Study on Dormancy Progression and Floral Primordia Abortion Occurrence in ‘Housui’ Japanese Pear Grown under Mild Winter Conditions

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A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
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List of abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CH	Chilling hours below 7.2 ^{o}C
CS	Consecutive seasons
CU	Chilling units or Saitama Model, proposed by Asano and Okuno (1990)
DVI	Developmental rate index (=∑ DVR) proposed by Sugiura et al. (1991) and Sugiura and Honjo (1997b)
DVR	Developmental rate model
EPAGRI	Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Agricultural Research and Rural Extension Enterprise of Santa Catarina State, Brazil)
FAA	Formaldehyde, acetic acid, and ethanol solution, in v/v
FOV	Field of view
FW	Fresh weight, in g

GDH	Growing degree hours, in ^{o}C
HPLC	High-performance liquid chromatography
ISA	Image sequence analysis
MOPS	3-(morpholin-4-yl) propane-1-sulfonic acid
MR	Magnetic resonance
MRI	Magnetic resonance imaging
NC	Natural condition
NMR	Nuclear magnetic resonance
NTP	Non-adenylic triphosphate nucleotide
PD	Relative proton density
PVPP	Polyvinyl-polypyrrolidone
ROI	Region of interest
SEM	Scanning electron microscope
SS	Single season
T_1	Longitudinal spin-spin relaxation time, in ms
T_2	Transverse spin-spin relaxation time, in ms

Chapter 1

General introduction

1.1. Pear production around the world

According to the Food and Agriculture Organization of the United Nations (FAO) report (2009), from 1961 to 1991, the world pear production increased around 100%, and from 1991 up to now the production increased other 100%, reaching around 20.0 million t in 2007 (Fig. 1). In 2005, the biggest producers of pear in the world were, in order: China, Italy, USA, Spain, Argentina, Germany, South Korea and Japan. In Europe, Italian production was around 1.5 million t until 1976, but decreased to 1.0 million t per year during following years. Spain showed a crescent production, whereas Germany had a constant production. In the Americas, the production in the USA remained around 0.8 million t from 1980s. Argentina increased their production of pear from middle to end of 1990s. In Asia, Japan have a stabilized production of pear, around 0.4 million t, whereas South Korea increased their production from the end of 1980s, and from last 2 years they become bigger grower than Japan. In 1961, China produced less than 10% of worldwide production, but from 1990s the production increased rapidly, and from 1997 they produce around 50%.

Brazil has only a small area of pear production (less than 2000 ha) and national consumption depends largely on imports. From the 1960s, pear production increased during 10 years, reaching 60000 t in 1969, but it decreased to 20000 t by the end of 1980s, when it stabilized until now. On the contrary, import quantity increased with

little fluctuations during all this period, showing the maximum value of 160000 t in 1998, corresponding to near 90% of the total consumption of this fruit (Fig. 2).

The worldwide biggest exporter of fresh pears is Argentina (FAO, 2009), a full member of the free trade region of South America called Southern Common Market (Mercosur) and which includes Brazil. This condition allows the Argentinean fresh pear to be exported with competitive prices to be offered to Brazilian consumers (Faoro, 2001). There are two possibilities for Brazilian pear producers: (1) to produce in large scale, with high production and low costs, destined to general markets and competing with imported fruits; or (2) to produce in small scales, with high quality fruits, aimed at upmakets with high prices. The second option seems to be the better choice since Japanese pears show high potential for high quality fruit market (Faoro, 2001).

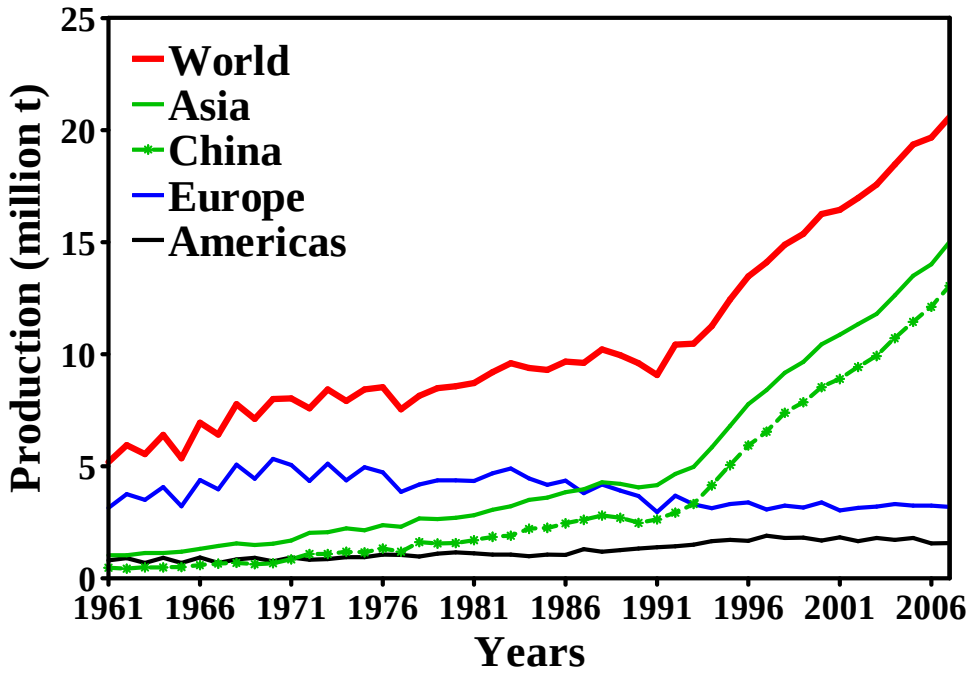

Fig. 1. Worldwide, continental and Chinese pear production, in million t, from 1961 until 2007 (FAO, 2009).

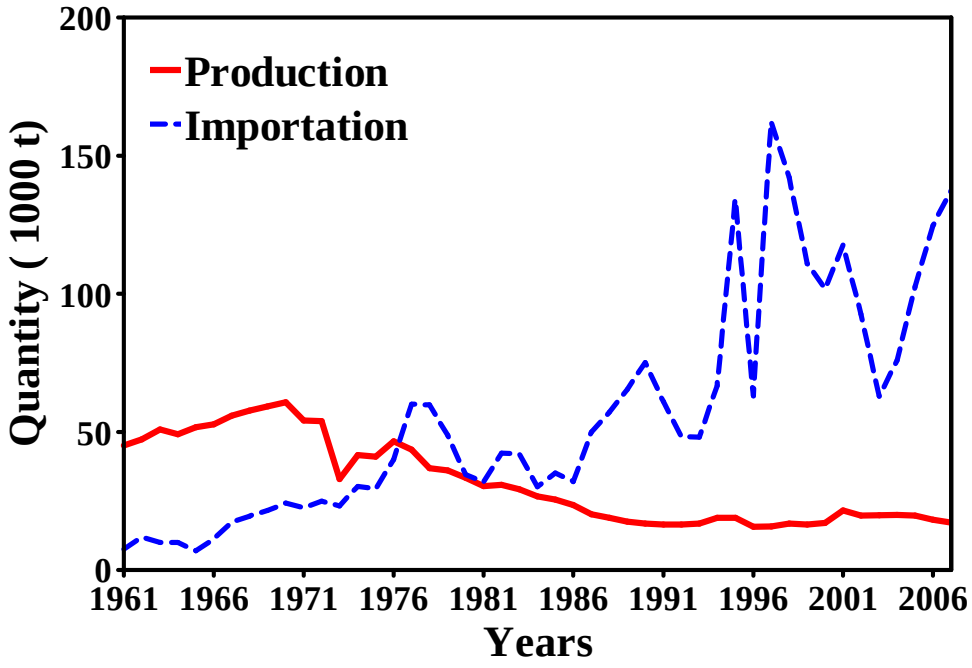

Fig. 2. Total pear production and importation quantity in Brazil, in 1000 t, from 1961 until 2007 (FAO, 2009).

1.2. History of Japanese pear cultivation

The *Pyrus* species was originated in Southeast China, and native Japanese pear [*Pyrus pyrifolia* (burm. f.) Nakai] cultivars share the same genetic background as the native East Asian pears (*P. serotina*, *P. ussuriensis* and *P. brestschneideri*) (Tamura, 2006). The Japanese pear is also called “Nashi”, “Nihon Nashi” or “Asian pear”, which is for *P. pyrifolia* Nakai and *P. serotina* Rehder.

An important step in modern Japanese pear cultivation occurs in the 19th century, when two high quality chance seedlings, ‘Choujuro’ (Fig. 3A) and ‘Nijisseiki’ (Fig. 3B), were found. These two cultivars comprised over 80% of pear production from 1920 to 1970. From 1971, ‘Choujuro’ was gradually substituted by two newly-bred cultivars, ‘Kousui’ (Fig. 3C) and ‘Housui’ (Fig 3D). Recently, new cultivars were bred and cultivated locally, such as ‘Saigyoku’ in Saitama Prefecture (Fig. 3E) and ‘Akemizu’ in Kanagawa Prefecture (Fig. 3F). In 2003, ‘Kousui’ and ‘Housui’ corresponded to 40% and 25%, respectively, of total production area in Japan, whereas ‘Nijisseiki’ was reduced to 14% and ‘Niitaka’ (Fig. 3G) represented less than 10% (Tamura, 2006).

Japanese pear fruits weight between 200 and 400 g, but some late-maturing cultivars such as ‘Atago’ (Fig. 3H) bear fruit of more than 1000 g. There are two types of fruit skin: russet (‘Housui’ and ‘Kousui’) or smooth and yellowish green (‘Nijisseiki’). Pulp of Japanese pear is crispy, juice-rich texture, between 11 and 13 ^{o}Brix and also contains stone cells and fibers. About 75% of fruits were harvested and marketed without storage during August and September, when is the maturation time of the two major cultivars ‘Kousui’ and ‘Housui’.

Japanese pear growing regions in Japan have an annual average temperature between 12 and 16 ^{o}C, rainfall between 750 and 1600 mm during growing season (Tamura, 2006). Some important and intensive cultivation practices in Japanese pear

crops are: training (horizontal trellis) and pruning (Fig. 4A), hand pollination, fruit thinning, bagging (Fig. 4B), harvesting, shipping, soil management (Fig. 4C), pest and disease control and forcing culture in some cases (Fig. 4D).

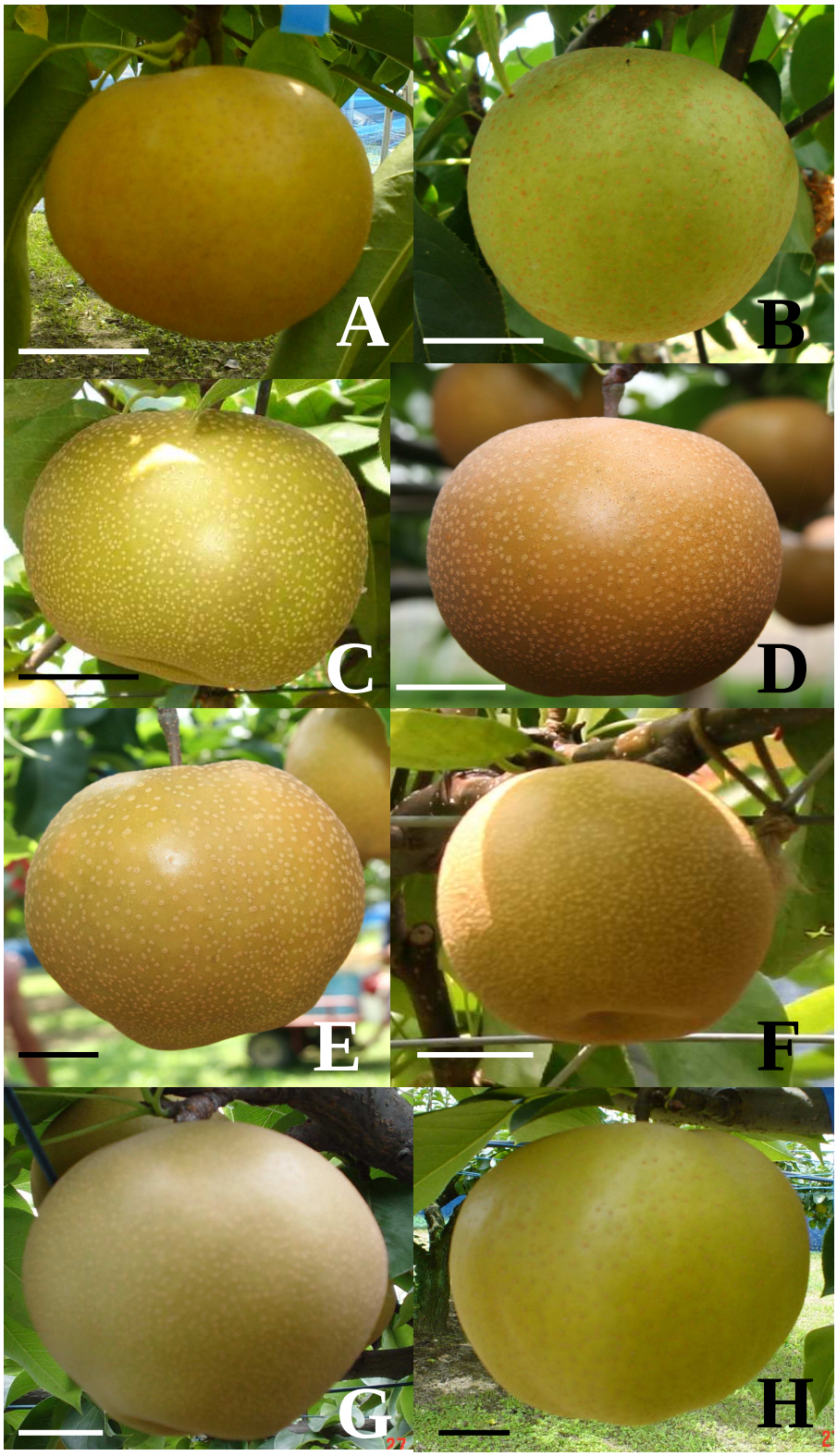

Fig. 3. Some cultivars of Japanese pear: ‘Choujuro’ (A); ‘Nijisseiki’ (B); ‘Kousui’(C); ‘Housui’ (D), ‘Saigyoku’ (E), ‘Akemizu’(F); ‘Niitaka’ (G); ‘Atago’ (H). The scale bars represent 30 mm.

Fig. 4. Some Japanese pear cultivation practices in Japan: after pruning in winter (A); bagged fruits (B); soil management during winter season (C); forcing culture by using heated greenhouse (D).

1.3. Japanese pear production in Brazil

Japanese pear production in Brazil started on the 1950s by the Japanese descendants living in São Paulo State, with the cultivars 'Choujuro', 'Okusankichi', and 'Imamura Aki'. On the 1990s, the commercial production of this kind of pear increased in Santa Catarina State (Fig. 5) with the cultivars 'Housui', 'Kousui', and 'Nijisseiki'. The major growers, in this case, produces Japanese pear in small scale (each one from 1.0 to 2.0 ha in average), as an important second income source. The market price is considerably high, when compared to other temperate fruits, such as apple. From last 5 years the production of high quality fruits increased in Southern Brazil especially that of Japanese pear, which are big, juicy, and very sweet fruits (Faoro, 2001).

Many production techniques developed in Japan are used in Brazil, such as horizontal trellis with wire or bamboo (Fig. 6A, B, C, D), artificial pollination, fruit thinning, orchard with anti-hail net (Fig. 6E); fruits packed individually by using an expanded polyethylene packing net in a 5 kg card-board box (Fig. 6F); fruit bagging to reduce chemical applications and to prevent damages from fruit flies (Fig. 6G), among others.

In Southern Brazil, climate conditions in autumn and winter seasons are very variable with great temperature fluctuations in the year and year-to-year (Petri et al., 2002) (Table 1). This region, where the Japanese pear crops are concentrated, includes São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul States. In Santa Catarina State (Latitude 26^{o}46' S, Longitude 57^{o}00' W, 920 m above sea level), the most important production area of this fruit crop, the average of accumulated chilling hours below 7.2^{o}C during the last 45 years is around 600 (Fig. 7).

Recently, due to some adaptation problems, 'Kousui' and 'Nijisseiki' have been substituted or top-grafted with 'Housui', which shows higher adaptability and are better

accepted by Brazilian consumers. Unfortunately, ‘Housui’ showed some physiological abnormalities, locally called “floral bud abortion”, and it has been cited insufficient chilling accumulation as one of the major factors.

Fig. 5. Geographic map of Latin America showing the Japanese pear production area in Southern Brazil (circle). Asterisk indicates the Santa Catarina State (Latitude 26°46' S, Longitude 57°00' W, 920 m above sea level), the most important production region of this fruit crop in Brazil.

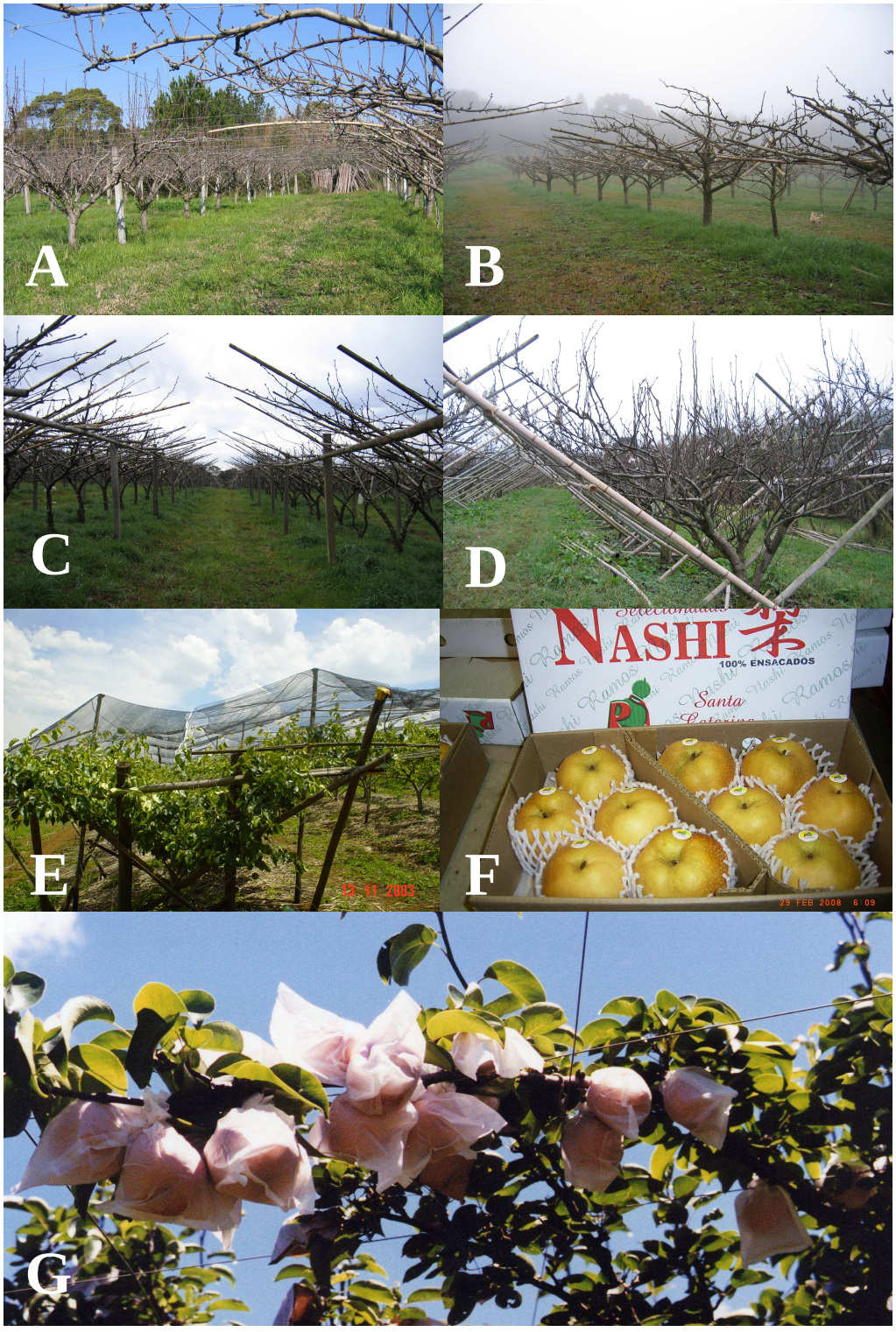

Fig. 6. Aspects of 'Housui' Japanese pear production in Southern Brazil: horizontal trellis with wire (A); without a fixed training system (B); different training systems using bamboos (C and D); orchard with anti-hail net (E); fruits packed individually by using an expanded polyethylene packing net in a 5 kg card-board box (F); bagged fruits (G).

