water (Faust et al., 1991; Liu et al., 1993; Sugiura et al., 1995). However, plants grown under mild and delayed winter conditions kept high levels of bound water (low T_2 values) in the floral primordia, bud base, and whole bud until the end of chilling treatment, and an abrupt change to free-state water during the afterward heating accumulation period was observed. Previous studies in peaches suggested a correlation between the level of bound water and cold hardiness (Erez et al., 1998; Faust, 1991; Parmentier et al., 1998) through the activation of dehydrin, a hydrophilic protein (Kalberer et al., 2006; Rowland & Arora, 1997; Yamane et al., 2006). Differences in T_2 values at the end of chilling accumulation (600 CH) is

probably resulted from reduction on percentage of normal primordia occurred during cold deprivation in buds grown under mild winter conditions (data not shown). Differences in T_2 signal intensity with time, as a consequence of environmental temperature variation, were detected in buds of plants grown for only one season under mild winter conditions, whereas response under several seasons tended to occur slowly and was specific to some portion of the bud, like in the duplicated inflorescence.

The proton density (PD), which is also called spin density, indicates the concentration of MRI-visible protons, which were associated with water protons (Brown et al., 1986). PD maps (Fig. 6B) showed a low water content in the bud scales under mild winter conditions, and a high water content in specific portions of the floral primordia (600 CH) and bud base (4000 GDH) in bud after continuous mild winter seasons. A possible loss of functionality in vascular connections or dysfunction of water channel proteins between bud base and primordia under mild winter conditions might be resulted in progression of floral primordia necrosis (Yamamoto et al. 2010a). This irregular distribution of water resulted in a high water mobility in morphologically modified portions of the bud. However, it was not possible to determine the cause and effect essay.

The highest incidence of flower bud abortion in Japanese pear under mild winter conditions was observed in lateral buds of young shoots (Klinac & Geddes, 1995), reason why this bud type was used in this experiment. It was demonstrated that lateral (axillary) buds have the greatest sensitivity to temperature-induced dormancy (Kalcsits et al., 2009). Bud scales, which are modified leaves responsible for enclosing and protecting buds of perennial plants, had high T_2 and PD values in the buds of plants grown under sufficient chilling conditions, during all dormancy stages of our experiment. In contrast, the low water mobility (T_2) and low water content (PD) detected by MRI and in the scales of buds exposed to mild and delayed winter conditions might have been related to an increase on sensibility of the floral primordia to external temperature oscillations and/or reduced protection from freezing during winter (Yamamoto et al., 2010a).

Changes of amplitude on NMR signal intensity is related to the number of protons, and is proportional to the water content per volume element (voxel) (Donker et al., 1997; Van der Weerd et al., 2002). Morphological images of longitudinal sections showed different signal intensities in buds of plants kept under delayed and warm winter conditions. Photographs and morphological images of buds showed a clear difference in conditions of these bud scales (Figs. 7, 8, 9). Yooyongwech et al. (2008b) reported that oscillation temperature conditions accelerate water movement in peach buds, but promoted an irregular bud growth. The effect of consecutive seasons of mild winter (CS) on reduction of water mobility and content in bud scales was also observed by a more accentuated reduction on signal intensity (Yamamoto et al., 2010a). An important detail in MR studies is that only detectable portions are used. Dehydrated bud scales or necrosed floral primordia, and also structures located in different plans from selected section is not considered.

Buds under normal winter conditions had all of floral primordia developed normally, only a single inflorescence was observed during the dormant period, and all primordia sprouted simultaneously (Figs. 9, 10). However, plants grown under mild winter conditions developed a second (sometimes a third) inflorescence in buds of Japanese pear, resulting in more than 8

floral primordia per bud in average (data not shown). In such artificial conditions of 80% of chilling fulfillment, a prolonged flowering period, with low number of opened flowers was observed, regarding of necrosis incidence in floral primordia. It was possible to reproduce, in this experiment, similar phenology observed in a warm winter conditions of Southern Brazil and described by several authors as “flower bud abortion” (Marafon et al., 2011; Petri & Herter, 2002; Petri et al., 2002; Petri et al., 2001; Trevisan et al., 2005; Verissimo et al., 2002).

4. Conclusions and future prospects

A cumulative effect of several seasons of mild winter conditions could be observed, where flower primordia necrosis occurrence were anticipated with delaying chilling accumulation. Moreover, a dehydration of bud scales, development of new inflorescences, different degrees of necrosis, and development of abnormal flowers were observed. MR images indicated that low levels of mobile water (T_2) and relative water content (PD) in bud scales might affect the sensibility of flower primordia to external temperature oscillations under mild winter conditions. Another possible consequence of such changes on water dynamics is a reduction on protection from freezing temperatures (hardening level) during winter. Floral primordia necrosis might reallocate water, by affecting its absorption potential, and other functional elements (carbohydrates or plant hormones) for development of new inflorescences.

Studies on hydraulic conductivity are focused on situations of severe winters, generally observed in the Northern hemisphere, where sap freezing occurs in xylem. But, how is the functioning of winter embolism in deciduous woody plants in regions where winter is more affected by global warming?

The advanced measurement techniques above mentioned, which can facilitate investigations of water movement in intact plants, seems to be a promising methodology to study in long-distance water transport and water dynamics in specific portions of plant tissues grown under conditions of global warming context. Unfortunately, major part of these studies were done in a typical temperate zones (Germany, Japan, Netherlands, USA, among others), where just recently faced the effect of global climate changes. In subtropical climates of Southern Brazil, for example, temperate zone fruit crops were produced under marginal conditions of mild winters. Actually, in such locations basically phenotypical observations and superficial determinations of water or carbohydrate contents were done experimentally. So, a lack of more specific studies, by using modern techniques described in this chapter under “natural” marginal conditions during dormancy phase, is nowadays observed. A multidisciplinary approach, including multi-institutional collaborations and international cooperation, is needed to study the consequences of global warming on water dynamics during dormancy phase in temperate zone fruit crops with economic importance.

Author details

Robson Ryu Yamamoto¹, Paulo Celso de Mello-Farias¹,
Fabiano Simões² and Flavio Gilberto Herter¹

¹Federal University of Pelotas. ‘Eliseu Maciel’ Faculty of Agronomy, Brazil,

²Rio Grande do Sul State University, Vacaria Uniduity, Brazil

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