Table 1. Climatic data of the Caçador Agriculture Experimental Station, Santa Catarina State, Brazil (Latitude 26°46' S, Longitude 57°00' W, 920 m above sea level). Data are average from 1961 to 2004. Source: EPAGRI

	Temperature (°C)			Humidity (%)	Rainfall (mm)
Month	**Max.**	**Average**	**Min.**	**Average**	**Average**
January	30.9	20.7	10.1	77.1	178.8
February	30.6	20.5	10.1	79.5	167.7
March	29.6	19.6	8.0	79.4	132.3
April	28.0	16.5	2.9	79.1	105.3
May	25.8	13.3	-1.3	90.3	125.4
June	24.7	11.9	-3.7	90.4	119.9
July	25.1	11.9	-3.4	79.0	114.5
August	27.7	13.2	-2.7	76.2	115.5
September	28.7	14.8	-0.7	76.8	149.8
October	29.6	16.8	3.0	75.9	181.9
November	30.4	18.6	5.2	72.9	141.0
December	30.8	20.0	8.4	74.7	143.3
Total					**1675.4**
Average	**28.5**	**16.5**	**3.0**	**77.6**	

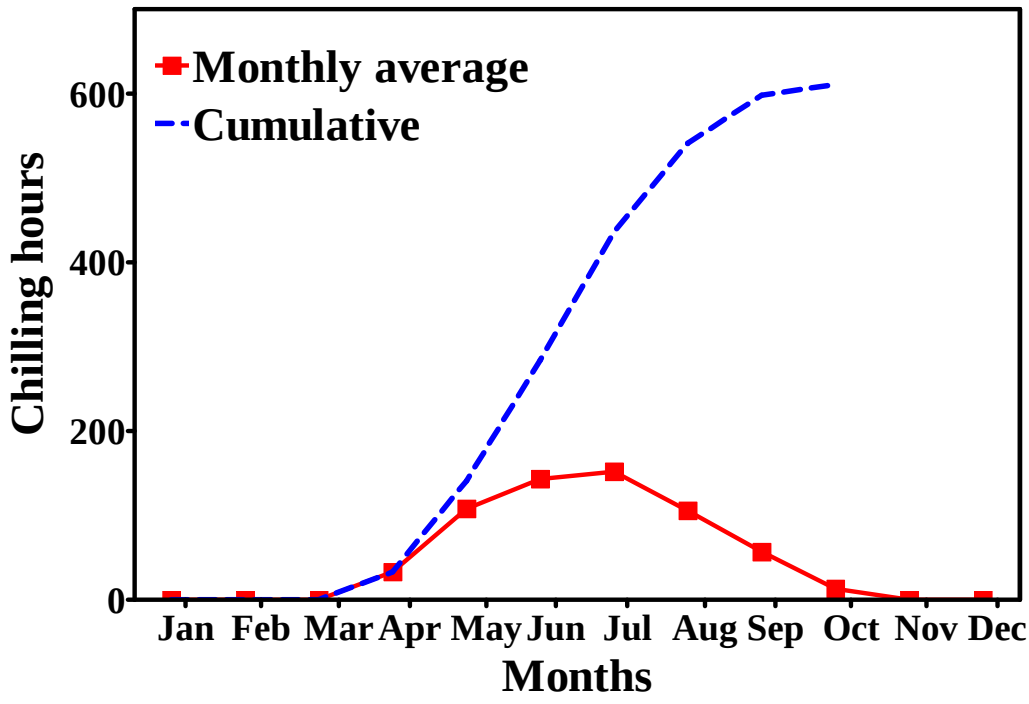

Fig. 7. Cumulative and monthly average of chilling hours below 7.2 ^{o}C in the Caçador Agriculture Experimental Station, Santa Catarina State, Brazil. Data are average from 1961 to 2004. Source: EPAGRI

1.4. Dormancy in temperate trees

Lang et al. (1987) defined dormancy as a state of reduced or stopped activity or development of specific plant tissues that will resume in the future, and it is a basic phase in their annual developmental cycle in temperate-zone deciduous fruit trees that allows the trees to survive unfavorable conditions during winter season (Erez, 2000a). In other words, dormancy constitutes an inability to resume growth (Rohde and Bhalerao, 2007)

For discussion of dormancy stages, we have chosen to utilize the terminology proposed by Lang et al. (1987), who classified the various stages of dormant bud as: **paradormancy** (correlative inhibition or apical dominance), when the growth cessation and dormancy induction is regulated by physiological factors outside the affected structure; **endodormancy** (winter or deep dormancy), when the dormancy causing factor resides within the bud (chilling responses, photoperiodic responses); and **ecodormancy,** which is regulated by environmental factors (extreme temperatures, nutrient deficiency, water stress). In paradormancy, axillary buds can be forced to grow by removing the terminal bud, which exerts apical dominance (Rowland and Arora, 1997). The temperature-related stages of dormancy, endo- and ecodormancy, are showed on Fig. 8. The endodormancy stage is induced in fall and is released by chilling temperature in winter, but the transition from endo- to ecodormancy stage is not visible. After accumulation of sufficient chilling, endodormancy concludes giving its way to ecodormancy. At this later stage, resumption of growth mostly depends on heating accumulation, so that bud growth speed increases considerably due to the warm temperatures (Kester and Gradziel, 1996).

By preparing the plants to unfavorable conditions, it enables tree to accumulate reserves, mostly carbohydrates, to drop sensitive organs, e.g. leaves, to develop organs

to protect the meristems and to resist harsh conditions by develop cold hardiness (Erez, 2000b). Budbreak in perennial fruit trees is affected by two temperature dependent processes: a) accumulation of chilling temperatures to the level required for dormancy completion; and b) accumulation of heat-units required for the buds to bloom and foliation (Naor et al., 2003).

Cold temperatures are generally considered as the environmental cue responsible for the initial induction (in autumn) and subsequent alleviation (in winter) of chilling requirement during bud dormancy (Crabbé and Barnola, 1996). Ranges of cold temperatures which are effective on chilling accumulation differ among species and cultivars (Naor et al., 2003). Even within a cultivar there are wide differences in chilling requirements between bud types and localization (Hauagge and Cummins, 1991; Erez, 2000a).

In horticulture, one of method utilized to calculate the progression of endodormancy and ecodormancy stages are based on exposed temperatures and correlated to accumulation of chilling and heating amounts, but it does not take into account the physiological status of the plant. Because these models are not based on physiological processes, their accuracy may be confined to specific environments (Hanninen, 1995).

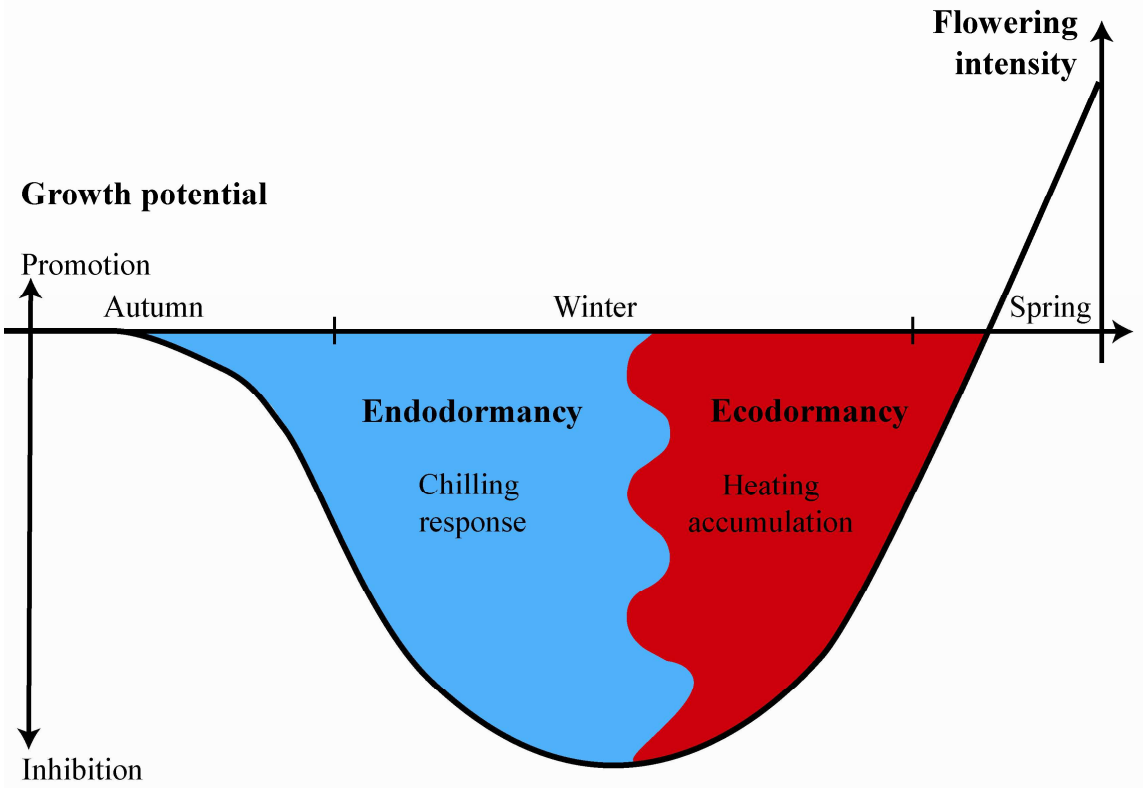

Fig. 8. Scheme of temperature-related stages of dormancy, endo- and ecodormancy, growth and flowering potential, and both chilling and heating requirement during autumn-winter season in temperate-zone fruits. Modified from Saure (1985), Lang et al. (1987), Welling and Palva (2006).

1.5. Production of temperate fruits under mild winter conditions

In temperate regions, chilling requirements are largely met before terminate of the cold season. However, in subtropical or tropical areas, temperate-zone deciduous fruit trees usually suffer from problems of bud break and flowering (Edwards, 1987). The impact of an incomplete dormancy release on a modern fruit production system is very heavy (Erez, 2000b). Insufficient chilling accumulation during dormancy stage leads to three major effects, whose intensities will depend on the level of chilling deficiency: a) poor bud break, poor foliage development, sparse bloom, and frequently abnormal flower; b) delayed foliation and bloom; and c) poor fruit set, reduced leaf area due to secondary dormancy (Erez, 1987).

Biological approaches during dormancy stage indicated that under cold temperatures growth potential in flower buds of peaches remained high, but warm temperature conditions did not permit to recover growth capacity, resulting in necrosis and death of the floral primordia (Bonhomme et al., 1997). Another study in peaches grown under partial chilling deprivation (1/3 and 2/3 of chilling requirements), flower buds showed different pattern of bud break. Under 2/3 of chilling requirements satisfied, flower buds showed higher percentage of bud break when compared to another treatment with lower chilling requirements satisfied. But in this study it was not considered about differences in temperature during chilling treatment (Leite et al., 2004).

In warm winter climates, floral bud initiation can be a long drawn out process, and high temperature seems to adversely affect bud development, and leaf buds often break at the same time flower buds open and there is a direct competition for the meager supply of stored carbohydrates (Faust, 2000).

Recent climate changes and consequent global warming is likely to change the

environment in which crops are currently planted. Temperatures are predicted to increase, affecting plant growth (Richardson et al., 2004), and possibly spreading the dormancy-related problems to plants grown on subtropical and temperate zones.

1.6. “Floral bud abortion” in Japanese pear crops

In regions with mild winter, one of the most important problems in Japanese pear cultivation, as well as other deciduous fruit trees, is shortage of chilling temperatures in order to satisfy the chilling requirement for budbreak, resulting in low percentage of blooming (Petri and Herter, 2002).

“Floral bud abortion” has been considered as a limiting factor in Japanese pear production, assuming great importance on the development of the pear crop in Southern Brazil (Herter et al., 1994). It is characterized by floral primordia abortion, in different levels: with total destruction of buds and consequently no flowering (Fig. 9A, B) or partial destruction, resulting in few number of opened flowers and different development velocity (Fig. 9C, D), resulting in low productivity (Herter et al., 2002). In same region, but under favorable conditions for dormancy development and release (such as occurred in 2008 season), high percentage of opened flowers and consequently low incidence of abortion were observed (Fig. 10A, B).

A similar disorder occurs in New Zealand, known as “budjump”, and has been noted in all growing areas. In this case, affected trees seem to develop normally until late winter-early spring, when large numbers of floral buds abort and drop from the tree or disintegrate when touched (Kingston et al., 1990).

In Thailand, Asian pears are growing satisfactorily under highland conditions. However, insufficient chilling accumulation some times causes improper and irregular breaking of dormancy, which resulted in an abnormal flowering and fruit set (Rakngan, 1995).

Possible causes of “floral bud abortion”, its intensity and time at which it happens, are related to the amount of chilling accumulation and the occurrence of thermal fluctuations (Herter et al., 1994). It has been suggested that, among

ecophysiological factors are those related to the climate, mainly temperature fluctuation and low chilling accumulation; nutritional factors, with emphasis on the micronutrients and carbohydrates; flower development and morphology of buds and water stress (Herter et al., 2001).

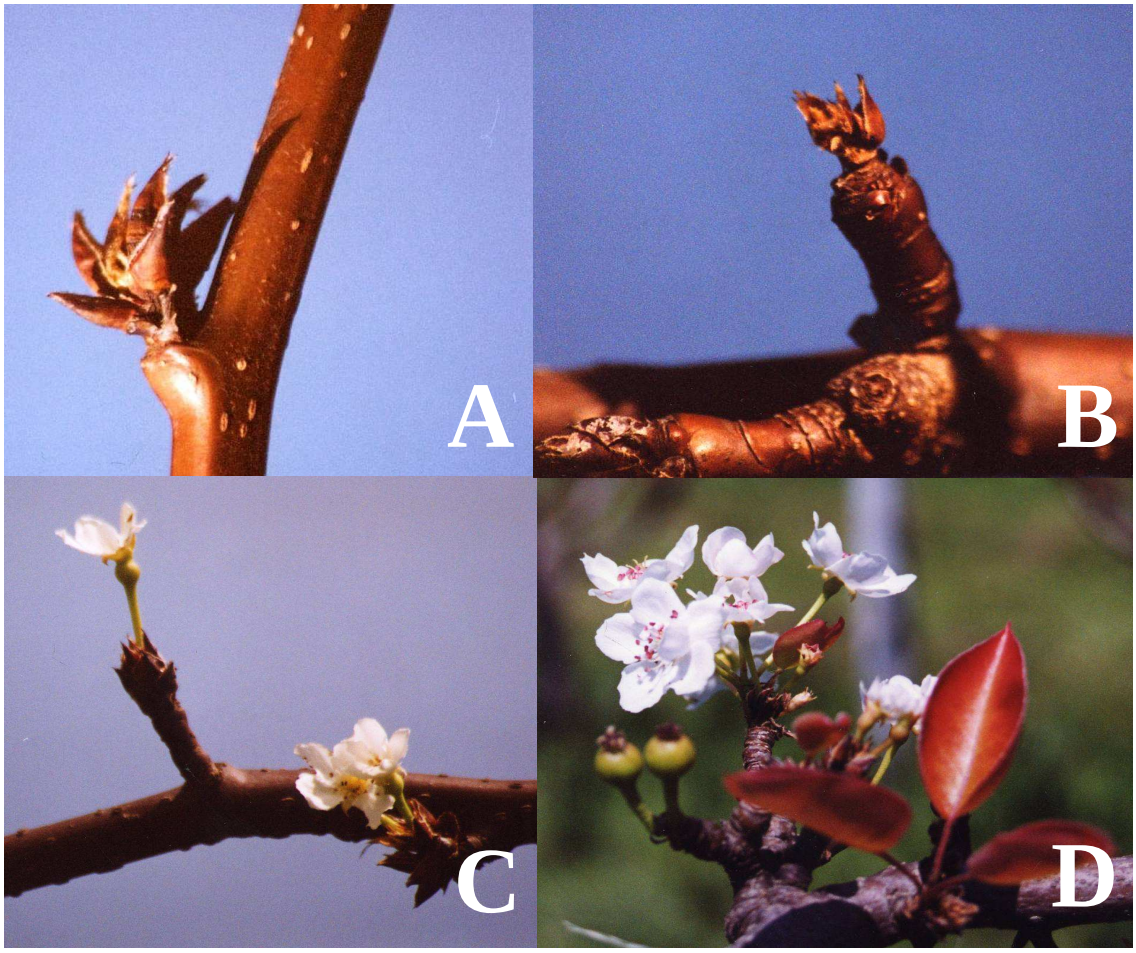

Fig. 9. Flowering situation in conditions of Southern Brazil: complete “floral bud abortion” in mixed buds of one year old shoot (A); complete “floral bud abortion” in spur buds (B); spur mixed buds with few number of opened flowers (C); mixed buds with flowers in different developmental stages, fruits and leaves (D) during 2003 season.

Fig. 10. Flowering situation in conditions of Southern Brazil: apparently normal flowering (A) and tree with apparently normal flowering (B) during 2008 season.

1.7. Carbohydrate metabolism and water dynamics in buds during dormancy stage

Changes in carbohydrate metabolism and water status have been received special attention in recent studies on mechanism of dormancy control in buds (Young et al., 1987; Rakngan et al., 1996; Marquat et al., 1999; Leite et al., 2004; Anderson et al., 2005; Bonhomme et al., 2005; Leite et al., 2006; Morin et al., 2007).

In plant life, carbohydrate production through photosynthesis is the most fundamental activity. They are important to provide energy during important key stages of plant growth, such as the period between reproductive organ formation and fruit set (Candolfi-Vasconcelos and Koblet, 1990). In other species, sugar is known to regulate (i) sink-source interactions in whole plant under normal or stress conditions (Roitsch, 1999) and (ii) plant development and gene expression (Gibson, 2004). Photosynthetic activity level and carbohydrates distribution are crucial to obtain high productivity in fruit crops (Faust, 2000). Gibson (2004) cited influences of sugar on alterations on levels, localization and/or transport of different phytohormones and also on phytohormone-response pathways by affecting the expression and/or activity levels of components of those pathways.

In the past, determination of water content was possible only by correlating fresh and dry weight of some specific part of plants. But recently, it became possible to determine water dynamics by using magnetic resonance imaging (MRI) techniques. During endodormancy, water is associated to macromolecules and it was detected as bound water (low transverse spin-spin relaxation time, T_2). On contrary, free state water was found at the end of dormancy. These results were found in apple (Faust et al., 1991, Liu et al., 1993), peach (Sugiura et al., 1995; Erez et al., 1998; Yooyongwech et al., 2008a), and blueberry (Rowland et al., 1992).

1.8. Objective of this study

Brazil has a potential market for pears, since 90% of consumed fruits are imported. A small area located in Southern Brazil initiated recently a commercial production of Japanese pear, but growers are facing some climatic adaptation problems, called “floral bud abortion”. The most accepted cultivar in the local market, ‘Housui’, showed high incidence and severity of that physiological disorder. Studies on dormancy progression have been done, but usually researchers compared plants growing under natural condition and under totally cold deprived condition.

The main objective of this study was to investigate the effect of artificial mild winter conditions on dormancy progression and “floral primordia abortion” occurrence in ‘Housui’ Japanese pear.

Potted plants of ‘Housui’ Japanese pear were submitted to similar amount of chilling accumulation than those observed in Brazil, corresponding to 80% of theoretical requirement. Chilling treatment started at three different times as described in Chapter 2. The experiment was repeated for four consecutive seasons, from 2005-2006 until 2008-2009, by submitting the same group of plants to same temperature treatment, in order to verify the effect of chilling exposure time and successive mild winters on “floral primordia abortion” incidence and severity. Some parameters, such as determination of dormancy progression by using different mathematic models (Chapter 2), morphological and phenological observations (Chapter 3), carbohydrate contents (Chapter 4), and water dynamics (Chapter 5) on lateral buds of current year shoots were analyzed and discussed.

Chapter 2

Determination of dormancy progression in Japanese pear grown under mild winter conditions

2.1. Introduction

Recently, climate changes and global warming resulted in an increase of temperature, affecting directly the dormancy progression by low and/or slow accumulation of chilling temperatures, which resulted in problems on temperate fruit production.

Nishimoto et al. (1995) cited the value of 750 chilling hours (CH) as the chilling amount necessary to release the endodormancy stage of both ‘Housui’ and ‘Kousui’ Japanese pear mixed buds. But in this experiment we submitted the ‘Housui’ potted plants to 600 CH, the average amount of last 45 years observed in the Japanese pear production area of Santa Catarina state, located in Southern Brazil (Petri and Herter, 2002) (Fig. 7).

This experiment was done in order to analyze the effect of chilling accumulation below the theoretical requirement, and also submitted to chilling temperatures at different times on dormancy progression of ‘Housui’ Japanese pear potted plants. Effect of several seasons (from 2005-2006 to 2008-2009) under similar condition was also studied. For determination of dormancy progression, three different mathematical models were utilized: CH, chilling unit (CU), and developmental rate (DVR) models. After chilling accumulation, potted ‘Housui’ plants were moved to a greenhouse for heating accumulation until flowering.

2.2. Materials and methods

One-year-old plants of the Japanese pear cultivar ‘Housui’ grafted on *Pyrus pyrifolia* were obtained from a commercial nursery. Each was transplanted into a 30-cm-diameter plastic pot in April 2005 and grown at the Agricultural and Forestry Research Center, University of Tsukuba, Japan (36° N, 140° E). Forty-five plants were divided into three chilling treatments (Fig. 11), with 15 plants for each treatment: chilling accumulation initiated under natural conditions (Treatment 1); one month of cold deprivation before expose to chilling temperatures (Treatment 2); and two months of cold deprivation before exposure to chilling temperatures (Treatment 3). Same groups of 15 plants was submitted to same treatments for four consecutive seasons, from 2005-2006 until 2008-2009. Fifteen potted plants with same age grown under natural conditions of field (NC) throughout four consecutive seasons from 2005-06 were used as control. For cold deprivation, a heated greenhouse with minimum temperature set at 16 °C was utilized to avoid any effect of chilling temperature on endodormancy induction. Maximum temperature was not controlled and plants were submitted to natural photoperiod.

Field and greenhouse air temperatures were recorded by a data logger (TR-51A, T and D Co., Matsumoto, Japan). For chilling treatment, each hour of temperature below 7.2 °C under field conditions was counted until reach 600 CH, in order to expose potted plants to similar amount of chilling hours as occur in Southern Brazil (Fig. 7). Chilling calculation initiated when two consecutive hours below 7.2 °C were detected in field.

The CU model was proposed by Asano and Okuno (1990) for determination of endodormancy depth in Japanese pear. According to this method, also called the

Saitama model, the temperature ranges and correspondent chilling units were calculated considering also the seasonal factor (Table 2).

Sugiura et al. (1991) and Sugiura and Honjo (1997b) proposed the DVR model, which characterizes the relationship between dormancy developmental rate and temperature range. The developmental phase can be expressed as a function of temperature based on the developmental stage index (DVI), by addition of hourly temperature DVR (DVI=∑DVR). The developmental rate model is able to express different stages of dormancy in a uniform format (Sugiura and Honjo, 1997a). For the endodormancy stage, temperature ranges proposed by Sugiura and Honjo (1997b) and correlated hours necessary for breaking were adopted according to the Table 3 for determination of DVI. When DVI value reaches 1.0, the endodormancy stage is theoretically considered broken.

After the chilling treatment, potted plants were moved into a heated greenhouse for heating accumulation until flowering, with minimum temperature set on 16 °C. For quantification of heat-unit accumulation, the number of growing degree hours (GDH) was calculated by subtracting 5 °C (adopted as the minimum temperature that could promote plant growth) from each hourly temperature and then adding the readings (Scalabrelli and Couvillon, 1986). The ecodormancy was considered released when buds reached the “green tip” stage (Fig. 12) (Tamura et al., 1993) and the number of opened flowers was determined when petals were totally opened.

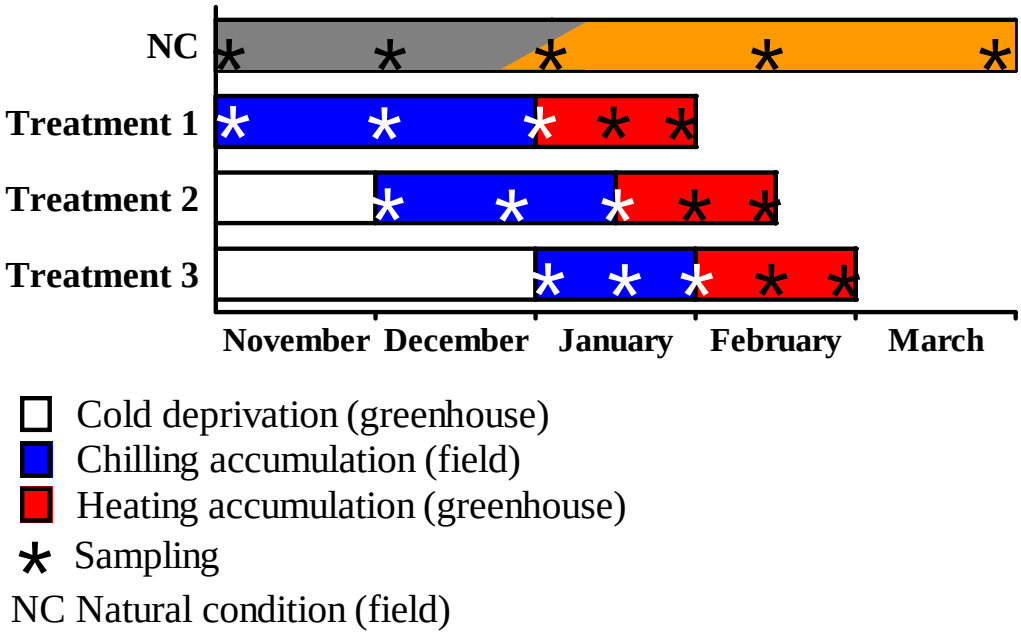

Fig. 11. Experimental design from 2005-2006 to 2008-2009 seasons. Both cold deprivation and heating accumulation were performed in a heated greenhouse with the minimum temperature set at 16 °C. For chilling accumulation, potted plants were moved to field and subjected to 600 h below 7.2 °C. They were then transferred to greenhouse for accumulation of heating until reached the "green tip" stage, indicating the release of ecodormancy stage. NC plants were kept under field conditions.

Table 2. The chilling unit model (CU) or Saitama method, with temperature ranges and correspondent hourly chilling units for determination of floral bud endodormancy progression for ‘Housui’ and ‘Kousui’ Japanese pear, proposed by Asano and Okuno (1990).

	Chilling Units*	
Temperature (^{o}C)	Until 19th November	After 20th November
t ≦ 10.0	0.4	1.0
10.0 < t ≦ 12.0	0.24	0.6
12.0 < t ≦ 15.0	0	0
15.0 < t ≦ 18.0	-0.2	-0.5
18.0 < t	-0.4	-1.0

* Seasonal factor: 40% of chilling units until 19th November; 100% after 20th November;

Table 3. The developmental rate (DVR) model, with temperature ranges, hours of temperature necessary to break endodormancy and relation of DVR per hour, proposed by Sugiura and Honjo (1997a, b; with modifications). The developmental stage index (DVI) was obtained by adding each hourly DVR (∑DVR).

Temperature (^{o}C)	Hours*	DVR (h^{-1}**)
$t \leqq -3$	0	0
$-3 < t \leqq 0$	1500	0.000666 (=1/1500)
$0 < t \leqq 3$	750	0.001333 (=1/750)
$3 < t \leqq 6$	750	0.001333 (=1/750)
$6 < t \leqq 9$	750	0.001333 (=1/750)
$9 < t \leqq 12$	1160	0.000862 (=1/1160)
$12 < t$	0	0

* hours necessary for break endodormancy

** DVR (h^{-1}) = 1/Hours necessary for endodormancy break

Fig. 12. Mixed bud of current season's shoot at the "green tip" stage. The scale bar represents 3 mm.

2.3. Results

Field temperature data from November to March is showed in Fig. 13. Among seasons of study, 2005-2006 season showed the minimum temperatures. The 2006-2007 season was characterized by a relative high average temperatures, whereas 2007-2008 and 2008-2009 seasons had great variations between maximum and minimum temperatures during January and February, but in average 2007-2008 season was colder than the last season of experiment. The accumulation pattern of CH, CU, and DVI from November to March showed differences among seasons in CH data, whereas CU and DVI did not showed great differences (see Appendix 1).

In our experiment we adopted the start of chilling accumulation when two consecutive hours below 7.2 °C were detected under field conditions. Treatment 1 initiated on October 28th 2005 (first season), November 8th 2006 (second season), November 4th 2007 (third season), and November 1st 2008 (forth season). Treatments 2 and 3 started one and two months, respectively, after start the Treatment 1 in each season.

At the beginning of chilling accumulation of Treatment 1 (November), field temperature (Fig. 14) was falling toward chilling temperature range (7.2 °C), but the first season of experiment (2005-06) showed the lowest temperatures during mostly of period. In the same first season, Treatment 2 showed the lowest field temperatures during all the period. In Treatment 3, the first season showed low temperatures with some fluctuations, whereas 2007-08 was cold with constant temperatures.

Large differences in the time taken to accumulate 600 CH were observed: the first season took 47 days to accumulate 600 CH, whereas in the second and fourth seasons it took 16 days longer (Fig. 15 and Table 4). Because winter 2005-2006 was cold (Fig. 14) during the chilling accumulation of Treatment 2, it took only 30 days to

accumulate the same amount of chill (Fig. 15 and Table 4). In the subsequent three seasons took 6 to 10 days more than the first season. Treatment 3 started during the depth winter (January), when more temperatures were effective on chilling accumulation (Fig. 15). In this treatment there were, therefore, only small differences between four seasons (time taken to accumulate 600 CH: 29 to 36 days among four seasons of study). Chilling accumulation curves for each year were therefore more distinct in Treatment 1 but strongly resembled each other in Treatment 3.

The DVI data showed that all treatments of the first season (2005-2006) and all seasons of Treatment 3 did not reached 1.0. For Treatment 2, only 2006-2007 season reached value higher than 1.0 (Fig. 16). DVI curves were resembled among seasons in all three treatments.

The chilling unit accumulation curves among treatments were showed in Fig. 17. Treatment 1 showed the most variable values for CU accumulation among seasons, reaching 660 CU during the coldest season (2005-2006) and 1000 CU in the 2008-2009 season. Treatment 2 had two distinct groups: 2005-2006 and 2007-2008 seasons accumulated less than 800 CU, whereas other two seasons reached values higher than 850 CU. 2006-2007 accumulated more CU than other three seasons, but with little difference among them. Treatment 3 showed identical velocity and pattern of CU accumulation and duration varied one week among seasons (Table 4). The amount of CU accumulated was less than 700 during 2005-2006 season for all treatments. On contrary, the second season (2006-2007) reached more than 800 CU in all treatments. 2008-2009 season accumulated the highest amount of chilling units (near 1000 CU) among all treatments of all seasons.

After 600 CH accumulations, plants were transferred to a heated greenhouse to complete the process of dormancy release. The ecodormancy stage was considered

release when the “green tip” was observed in mixed buds (Tamura et al., 1993), and occurred after 25 (Treatment 3) to 32 days (Treatment 1) of heating accumulation during 2005–06 season. In average, Treatment 1 accumulated 6779.0 GDH after received 600 CH, showing higher values compared to Treatment 2 and 3 (5956.5 and 6475.75, respectively) (Table 4). In same season, the duration of heating accumulation in Treatment 3 was shorter than other two treatments in most of seasons, except during 2007-2008 season, when Treatment 2 spent 16 days until reached the “green tip” stage (Treatment 3 spend 20 days). In 2008-09 season, which corresponded to forth consecutive seasons of mild winter conditions, “green tip” was observed after 18 (Treatment 3) to 23 days (Treatments 1 and 2) under forcing conditions.

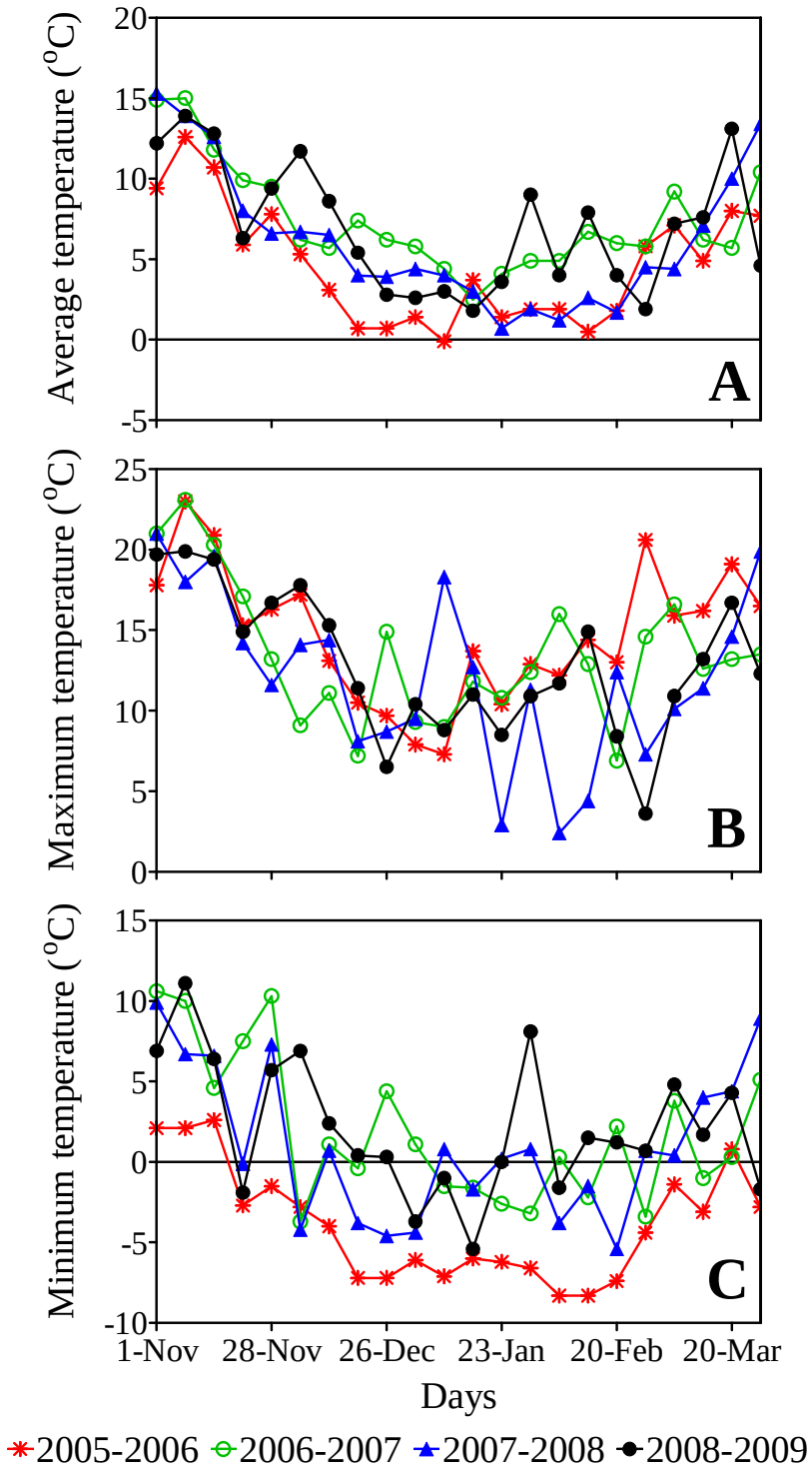

Fig. 13. Field average temperature (A), maximum (B) and minimum temperature (C), from November to March during four seasons of experiment (from 2005-2006 to 2008-2009). Temperature data are plotted as weekly averages.

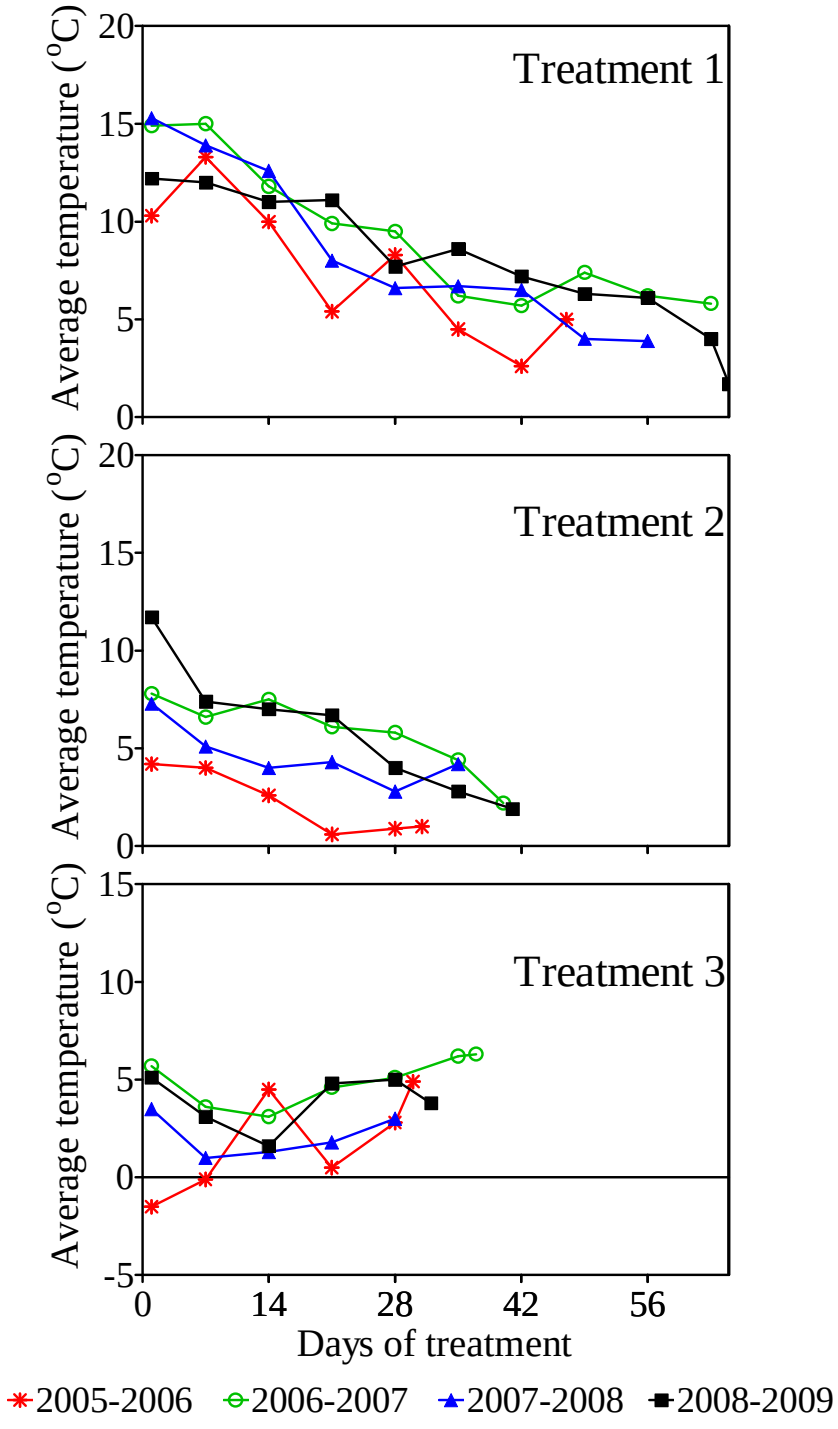

Fig. 14. Average air temperature at field during chilling accumulation period of four seasons of experiment in all treatments 1 to 3.

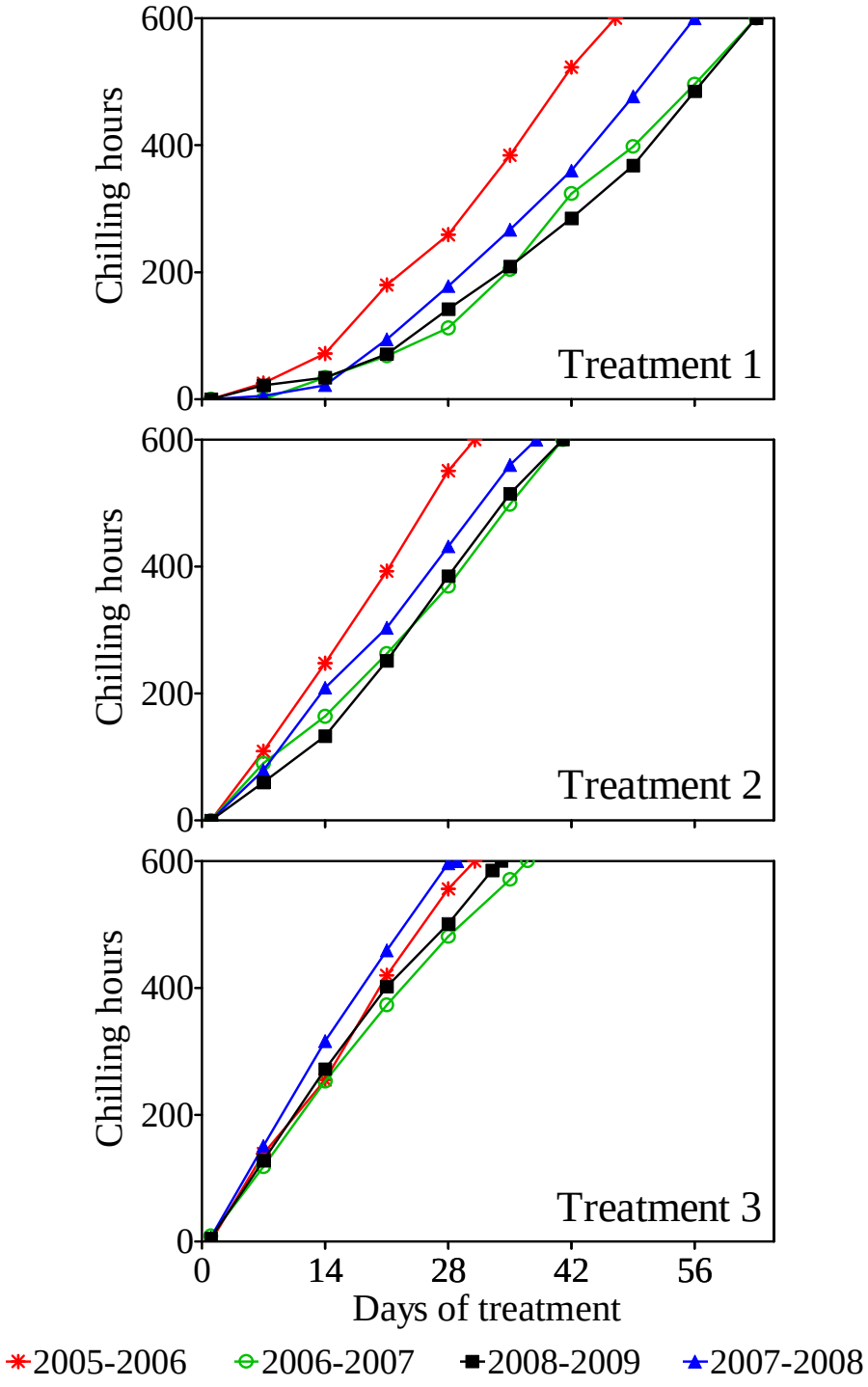

Fig. 15. Progress of CH accumulation until reached 600 CH (below 7.2 °C) during chilling accumulation period of four seasons of experiment in all treatments 1 to 3.

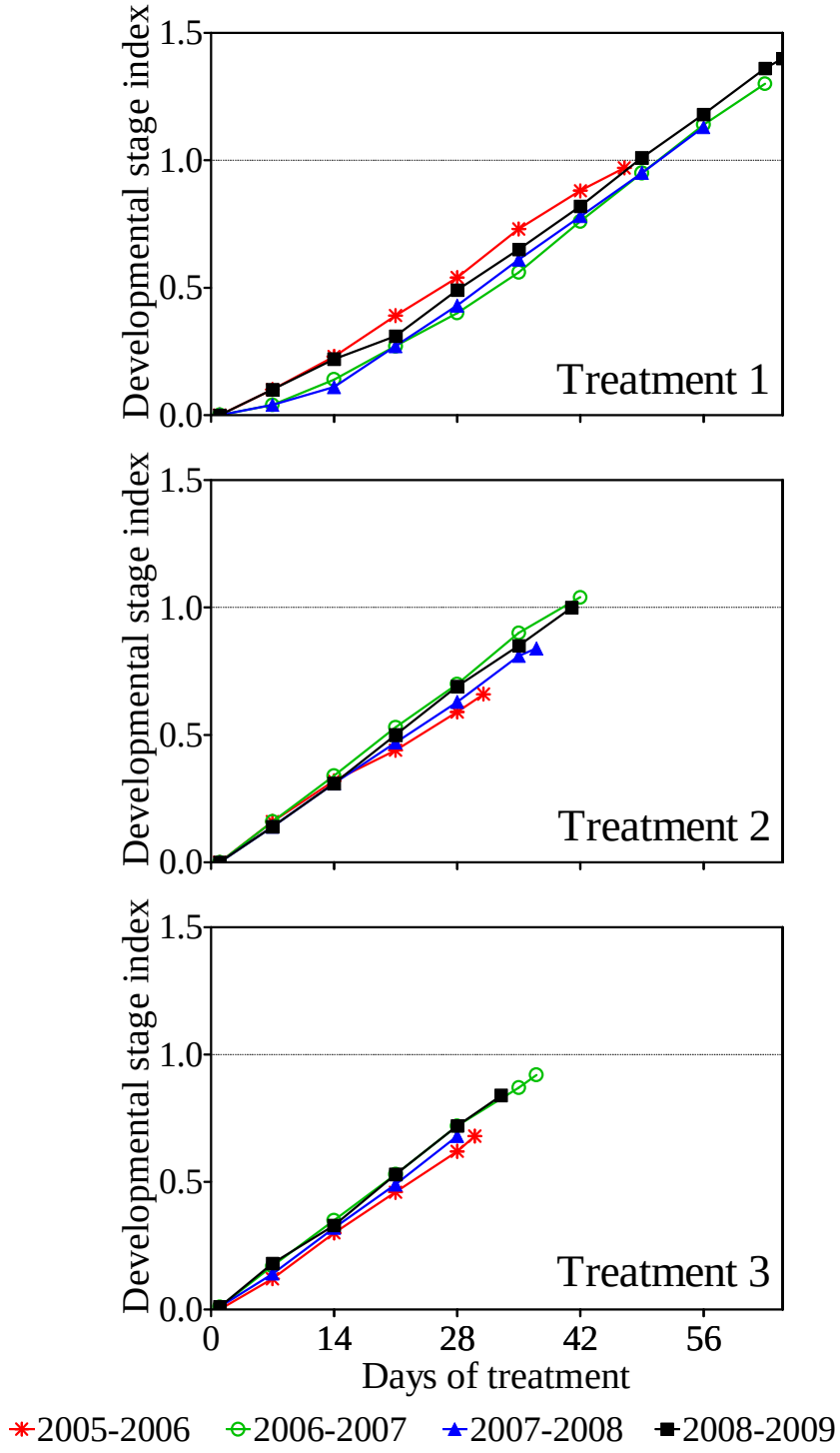

Fig. 16. Progress of DVI accumulation until reached 600 CH (below 7.2 °C) during chilling accumulation period of four seasons of experiment in all treatments 1 to 3. Dotted line indicates the theoretical requirement of chilling amount (DVI = 1.0) to release the endodormancy stage.

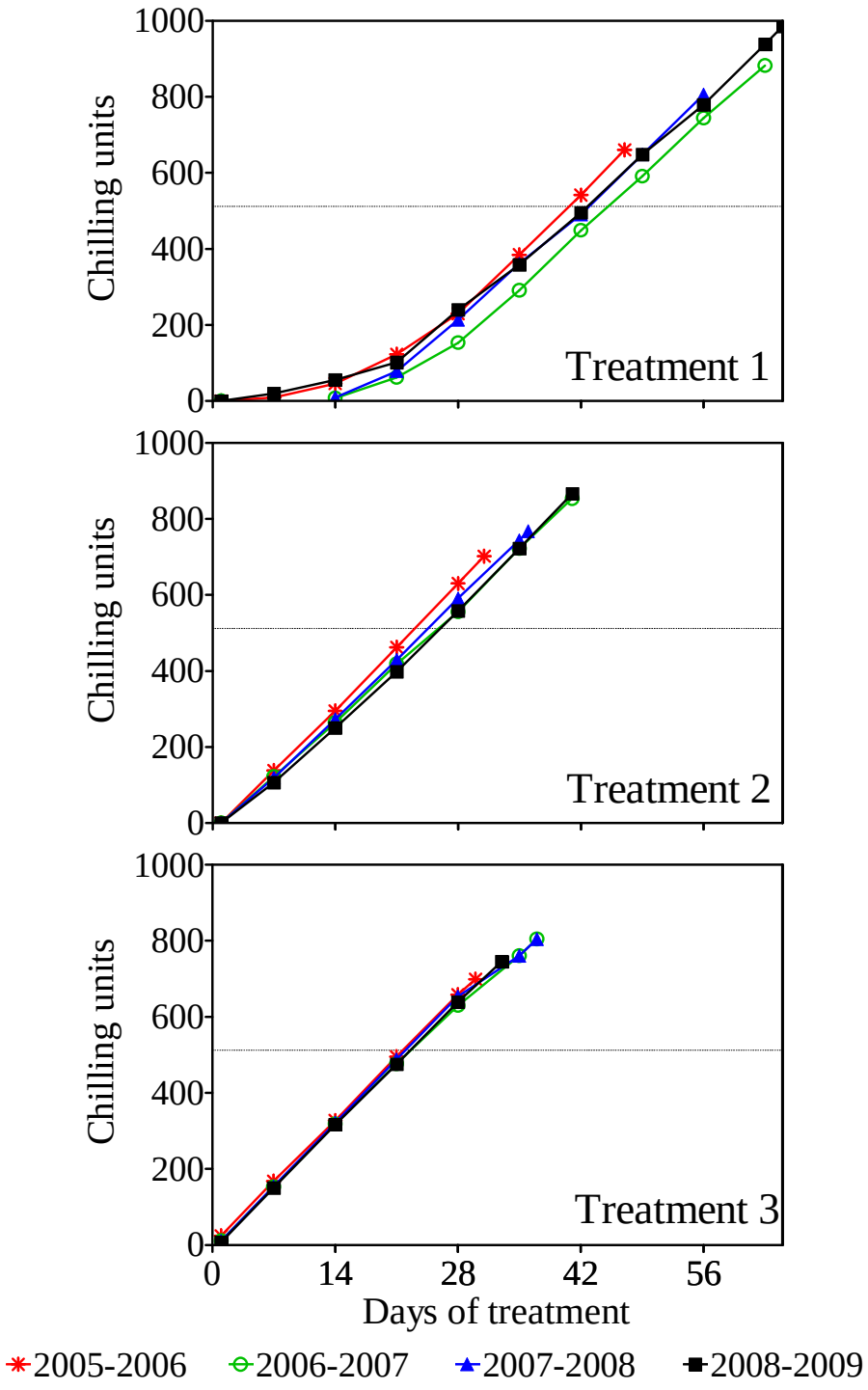

Fig. 17 Progress of CU accumulation until reached 600 CH (below 7.2 °C) during chilling accumulation period of four seasons of experiment in all treatments 1 to 3. Dotted line indicates the theoretical requirement of chilling amount (512 CU) to release the endodormancy stage.

Table 4. Relationship among treatments, seasons, duration of chilling treatment to accumulate 600 CH, duration of heating treatment and accumulated GDH before reach the "green tip" stage on 'Housui' Japanese pear potted plants.

Treatment	Season	Days of chilling treatment*	Days of heating treatment**	Accumulated GDH
1	2005-2006	47	32	8020
	2006-2007	63	18	5770
	2007-2008	56	31	7256
	2008-2009	63	22	6070
	Average	**57**	**25**	**6779.00**
2	2005-2006	30	29	7239
	2006-2007	40	17	5483
	2007-2008	36	16	5039
	2008-2009	38	23	6065
	Average	**36**	**22**	**5956.50**
3	2005-2006	30	25	7827
	2006-2007	36	16	5145
	2007-2008	29	20	6825
	2008-2009	33	18	6103
	Average	**32**	**20**	**6475.75**

***** Days of chilling temperatures necessary to accumulate 600 CH;

** Days of heating temperatures necessary to release the ecodormancy stage ("green tip");

2.4. Discussion

The amount of chilling necessary to release endodormancy in Japanese pear mixed buds has been estimated in many studies, which have yielded different values: 720 h at 10 °C for ‘Housui’ (Asano and Okuno, 1990), 756 h below 7.2 °C for ‘Housui’ and ‘Kousui’, and 721 h below 7.0 °C for ‘Housui’ (Nishimoto et al., 1995), 750 h between 0 and 6 °C in ‘Kousui’ (Sugiura and Honjo, 1997b), and 780 h at 6 °C for ‘Kousui’ (Sugiura et al., 2003). In our study, we adopted the amount of 750 h below 7.2 °C (CH) as the chilling requirement of ‘Housui’ to release the endodormancy stage. By submitting the potted plants to 600 CH, we imposed to a theoretical 80% of chilling requirement.

As a consequence of global warming, increases in temperature from September to February have been noted in Japan and have resulted in variations in the times of endodormancy break and full bloom in Japanese pear ‘Kousui’ (Ito and Ichinokiyama, 2005). Even in Japan, which is in a temperate climatic zone, our results showed great differences in chilling accumulation among years as a result of temperature fluctuations during autumn–winter. These variations resulted in differences on duration to accumulate 600 CH among treatments and seasons (Table 4).

The theoretical values of CH and DVI necessary for endodormancy completion of ‘Housui’ Japanese pear (750 CH and DVI = 1.0, respectively) were not adapted to our experimental conditions, since dormancy was released and flowering occurred with accumulation of values lower than the requirement in all treatments of all seasons. Our results indicate that 600 CH below 7.2 °C brought about the release of dormancy stage in lateral buds of potted ‘Housui’, contradicting the results obtained by Nishimoto et al (1995). Independently of chilling exposure time (treatments), the endodormancy stage were released even with DVI values lower than 1.0.

Tamura et al. (2001) determined the amount between 800 and 1000 CU by using the Saitama model for breaking leaf bud endodormancy in ‘Housui’, but leaf buds have higher chilling requirement than floral buds in Japanese pear (Tamura et al., 1993, 1995). According to Asano and Okuno (1990), 512 CU was sufficient to release the floral bud endodormancy in both ‘Housui’ and ‘Kousui’ cultivars. In this experiment, all of treatments of all seasons reached the requirement of CU to release the endodormancy stage (Fig. 17). These observations indicated that Saitama method can be used even under mild winter conditions to determine the chilling accumulation with the amount of 512 CU might be the requirement of ‘Housui’ even under such conditions.

After chilling, plants were moved into a heated greenhouse, with the minimum air temperature set at 16 °C, to release ecodormancy and induce flowering. Among four seasons of study, the coldest season (2005-2006) required few days to accumulate 600 CH in Treatments 1 and 2, but needed more days to accumulate heating temperatures for flowering. On contrary, relatively warm winters (2006-2007 and 2008-2009 seasons) accumulated chilling amount slowly but spent few days to accumulate GDH. A correlation between duration of chilling and heating treatment was observed in this experiment. Independent of treatments, long duration of chilling resulted in short duration of heating until dormancy release (Table 4). The exception was observed in Treatment 2 of 2007-2008 season, when a middle duration of chilling accumulation resulted in the shortest heating duration. Differences on duration of the ecodormancy stage between seasons indicate that successive conditions of mild winters reduced the heating requirements, as observed previously in peaches (Citadin et al., 2001).

Chapter 3

Floral primordia abortion occurrence and abnormalities at flowering in Japanese pear grown under mild winter conditions

3.1. Introduction

Japanese pear shows adaptation problems, especially during dormancy and its release, when grown in regions with mild winters, such as New Zealand (Kingston et al., 1990; Klinac and Geddes, 1995), Thailand (Rakngan et al., 1995), and Brazil (Petri et al., 2002; Petri and Herter, 2002), or under forcing conditions in greenhouses (Gemma, 1994). In New Zealand, floral buds of 'Housui' are highly susceptible to a disorder called "budjump", incidence of which in winter 1988 was near 95% (Klinac and Geddes, 1995). In this region, affected buds were aborted and drop from trees during late winter-early spring when touched (Kingston et al., 1990). In some locations of Southern Brazil, over 60% "flower bud abortion" was found in 2001 (Petri et al., 2002) and more than 90% in 1999, resulting in low numbers of flowers at budburst and consequently low production (Veríssimo et al., 2002). Another consequence of low chilling is a prolonged and poorly synchronized flowering, which results in poor uniformity of fruit size at harvest. In 2008, it was noticed some physiological disorder in 'Housui' Japanese pear cultivated under greenhouse in Fukuoka Prefecture and other regions of Southwest Japan. Detected symptoms were necrosis in both buds and shoot, and increases in temperature during dormancy stage have been cited as a major factor (Honjo, 2007).

The aim of this study was to observe the effect of low chilling response, submitted at different times, and also the effect of consecutive seasons of low chilling accumulation on floral primordia abortion occurrence in 'Housui' Japanese pear mixed

buds. Abnormalities at budburst and flowering, similarly to those observed in Brazil or New Zealand, were also expected to occur.

This experiment was conducted simultaneously to the experiment described on Chapter 2, reason why it was adopted the classic CH model as a parameter for dormancy progression and time for samplings. Moreover, the Saitama method and DVI model, which were developed in Japan for Japanese pear, was not frequently adopted overseas.

3.2. Materials and methods

For this experiment, samples were obtained from plants submitted to same temperature conditions and treatments as described in Chapter 2 (Fig. 11). Briefly, potted plants of 1-year-old ‘Housui’ Japanese pear were submitted to the amount of 600 CH at different times. Once each during summer and winter, current year shoots were reoriented to horizontal position to promote floral bud formation (Ito et al., 2004). Opened flowers were not pollinated artificially and occasional fruits were thinned manually every season. At the sampling of dormant bud, a vegetative bud localized in a lower portion of shoot was kept in order to promote the development of new shoot during the next growing season. Forty-five plants were divided among three different treatments, according to the time to initiate the chilling accumulation: chilling accumulation without cold deprivation (Treatment 1), one month of cold deprivation before exposure to chilling temperatures under field condition of Tsukuba, Japan (Treatment 2), and two months of cold deprivation before started the accumulation of 600 CH (Treatment 3). Fifteen potted plants with same age grown under natural conditions of field (NC) throughout four consecutive seasons from 2005-06 were used as control. After exposure to 600 CH, plants were transferred to a heated greenhouse, with minimum temperature set at 16 °C, for heating accumulation until flowering.

For phenological observations, lateral mixed buds with bud cushions were collected randomly from the current season’s (one-year-old) shoots (excluding terminal buds) of Japanese pear ‘Housui’ during four consecutive seasons, from 2005-2006 until 2008-2009 (Fig. 18). Buds were collected from all treatments before any chilling accumulation (0 CH), after received 300 CH (middle of chilling accumulation), 600 CH (transition from chilling to heating accumulation), 4000 GDH (middle of heating accumulation), and 8000 GDH (just before flowering). From NC plants, buds were

collected before any chilling accumulation, middle of endodormancy, transition from endo- to ecodormancy, middle and before release the ecodormancy stage, and plotted as 0 CH, 300 Ch, 600 CH, 4000 GDH, and 8000 GDH, respectively.

Phenological observations were made immediately after sampling in all seasons, in order to determine the incidence and severity of floral primordia abortion in lateral mixed buds of ‘Housui’ Japanese pear according to progress of dormancy stage. After remove all 12 scales, observations were done under a light microscope (SZX-12, Olympus Co., Tokyo, Japan) and a digital microscope (VHX-900, Keyence Co., Osaka, Japan). An arbitrary scale was adopted to determine the severity of floral primordia abortion (Fig. 19A): grade 0 for normal primordia (green), 1 for the beginning of primordia necrosis (yellow), 2 for primordia with partial necrosis (some black portions), and 3 for completely necrosed primordia (totally black). Data were presented as a percentage of total primordia, obtained from 10 lateral buds. In each treatment of all studied seasons, number of flowers was also determined when petals were completely opened.

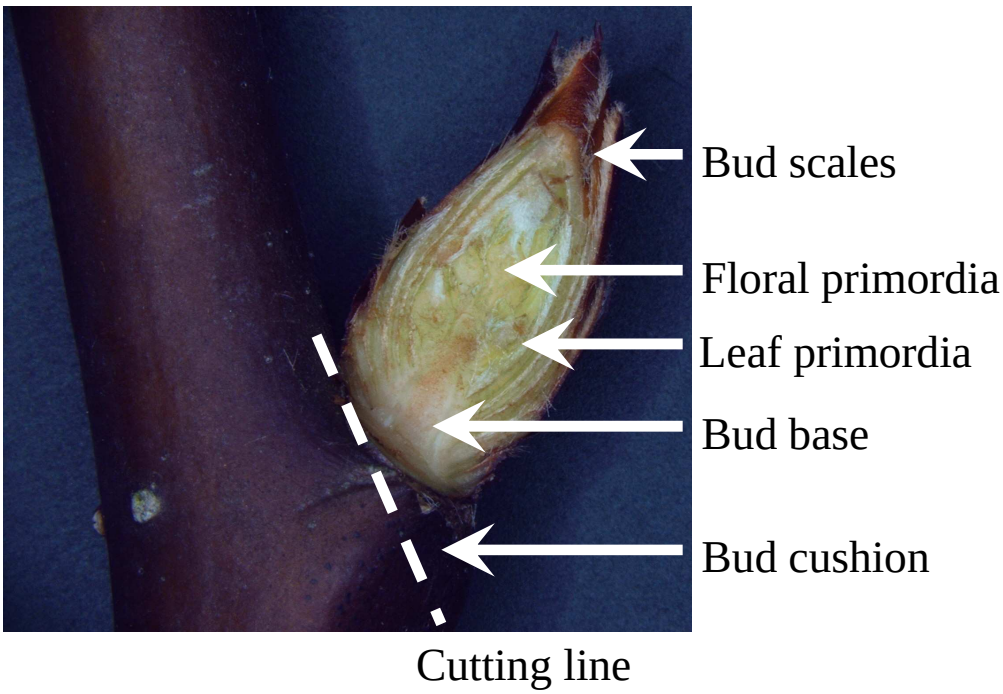

Fig. 18. Scheme of sampled material: lateral mixed buds of current-year shoot (except terminal bud) with bud scales, floral and leaf primordia, bud base and bud cushion.

3.3. Results

Symptoms of floral primordia abortion were observed in all treatments of all studied seasons, but with different severity, whereas NC plants did not showed abnormalities during dormancy progression (Fig. 19B). In treatment 1, mixed buds showed a great reduction in percentage of normal primordia at the end of heating accumulation period. Keeping plants under cold deprivation for 1 month (treatment 2) resulted in a gradual increase in abortion, which initiated during the chilling treatment in the first two seasons, whereas completely necrosed primordia were found just before initiate the chilling treatment in subsequent two seasons. Treatment 3 showed a similar trend to that in Treatment 2, but necrosis symptoms were observed at higher levels when the chilling accumulation started after two months of cold deprivation. Completely necrosed primordia (grade 3) were observed in Treatment 1 when initiated the heating accumulation in 2006-2007 season. The incidence of necrosis increased with increasing delay to initiate the chilling accumulation (Treatments 2 and 3 vs. Treatment 1).

During dormancy progression, it was found abnormalities in buds at different levels: few primordia with necrosis symptoms in low or initial levels (Fig. 20A), buds with partially necrosed primordia (Fig. 20B, E), buds with inflorescence duplication (Fig. 20C, D, E), buds with completely necrosed primordia (Fig. 20C, E), and completely necrosed buds (Fig. 20F).

There was no difference between seasons and also among treatments in the percentage of bud break, which was higher than 95%. At flowering, buds of treated plants showed less than 50% of opened flowers in comparison to plants grown under NC (Fig. 21). Among treatments, a significant increase ($P<0.05$ by *t*-test) on number of flowers was observed only Treatment 1 of 2007-08 and 2008-09 seasons in comparison to previous two seasons. Plants grown under mild winter conditions showed differences

on number of opened flowers (Fig. 22), variations on pedicel length (Fig. 22A, B, D, E), and also flowers at different stages of development (Fig. 22D, E).

Under NC (all time at field conditions), ‘Housui’ showed green scales and primordia until the end of dormancy stage (Fig. 23A). At the flowering stage, primordia had similar pedicel length, and a uniform flower development could be observed (Fig. 23B, C, D, E).

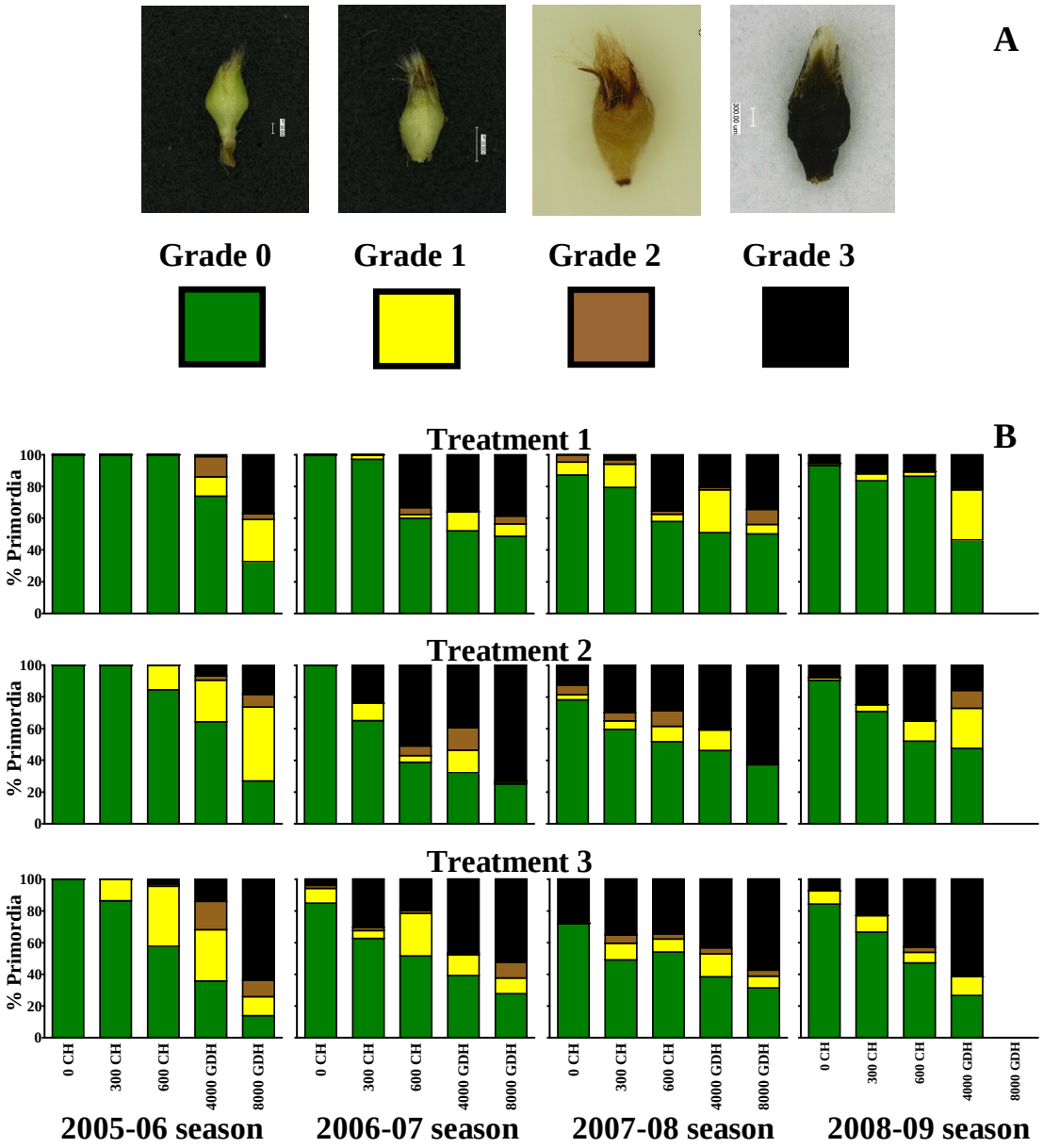

Fig. 19. Scale adopted to evaluate the severity of floral primordia abortion: grade 0, normal primordia (green); grade 1, yellow primordia; grade 2, primordia with partial necrosis; grade 3, completely necrosed primordia (A). Incidence and severity of floral primordia abortion in mixed buds of 'Housui' Japanese pear grown with low chilling, from four consecutive seasons (from 2005-06 to 2008-09), expressed as percentages of primordia (B). (n = 10). CH, chilling hours; GDH, growing degree hours.

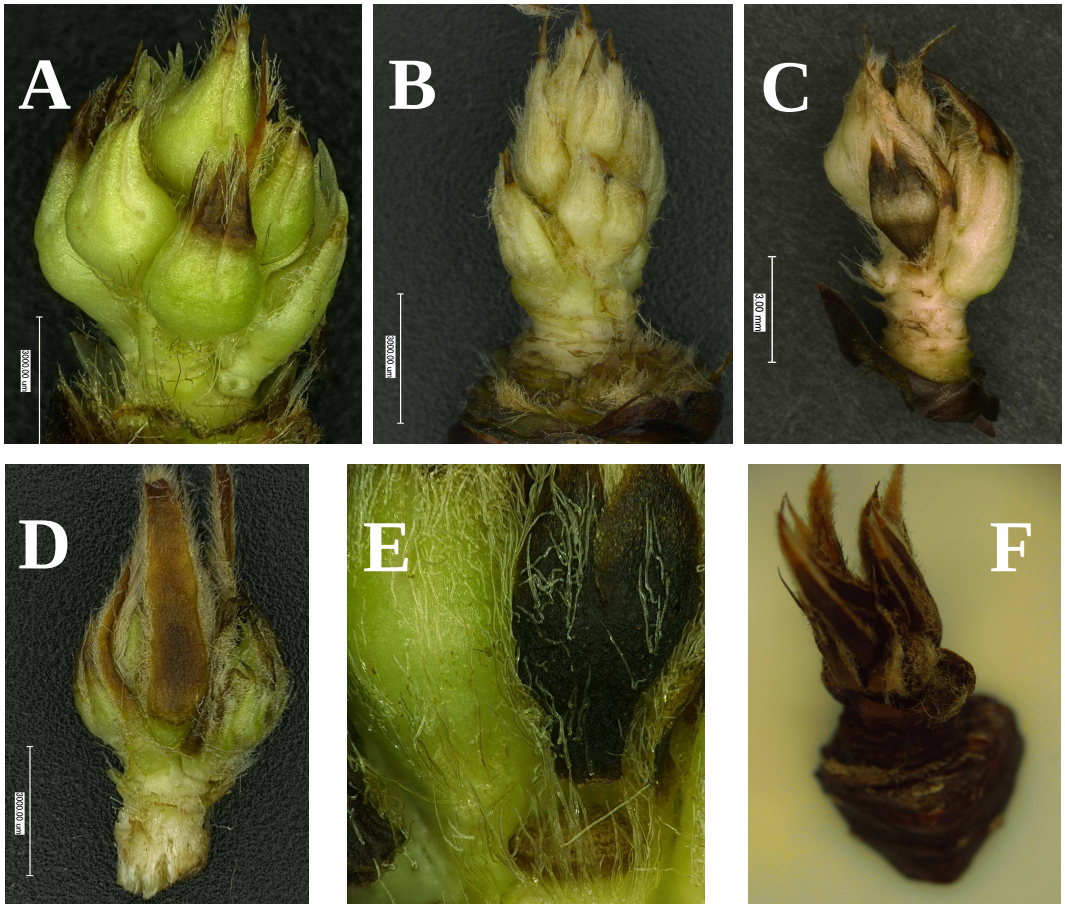

Fig. 20. Some of abnormalities found during dormancy progression in mixed buds of ‘Housui’ Japanese pears grown under mild winter conditions for consecutive seasons: beginning of floral primordia necrosis in bud of treatment 3, 2008-2009 season (A); advanced desiccation of bud scales and yellow colored primordia, but without completely necrosed, in the same bud of treatment 3, 2008-2009 season (B); necrosed primordia and development of a second inflorescence in bud of treatment 3, 2008-2009 season (C); inflorescence duplication with inner scales during heating accumulation, 2008-2009 season (D); completely necrosed primordia and abscission layer in bud of treatment 3, 2008-2009 (E); completely necrosed mixed bud of treatment 3, 2005-2006 season (F). The scale bars represent 3 mm.

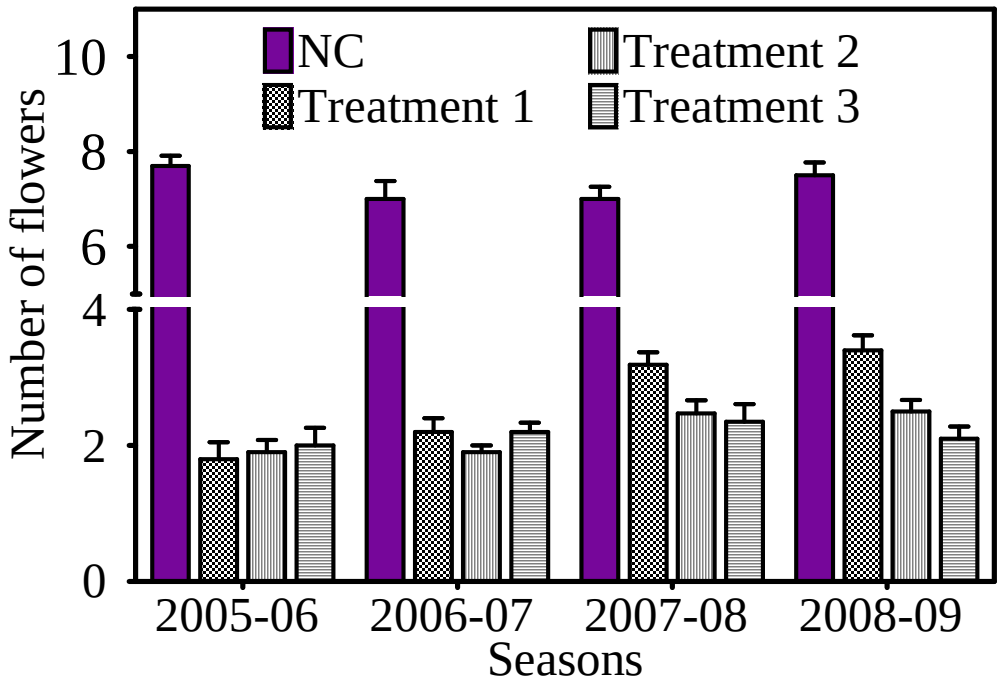

Fig. 21. Average number of opened flowers per bud, from 2005-06 to 2008-09 seasons in the three treatments and natural conditions (NC). The vertical bars are mean ± SD (*n* = 10).

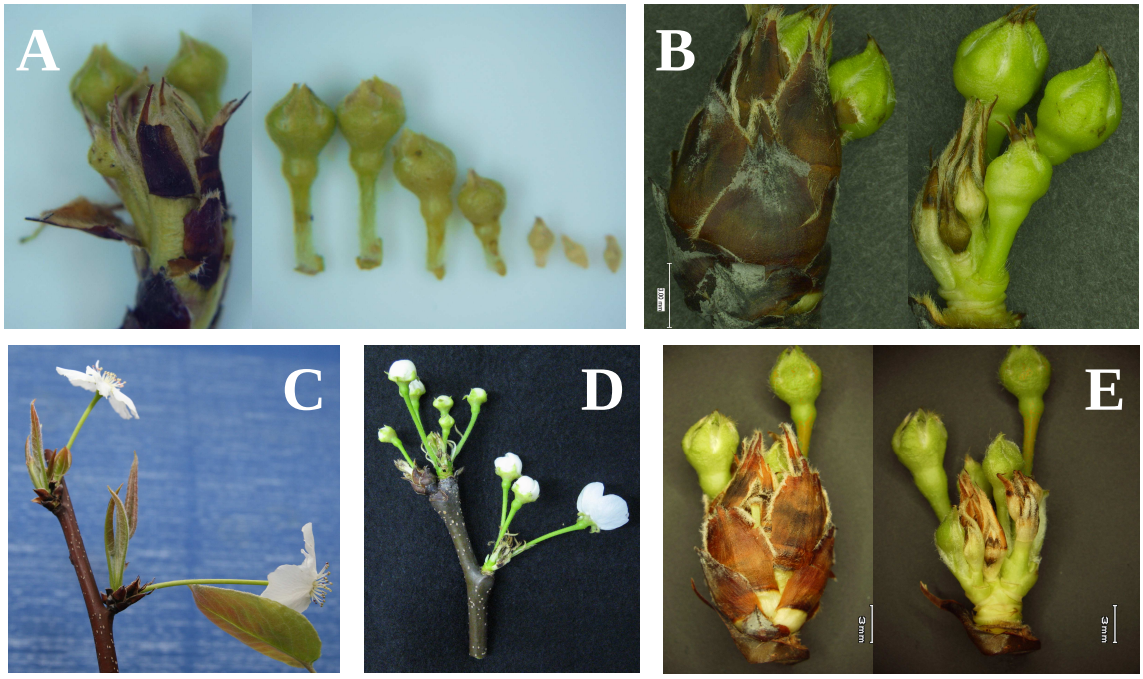

Fig. 22. Abnormalities at flowering found in mixed buds of 'Housui' Japanese pear grown under mild winter conditions for consecutive seasons: bud with visible flowers just after release (left) and flowers with different pedicel lengths in plants of treatment 1, 2005-2006 season (A); bud with visible flowers just after release (left) and primordia with different grade of necrosis in plants of treatment 3, 2008-2009 season (B); lateral mixed buds of current year's shoot showing one opened flower and vegetative growth in plants of treatment 2, 2007-2008 season (C); current year's shoot of treatment 3, 2005-2006 seasons, with terminal and lateral mixed buds at the balloon stage showing different number of flowers (D); bud of treatment 1, 2008-2009 season, showing visible flowers just after release (left) and duplicated inflorescence with some necrosed primordia (E). The scale bars represent 3 mm.

Fig. 23. Some photographs showing mixed buds of ‘Housui’ Japanese pear potted plants with same age of those materials utilized in our experiment and growing under normal conditions of winter in 2008-2009 season: bud at the “green tip” stage, after remove all scales (A); bud of current season’s shoot with visible flowers just after release showing flowers with similar size (B); current year’s shoot with flowers at “balloon” stage (C and D); current year’s shoot with flowers at “full bloom” stage (E). The scale bars represent 3 mm.

3.4. Discussion

Samples were collected before started the chilling accumulation (0 C), middle of chilling accumulation (300 CH), transition from chilling to heating accumulation (600 CH), theoretical middle of heating accumulation (4000 GDH), and just before flowering (8000 GDH), in order to verify the incidence and severity of floral primordia abortion in lateral buds of ‘Housui’ Japanese pear. In 2008-2009 season samples were not taken at 8000 GDH because the plants had already flowered.

According to our results, the amount of 600 CH below 7.2 ^{o}C brought about the release of dormancy stage in mixed buds of potted ‘Housui’, as discussed in previous chapter. However, floral primordia abortion and inflorescence duplication during dormancy progression and at flowering were observed in all cold deprived treatments. The time to initiate the chilling accumulation should be considered as one of the most important factors affecting the incidence and severity of floral primordia abortion in ‘Housui’ Japanese pear (Fig. 19). In a season, with delaying start of chilling accumulation, incidence of floral primordia abortion tended to occur earlier with higher severity than those plants submitted to normal exposition to chilling temperatures. On the contrary, plants grown under NC, with enough accumulation of chilling amount, did not showed remarkable abnormalities.

In a same treatment, the effect of consecutive seasons of mild winter conditions could be observed especially until 2007-2008 season. In this period, an anticipation and intensification of abnormalities were observed in the course of seasons. But, 2008-2009 season showed lower levels of abnormalities on floral primordia in the initial stage in comparison with the same sampling stage of previous season. It is possible that ‘Housui’ Japanese pear trees spent three seasons to adapt their physiological functions to conditions of mild winter, with lower influence of seasonal temperature variations. After

that (2008-2009 season), plants might be more affected by external temperature fluctuations.

Among treatments, trees submitted to cold deprivation (Treatments 2 and 3), showed completely necrosed primordia at the start of chilling accumulation (0 CH) after the second season. These observations can be related to degree of adaptation to cold temperatures (hardening), which is induced gradually by decreasing of temperature and photoperiod, resulting in a plant more resistant to extreme environmental conditions such as cold temperatures (Weiser, 1970; Bigras, 1996). In Treatment 1 temperature decreased gradually, whereas other treatments had an abrupt decrease of temperature, from more than 16 ^{o}C to lower than 6.3 ^{o}C (average of Treatment 3). This observation is not so evident in 2008-2009 season, when all treatments showed similar levels of floral primordia abortion at the start of chilling accumulation. It is possible that, after three seasons of mild winter conditions 'Housui' became more adapted to such condition.

Under natural conditions of chilling and heating accumulation (field), 'Housui' potted plants showed normal bud development: at the "green tip" stage, floral primordia were still green (Fig. 23A). Plants growing under mild winter conditions showed high percentage of abnormal primordia (partially or totally necrosed), and eventually with development of new inflorescence (Fig. 20).

According to Petri et al. (2002), in conditions of Southern Brazil floral bud abortion disorder occurs just before flowering, resulting in a reduction in the number of flowers, which are small and deformed, with no leaves, or leaves that are small. Experiments performed in that region have also shown variations in flower pedicel length, as well as inflorescence duplication (Veríssimo et al., 2002). If any abnormality on floral primordia was observed in Brazil at the beginning of chilling accumulation, it is possible that the phenomenon observed there is caused mostly by low chilling

accumulation. A gradual decrease of temperature during autumn-winter (similar to treatment 1 in our experiment) might result in low incidence and severity of floral primordia abortion, but caused a reduction in the number of flowers and leaves in southern Brazil. In New Zealand, a similar disorder called “budjump” has been described as ranging from a reduction in the number of opened flowers per cluster to complete necrosis, but it is more severe in the lateral buds of young shoots (Klinac and Geddes, 1995). We found similar symptoms to those observed in New Zealand: inflorescence duplication, differing severities of floral primordia abortion, few flowers per bud, and totally necrosed buds (Fig. 20F). “Budjump” in New Zealand appears to be an abnormality more severe than that occurred in Brazil, and seems to be a consequence of delayed exposition to low chilling accumulations (equivalent to treatment 3 in our experiment, but probably without abrupt decrease of temperature).

Chapter 4

Carbohydrate concentration in mixed buds of Japanese pear grown under mild winter conditions

4.1. Introduction

Dormancy induction, progression, and its release is a complex process, composed by several molecular and biochemical changes such as carbohydrate metabolism (Maurel et al., 2004a, b), cold-induced gene expression and cold stress tolerance (Tabaei-Aghdaei et al., 2003), osmoregulation in cold-acclimated plants (Stitt and Hurry, 2002), among others.

Carbohydrate contents of buds of plants grown under cold-deprived conditions or warm winters have been investigated in peach (Marquat et al., 1999; Bonhomme et al., 2005; Leite et al., 2006), grape (Lebon et al., 2005), fig (Kawamata et al., 2002), and Japanese pear (Rakngan et al., 1996). Bonhomme et al. (2005) compared carbohydrate concentrations in buds and nearby structures of peach plants grown under natural conditions and in a totally cold-deprived environment (heated greenhouse with temperature between 15^{o}C and 25^{o}C, in order to avoid any effect of chilling on dormancy).

The objective of this study was to determine the carbohydrate content in lateral buds of ‘Housui’ Japanese pear potted plants grown under mild winter conditions, in order to observe a possible effect of low chilling accumulation, different starting time of chilling accumulation, several consecutive seasons, and a possible correlation with floral primordia abortion.

4.2. Materials and methods

Plants subjected to temperature conditions as described in Chapter 2, from 2005-2006 until 2008-2009 season (Fig. 11). Same groups of plants were submitted to same treatment during all seasons. Mixed buds from current season's (1-year-old) shoots (excluding terminal buds) of Japanese pear 'Housui' (Fig. 16) were collected in two seasons, 2005-2006 and 2008-2009. Samplings were done after plants had received 300 CH, 600 CH, 4000 GDH, and 8000 GDH, similarly to the method described in Chapter 3. To determine the carbohydrate content, samples from plants were frozen in liquid nitrogen and kept in an ultra-freezer (–85 °C).

For determination of sugar content, approximately 1 g of frozen buds with bud cushion and scales was weighed accurately, ground with liquid nitrogen in a mill (ForceMill, Osaka Chemical Co., Osaka, Japan), and extracted twice with 20 mL of 80% ethanol. One milliliter of 1% pentaerythritol was added as an internal standard. The extracts were evaporated in a vacuum at 40 °C. Proteins were removed with a mixed cellulose ester filter, and the final volume was adjusted to 10 mL with deionized water. Polyphenols were removed by adding 3 mg polyvinyl-polypyrrolidone (PVPP), and purified through a membrane filter (0.45 µm). Twenty microliters of extract solution was injected into a high-performance liquid chromatography (HPLC) apparatus fitted with a packed column (SCR-101C, Shimadzu Co., Kyoto, Japan) and refractive index detector (RI-8000, Toyo Soda Inc., Tokyo, Japan). The equipment was set at a flow rate of 0.8 $mL \cdot min^{-1}$, column temperature of 80 °C, and ultrapure water (18 mΩ) was utilized as the mobile phase.

For starch quantification, the pellet remaining after ethanol extraction for sugar determination was oven dried overnight at 60 °C. Approximately 100 mg was weighed accurately and put in a glass test tube. It was assayed by the amyloglucosidase/

α-amylase method (AACC method 76-13, Megazyme International Ltd., Wicklow, Ireland). After mixed sample with 0.5 mL of 80% ethanol, tube was stirred and 3mL of thermo stable α-amilase diluted in 3-(morpholin-4-yl) propane-1-sulfonic acid (MOPS) buffer was added. After incubated in boiling water for 6 min, 4 mL of sodium acetate buffer and 0.1 mL of amyloglucosidase solution was added. Incubation in a 50 °C water bath was followed by adjustment of volume to 10 mL and centrifuged. Three mL of glucose determination reagent (GOPOD reagent) was added before incubated for 20min in a 50 °C water bath. D-glucose and water were used as a standard and blank solution, respectively. Absorbance at 510 nm was determined by UV/VIS spectrophotometer (V-550, Jasco Co., Tokyo, Japan).

4.3. Results

Sugars detected in mixed buds of Japanese pear were glucose, fructose, stachyose, raffinose, sucrose, and sorbitol. Contents of glucose and fructose were pooled and expressed as hexose, and stachyose and raffinose contents were presented as oligosaccharide.

Sucrose concentrations showed little decrease in buds of the first season (2005-2006), whereas an accentuated decrease was observed during heat accumulation of 2008-2009 (forth season) in Treatments 1 and 3 (Fig. 24A). Concentration of oligosaccharides was higher in 2008-2009 season than 2005-2006 in all treatments, although difference was not great in Treatment 3 (Fig. 24B). Hexose concentrations increased during dormancy in all treatments of all seasons, except in Treatment 2 of 2008-2009 season. Sorbitol was the most abundant sugar in dormant buds (Fig. 24D). In 2008-2009 (after four seasons under mild winter conditions) sorbitol peaked at the end of chilling treatment (600 CH), and decreased during heating treatment. In contrast, during the first season it peaked during heating treatment. Variations in total sugar concentration (Fig. 24E) were lower during 2005-2006 season than 2008-2009 season. Starch concentration increased slightly during chilling and heating in Treatments 1 and 2 of 2005-2006 season, but decreased toward the end of heating in Treatment 3 (Fig. 24F). 2008-2009 season gave a reduction in starch concentration from the beginning to the middle (4000 GDH) of heat accumulation in all treatments.

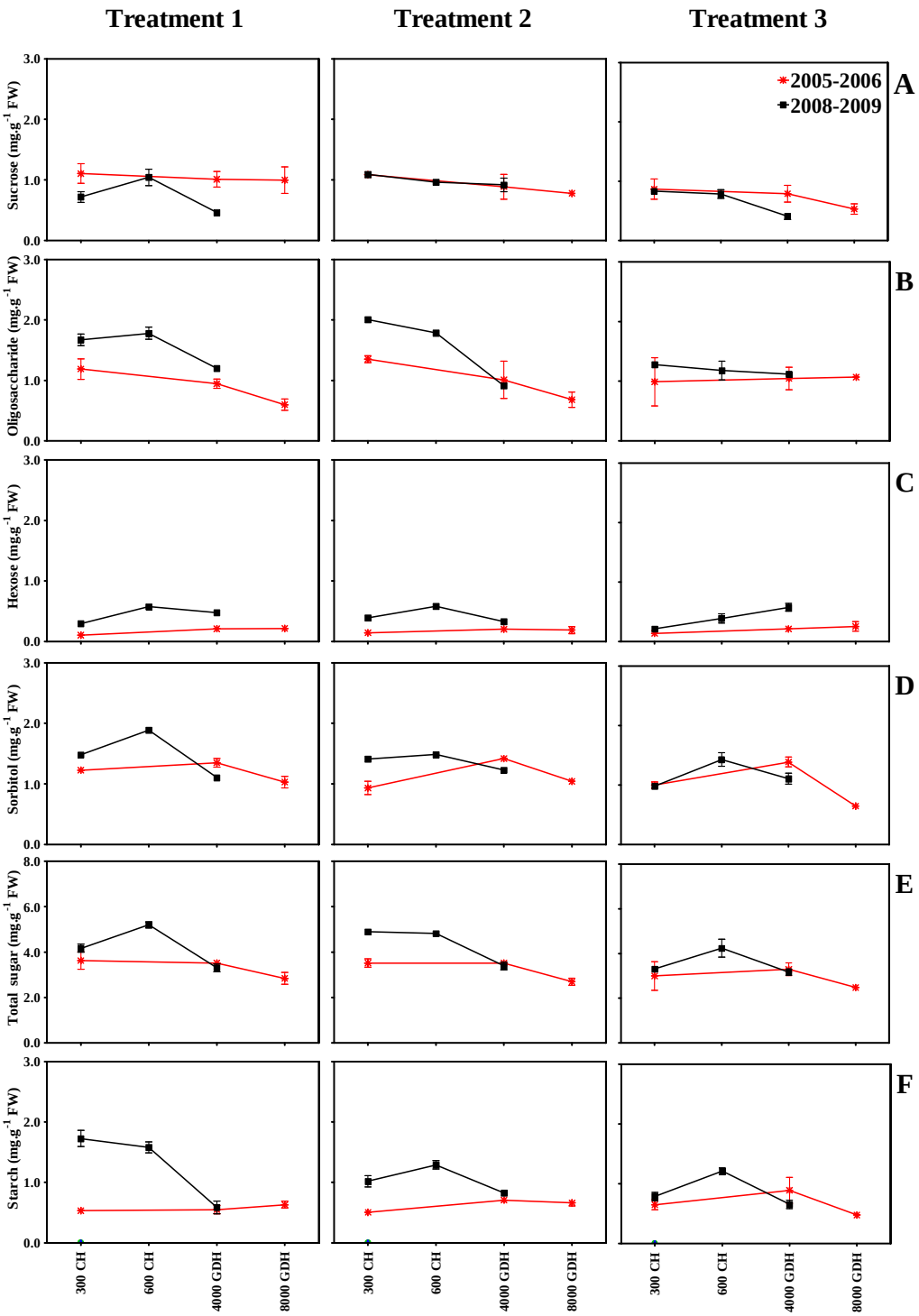

Fig. 24. Concentrations of sucrose (A), oligosaccharide (B), hexose (C), sorbitol (D), total sugar (E), and starch (F) in mixed buds of potted 'Housui' Japanese pear plants grown in each of three treatments. The experiment was conducted during four consecutive seasons, from 2005-2006 until 2008-2009. Samplings and extractions were done only in the first (2005-2006) and forth seasons (2008-2009), except at 600 CH in 2005-2006 (not analyzed). Means ± SE (n = 3 assays). CH, chilling hours; GDH, growing degree hours.

4.4. Discussion

Samples were collected at the middle of chilling accumulation (300 CH), transition from chilling to heating accumulation (600 CH), theoretical middle of heating accumulation (4000 GDH), and just before flowering (8000 GDH), in order to verify the variations on carbohydrate concentrations in lateral buds of 'Housui' Japanese pear. In 2008-2009 season samples were not taken at 8000 GDH because the plants had already flowered.

Peaks of carbohydrate concentrations during chilling accumulation in Treatment 1 were generally higher than those of cold-deprived plants, more so in 2008-2009 season (after four consecutive winters of low chilling accumulation), except for sucrose. Delays to initiate the chilling accumulation caused small fluctuations between the start and end of dormancy stage in carbohydrate concentrations. This result is similar to that of Leite et al. (2006), who found small variations in concentrations of several carbohydrates in peach floral buds under cold deprivation condition during dormancy.

During flower bud formation (June to September), Ito et al. (2002) observed differences in the activity of sugar-catabolizing enzymes between buds of two cultivars of Japanese pear. Marquat et al. (1999) showed that single-node peach buds have low sugar absorption potential during endodormancy, but increased potential at dormancy release. Bonhomme et al. (2005) reported that starch–sucrose interconversion did not occur in peach buds grown under NC or cold-deprived condition during dormancy. Under cold-deprived condition they also found an abrupt increase in carbohydrate concentrations near flowering stage in bud cushions and scales of floral buds, a slight increase in scales of leaf primordia and floral and vegetative primordia, and a continuous decrease in stems. We found that starch and sucrose concentrations showed variations during endo- and ecodormancy and an abrupt reduction just before flowering.

This may be explained by differences in absorption potential within mixed buds and nearby structures. Another important point is variations in numbers of floral primordia and inflorescences, as observed in previous chapter, which can affect carbohydrate metabolism, competition, and distribution, as a consequence of low chilling accumulation conditions.

Studies on plant sugar response are complicated by the fact that plants respond by several pathways. Sugars have been postulated to regulate synthesis and/or transport of phytohormones, to regulate expression of some components of phytohormone-response pathways (Gibson, 2004), and to regulate expression of specific genes (Sturm and Tang, 1999). On the other hand, Ito et al. (2004) suggested a possible involvement of plant hormones in the regulation of sugar metabolism. The oligosaccharides raffinose and stachyose are especially associated with cold hardiness and dormancy, and sucrose enhances both cold hardiness and desiccation tolerance in woody plants (Stushnoff et al., 1997). Changes in starch content (degradation) have also been implicated in deacclimatization or reduction in hardiness levels (Kalberer et al., 2006).

Soluble carbohydrates were responsible to provide a supply of energy and substrates for the early growth of shoots in spring (Yoshioka et al., 1988). And the trophic hypothesis asserts that bud break capacity is reflected in the ability to buds to acquire and use nutrients (Gendraud and Pétel, 1990).

Bonhomme et al. (2005) showed that under cold deprived conditions floral primordia abortion in peach buds occurred as a consequence of their incapacity to utilize available carbohydrates. These authors found a block on carbohydrate importation under cold deprived condition and a strong diversion towards structures close to primordia. High concentrations of carbohydrates in plants grown over consecutive seasons under low chilling accumulation conditions may have been related

to the source–sink imbalance that causes floral primordia abortion and alterations in growth pattern from the beginning of the hardening period.

It can be suggested that conditions of mild winters which ‘Housui’ potted plants were submitted in this experiment resulted in low levels available energy as a consequence of block in carbohydrate supply, which is necessary for metabolic processes, resulting in necrosis of floral primordia.

Chapter 5

Water dynamics in ‘Housui’ Japanese pear buds grown under mild winter conditions

5.1. Introduction

Dormancy in plants is an essential event to survive under unfavorable conditions, and it is closely related to changes on water movement (Welling and Palva, 2006). Water dynamics were assessed by using the nuclear magnetic resonance (NMR) spectroscopy in dormant buds of apple (Faust et al., 1991, Liu et al., 1993) and peach (Sugiura et al., 1995; Erez et al., 1998; Yooyongwech et al., 2008a). Magnetic resonance imaging (MRI) provides information on morphology and water dynamics, by the characterization of water status, in tissues or organs, allowing non-destructive (Van der Torn et al., 2000). The traditional method to assess water status is based on measurements on the proton relaxation times (T_2), and relatively high T_2 values indicate mobile of free water, while low values characterize presence of bound water (Erez et al., 1998; Yooyongwech et al., 2008a). While the values of T_2 are intrinsic and non-arbitrary, the proton density (PD) predominantly represents water molecules which were not incorporated to macromolecules (Van der Toorn et al., 2000).

However, there were few studies in Japanese pear relating water dynamics and a possible correlation with morphological changes, such as inflorescence duplication, on buds of whole plants growing under conditions of mild winter. We hypothesized that mild winter conditions promote an abnormal movement of water in dormant buds, resulting in incidence of floral primordia abortion. We also hypothesized that the time at which the low chilling accumulation is initiated over several seasons is related to

changes in dormancy progression and the occurrence of abortion in the mixed buds of Japanese pear.

The objective of this study was to study the effect of delayed mild winter conditions and consecutive seasons on water dynamics and analyzed a possible correlation with the floral primordia abortion on mixed buds of ‘Housui’ Japanese pear.

5.2. Materials and methods

One-year-old plants of the Japanese pear cultivar ‘Housui’ grafted on *Pyrus pyrifolia* were obtained from a commercial nursery. Each was transplanted into a 30-cm-diameter plastic pot in April 2005 and grown at the Agricultural and Forestry Research Center, University of Tsukuba, Japan (36° N, 140° E). Once each during summer and winter, current season shoots were reoriented to the horizontal position to promote floral bud formation (Ito et al., 2004). Opened flowers were not pollinated artificially and occasional fruits were thinned manually every season. At the sampling of dormant bud, a vegetative bud localized in a lower portion of shoot was kept in order to promote the development of new shoot during the next growing season. Each group of five plants was submitted to two months of cold deprivation before exposure to 600 CH under tow different regimes of seasons, according to Fig. 25A: for either a single season of 2008-09 (SS) or four consecutive seasons, from 2005-06 to 2008-09 (CS). After exposure to 600 CH (SS and CS), potted plants were transferred into a greenhouse for heating accumulation until flowering.

MRI measurements were made in 2008-2009. Three lateral buds with bud cushions were collected randomly from current season’s shoots (1-year-old, excluding terminal buds) of Japanese pear ‘Housui’ (Fig. 25B). Samples were taken from SS and CS after 1 month of cold deprivation (December); at the end of cold deprivation treatment (January) (SS only); after accumulate 600 CH and just before transfer to the greenhouse (February); and just before ecodormancy release (March). Samples from potted plants kept at all seasons under NC were taken in the middle of endodormancy (December); during the transition phase from endo- to ecodormancy (January), middle (February), and end of ecodormancy (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 ≈21 °C (Fig. 26A). Magnetic resonance (MR) 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. 26B, C). Morphological images and 32 sequential echo images in longitudinal sections of mixed 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 × 15 mm^2 or 18 × 15 mm^2, and the slice thickness of 0.5 mm. The sequential echo images were obtained with the repletion 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; Fig. 27A) of floral primordia (Fig. 27B), bud base (Fig. 27C), and whole bud (Fig. 27D) were determined manually in a longitudinal section at the central portion of mixed bud (that presented the highest bud base) and T_2 values of each ROI was also calculated. ROI of bud scales were obtained by subtracting floral primordia and bud base from whole bud (Fig. 27E). There were three replications in each analysis.

Phenological observations were made immediately after MRI measurement, in order to determine the incidence and severity of floral primordia abortion in mixed buds of ‘Housui’ Japanese pear and occurrence of multiple inflorescences. After remove all 12 scales, observations were made under a light microscope (SZX-12, Olympus Co., Tokyo, Japan) and a digital microscope (VHX-900, Keyence Co., Osaka, Japan). The same arbitrary scale described in Chapter 3 (Fig. 19) was used to determine the severity

of floral primordia abortion. Data were presented as a percentage of total primordia, obtained from the three lateral buds used for MRI analysis. Other five mixed buds were collected and fixed in FAA (10% formaldehyde, 5% acetic acid, and 85% ethanol solution, v/v) for morphological observations with scanning electron microscope (SEM; VE-8800, Keyence Co., Osaka, Japan), after removing all twelve scales.

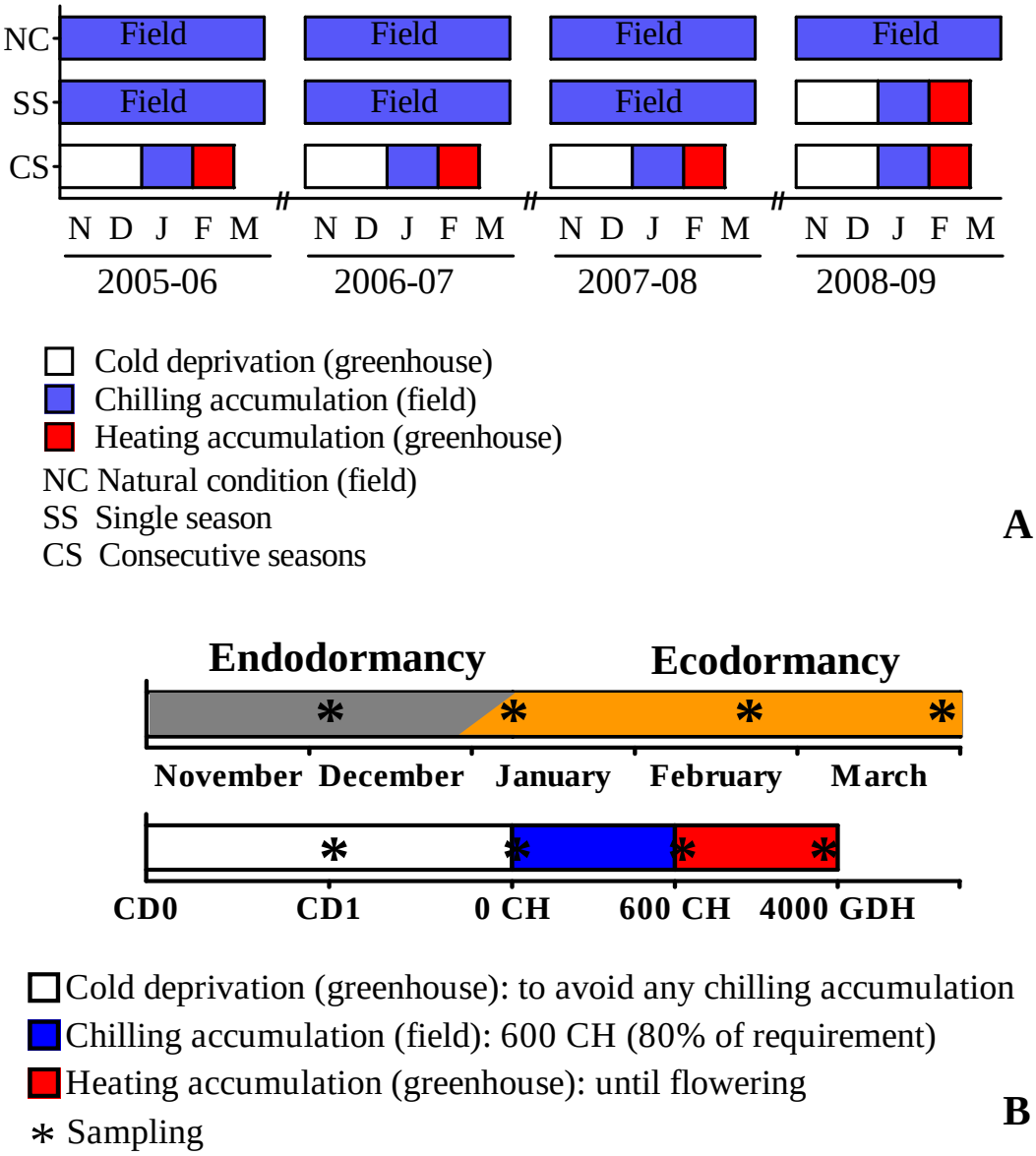

Fig. 25. Experimental design for MRI measurements. For chilling accumulation, potted plants were moved into the field and kept there until they had been subjected to 600 h below 7.2 °C. NC materials consisted of potted plants of same age kept at all times under natural field conditions, and samples were taken in the middle of endodormancy (December), at the beginning (January), middle (February), and end (March) of ecodormancy. The experiment was carried out in 2008-2009 season, and utilized plants growing under two regimes: only 2008-2009 season (SS) and four consecutive seasons from 2005-2006 to 2008-09 onward are marked as CS (A). Samplings and measurements were done according to (B).

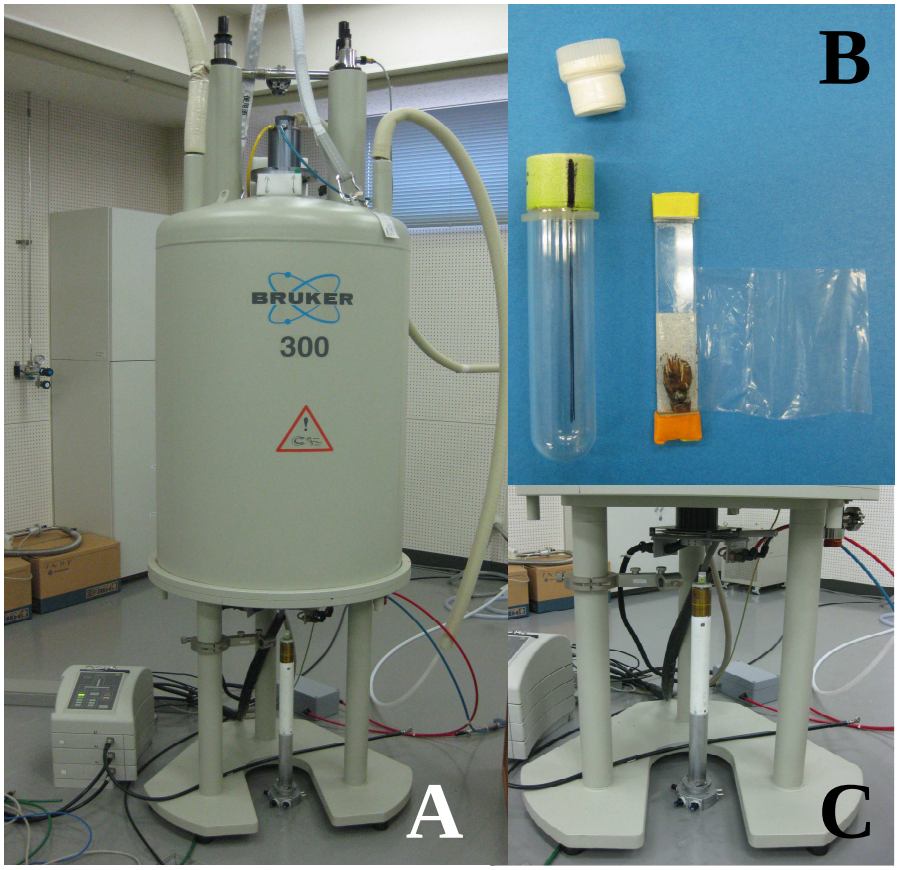

Fig. 26. Apparatus and preparation for water dynamic measurement by MRI: tank of NMR spectrometer (DRX 300WB) equipped with a 7.1 Tesla magnetic field tank (A); homemade plastic holder with sampled bud, which were putted in a 15 mm glass tube (B); MRI sample holder set in an active column of the NMR coil (C).

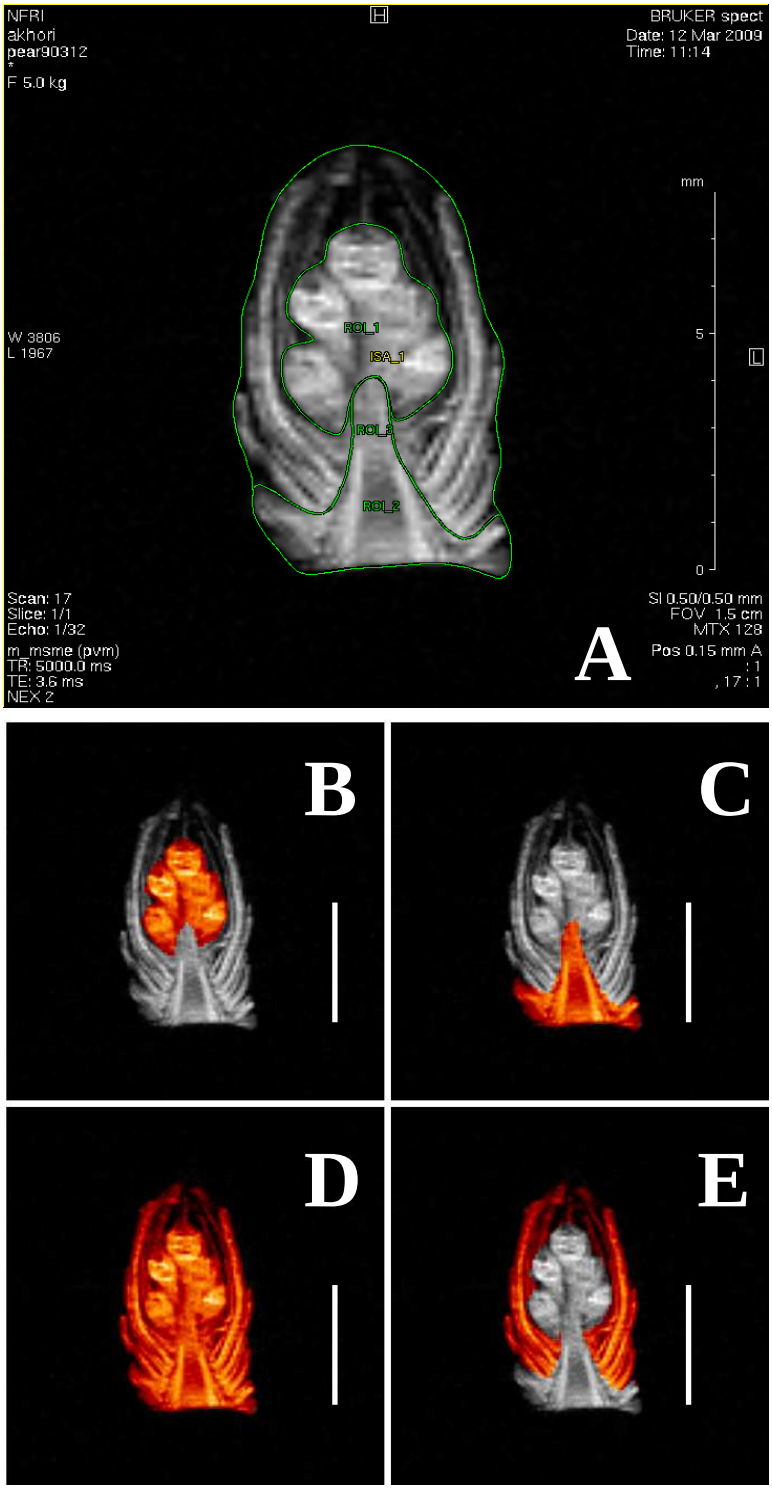

Fig. 27. MR morphological image of Japanese pear bud with regions of interest (ROI) determined manually (A); ROI of grouped floral primordia (B); ROI of bud base (C); ROI of whole bud (D); ROI of bud scales obtained by subtracting floral primordia and bud base from whole bud (E). The scale bars represent 5mm.

5.3. Results

Changes in T_2 values for the floral primordia, bud base, and whole bud were shown in Fig. 28. T_2 values of floral primordia increased considerably at the transition from endo- to ecodormancy stage (January) in buds grown under NC (Fig. 28A). Under both SS and CS, however, T_2 values of floral primordia increased only after initiated the heating accumulation (600 CH to 4000 GDH). These floral primordia showed an increase in primordia abortion after 1 month of cold deprivation, and by March normal primordia represented less than 50% under both SS and CS (Fig. 28B). NC buds had 100% normal floral primordia at all samplings. The bud base of plants under NC showed a gradual increase in T_2 values until the end of chilling accumulation (Fig. 28C). T_2 values in the bud base of buds under SS were lower than those of NC until after submitting plants to heating accumulation. After four CS mixed buds showed constant T_2 values until submitting plants to heating accumulation, when increased abruptly. Multiple inflorescences, an abnormality of the bud base, were observed under both regimes after 1 month under cold deprived conditions (Fig. 28D). Average T_2 values in whole bud increased gradually in plants kept under NC (Fig. 28E). In contrast, treated plants (SS and CS) had low values at the end of the chilling treatment (600 CH).

T_2 and PD maps (Figs. 29, 30) were almost entirely calculated in detected parts of the morphological images (Fig. 31). Water mobility, as determined by T_2 (Fig. 29), showed intermediate values (16 to 24 ms) in the floral primordia, bud base, and bud scales of NC on March. In contrast, SS (2008–09) at the end of chilling treatment (600 CH) had high T_2 values (over 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 (higher than 16 ms) in CS before flowering only in specific portions of bud base and scales near the bud base.

Relative amount of water, represented by PD maps (Fig. 30), gradually increased in the floral primordia of buds grown under 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 high (40-70 %) in the bud base, and high (until 80%) in the floral primordia. At 4000 GDH, PD in CS was higher in primordia and some specific portions of bud base than in the NC or SS.

Structural changes during dormancy until flowering were observed in morphological MR images of longitudinal sections at the central portion of 'Housui' mixed buds (Fig. 31). Mixed buds of plants grown under NC had single inflorescence during all dormancy stages, with a high and uniform signal intensity of scales and floral primordia. Bud scale and floral primordia configurations were remarkably different among buds under NC, SS (a single season of 2008–09), and CS (four consecutive seasons), from 2005–06 to 2008–09. Scales of buds under NC were sharply observed in MR images over all dormant stages. However, under both SS and CS, they were detected partially especially near the bud base. Inflorescence duplication was observed since chilling accumulation (0 CH) in SS. At this stage, signals from scales could not be detected. From the end of chilling accumulation primordia signals were not uniform, and numbers of primordia and inflorescences varied among samples under both SS and CS. CS showed also variations on primordia size especially during heating accumulation (600 CH and 4000 GDH).

Morphological comparison between buds of plants growing under CS and NC was done (Fig. 32). MR images showed some abnormalities in mixed buds, such as partially necrosed primordia (Fig. 32A, C) and multiple inflorescences when grown under CS (Fig. 32A, C, D, E). Under NC, however, normal number of floral primordia

had similar size, bud scales were distinctly detected, and only a single inflorescence was observed (Fig. 32B) even just before flowering (Fig. 32F). Differences on bud scales between mixed buds of plants grown under natural conditions and mild winter conditions could be observed also in photographs obtained from digital microscope (Fig. 33).

Photographs obtained from SEM and digital microscope showed morphological and phenological differences among buds growing under NC and artificial conditions during dormancy of both SS and CS (Fig. 34). Morphological differences among NC, SS, and CS during November were not found in SEM images. However, after one month under cold deprivation (December), SS and CS buds developed a new inflorescence, whereas the bud base of NC remained in normal conditions. Digital photographs obtained in February (after accumulated 600 CH) in both SS and CS showed a progression of floral primordia abortion and development of new inflorescences in different levels. Reduced number of opened flowers and variations on length of pedicels were observed at flowering in both SS and CS.

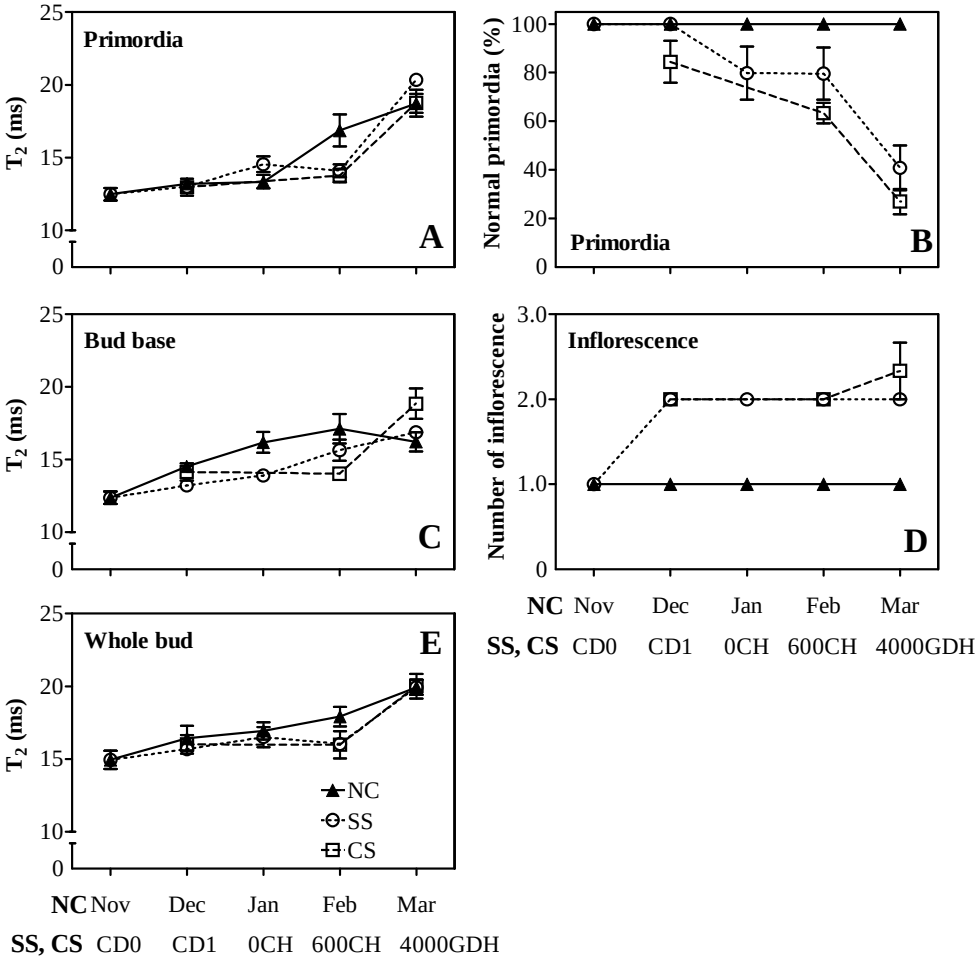

Fig. 28. Averages of T_2 values in ms (left column) of floral primordia (A), bud base (C), and whole bud (E), measured in mixed buds of 'Housui' Japanese pear grown under natural conditions (NC), one season of 2 months of cold deprivation before chilling treatment (SS), and four consecutive seasons of the same cold deprivation before chilling (CS). Percentages of normal primordia (grade 0) in analyzed buds (B) and number of inflorescences (D) are also shown. All samples were taken in 2008-2009. Means ± SE (n = 3). In plants grown under natural conditions (control), endodormancy was released in January. In the same month, chilling accumulation began in treated plants under field conditions (SS and CS). The ultimate measurement was taken at the end of March, before flowering.

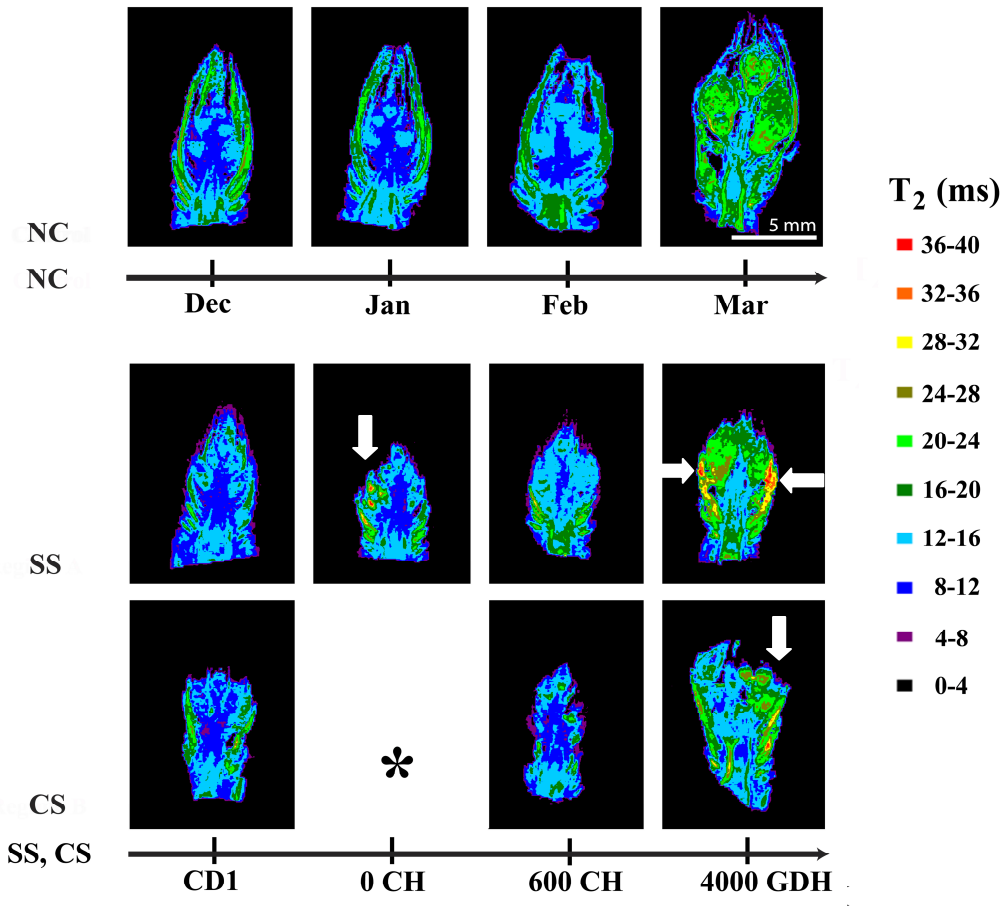

Fig. 29. Transverse relaxation time maps of mixed buds of Japanese pear 'Housui', obtained by magnetic resonance imaging. Control buds, obtained from plants kept always under field conditions (NC) during dormancy, were analyzed in mid-endodormancy (December), at the beginning of ecodormancy (January), in mid-ecodormancy (February), and at the end of ecodormancy (March) (top line in each panel). Mixed buds obtained from plants subjected to cold deprivation for 2 months followed by chilling over one season (2008-2009) are marked as SS (center line). Mixed buds of plants grown under similar cold deprivation-chilling conditions for four consecutive seasons from 2005-2006 onward are marked as CS (bottom line), except in January (not analyzed). All samples for MRI measurement were taken in 2008-2009. The arrows indicate significant changes on water dynamics. Asterisk: not analyzed.

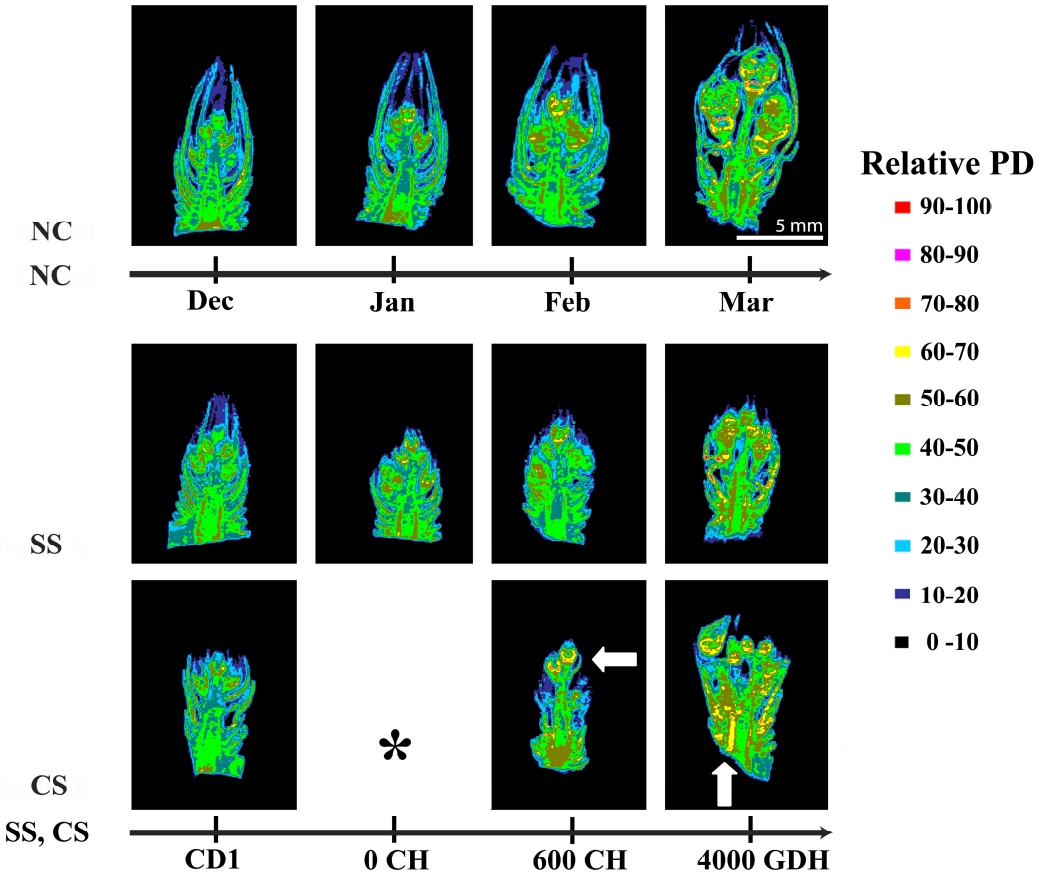

Fig. 30. Proton density (PD) maps of mixed buds of Japanese pear 'Housui', obtained by magnetic resonance imaging. NC buds, obtained from plants kept always under field conditions during dormancy, were analyzed in mid-endodormancy (December), at the beginning of ecodormancy (January), in mid-ecodormancy (February), and at the end of ecodormancy (March) (top line in each panel). Mixed buds obtained from plants subjected to cold deprivation for 2 months followed by chilling over one season (2008-2009) are marked as SS (center line). Mixed buds of plants grown under similar cold deprivation-chilling conditions for four consecutive seasons from 2005-2006 onward are marked as CS (bottom line), except in January (not analyzed). All samples for MRI measurement were taken in 2008-2009. The arrows indicate significant changes on water dynamics. Asterisk: not analyzed.

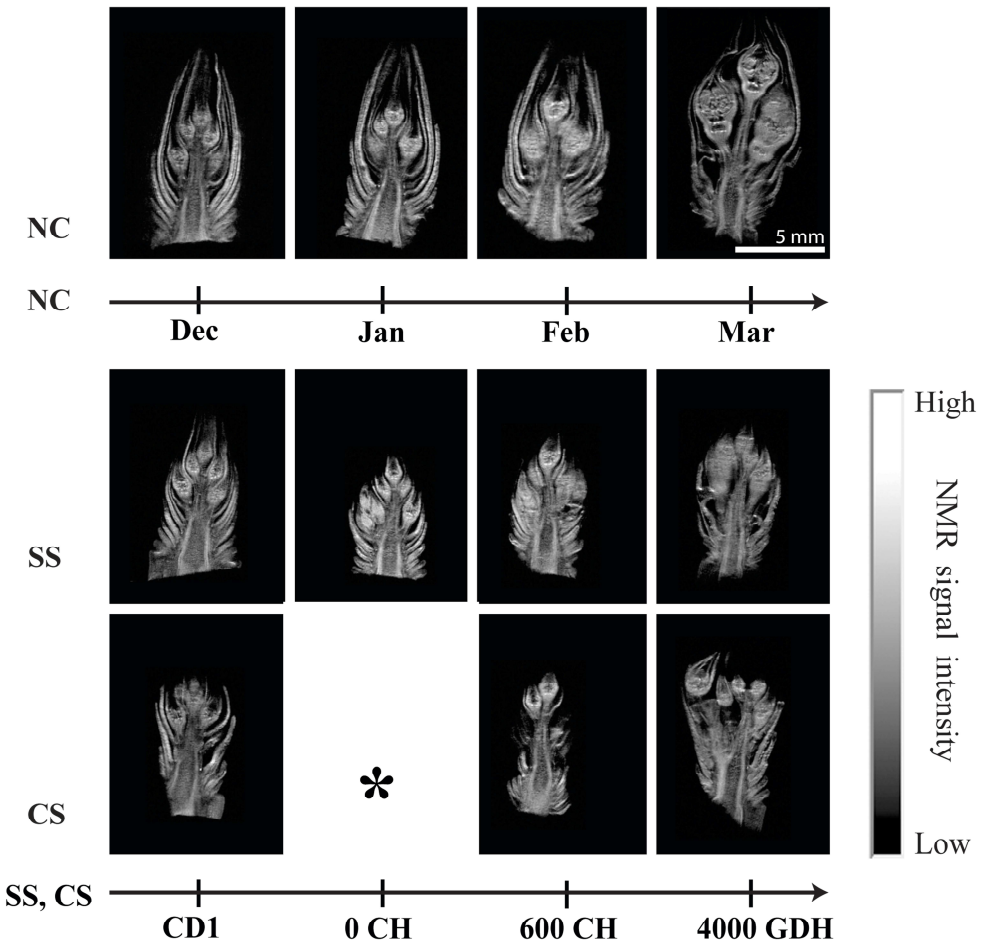

Fig. 31. Morphological images of mixed buds of Japanese pear 'Housui', obtained by magnetic resonance imaging. Control buds (NC), obtained from plants kept always under field conditions during dormancy, were analyzed in mid-endodormancy (December), at the beginning of ecodormancy (January), in mid-ecodormancy (February), and at the end of ecodormancy (March) (top line in each panel). Mixed buds obtained from plants subjected to cold deprivation for 2 months followed by chilling over one season (2008-2009) are marked as SS (center line). Mixed buds of plants grown under similar cold deprivation-chilling conditions for four consecutive seasons from 2005-2006 onward are marked as CS (bottom line), except in January (not analyzed). All samples for MRI measurement were taken in 2008-2009. Asterisk: not analyzed.

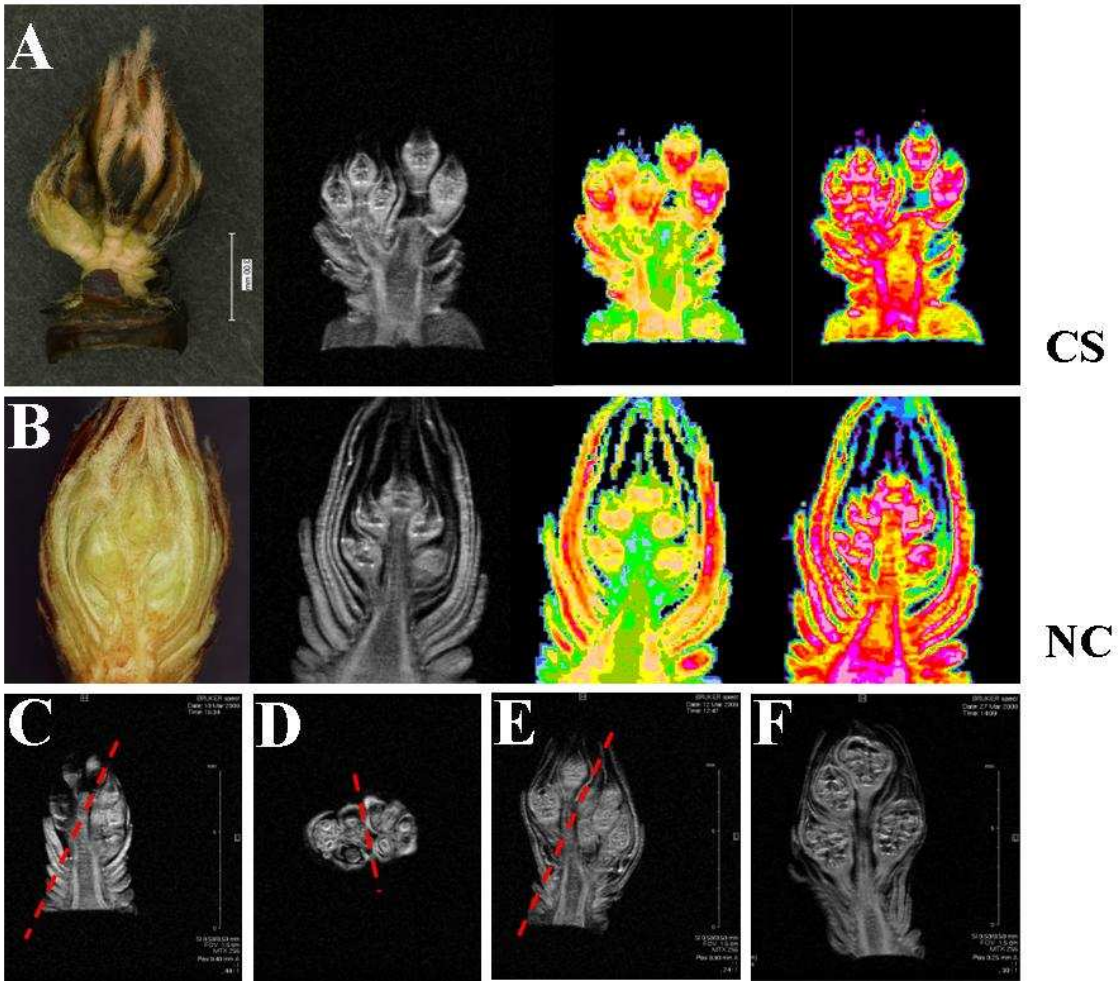

Fig. 32. Some of abnormalities found in the mixed buds of 'Housui' Japanese pear during the experiment in comparison to normal buds: (from left to right) photograph obtained by digital microscope, morphological image, T_2 relaxation time map and relative proton density maps obtained by MRI of a partially necrosed bud of CS during heating accumulation (A) and a normal bud collected from control plants on December (B); longitudinal and axial MR image of SS collected on February showing partially necrosed primordia and the development of multiple inflorescences, separated by the dotted line (C and D, respectively); longitudinal MR image of apparently normal bud sampled from plants grown under SS on March, with two inflorescences, separated by dotted line (E); longitudinal MR image of normal primordia sampled from plants under NC on March (F). The scale bars represent 3 mm in A and 8mm in C, E, and F.

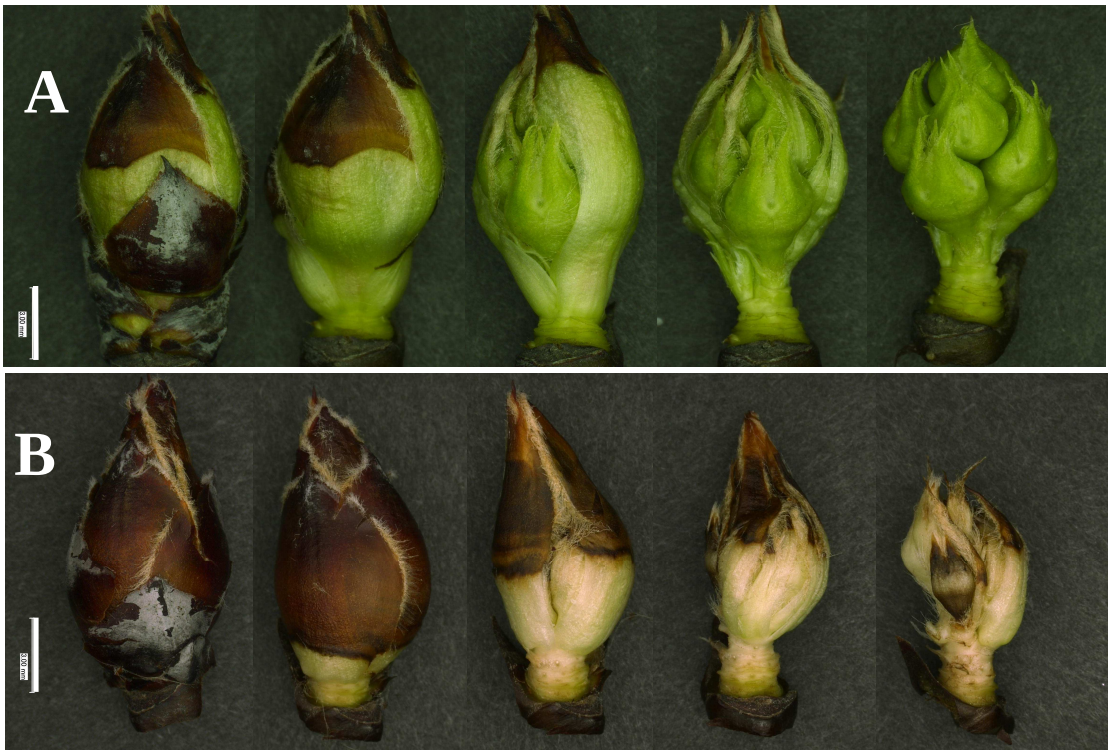

Fig. 33. Photographs obtained from digital microscope of mixed buds sampled on February from plants grown under natural conditions (A) and a single season of 2008-09 under artificial mild winter conditions (B). From left to right: normal bud; after removed 4 scales; after removed 8 scales; after removed all 12 scales; after removed inner scales. The scale bars represent 3 mm.

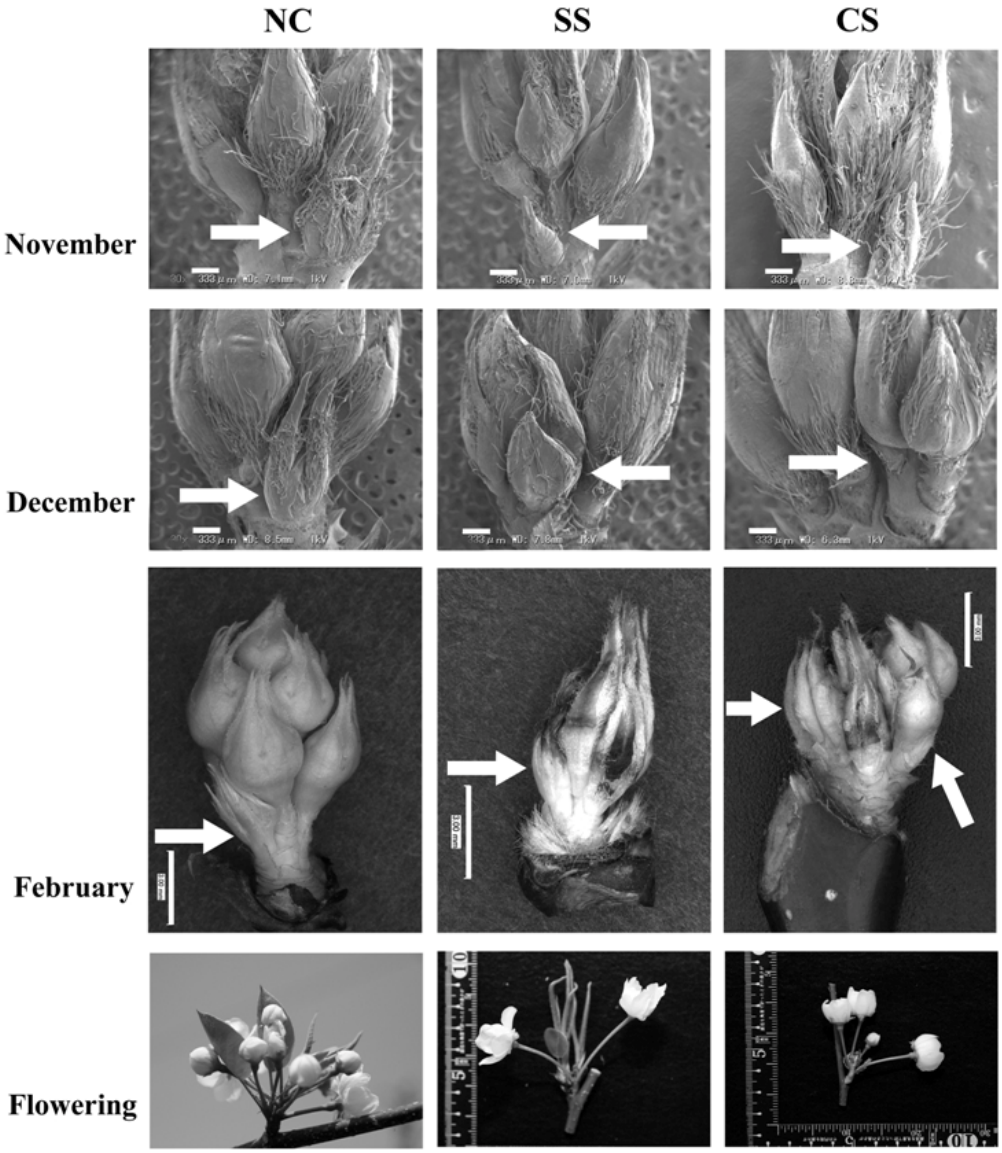

Fig. 34. Morphological and phenological images obtained during the experiment 2. Microimages of mixed buds obtained from SEM, indicating a normal development before any chilling accumulation (November), and an inflorescence duplication observed after one month of cold deprivation conditions in SS and CS (December). Photographs of buds during the transition from chilling to heating accumulation (February) in treated plants (SS and CS), obtained from digital microscope, showing some primordia abortion. The arrows indicate the leaf primordia in NC buds and inflorescence duplication in both SS and CS. Photographs at flowering in mixed buds indicate few numbers of opened flowers in both SS and CS. The scale bars represent 333 µm in SEM images (November and December) and 3 mm in digital microscope images (February).

5.4. Discussion

The state of water plays a critical role during the dormancy stage, and bound water was coupled to the endodormancy (Faust et al., 1991, 1997; Liu et al., 1993). A decrease in osmotic potential of tissues during cellular dehydration, to provide protection against the cold, results from the accumulation of soluble sugars and other compounds in cells (Ingram and Bartels, 1996). In this experiment, plants grown under conditions of sufficient chilling accumulation (NC) showed a gradual increase of free water (high T_2 values) in the whole bud, indicating a normal process of dormancy release (Fig. 28). However, plants grown under conditions of low and delayed chilling accumulation (SS and CS) kept high levels of bound water (low T_2 values) in the floral primordia (Fig. 28A), bud base (Fig. 28C), and whole bud (Fig. 28E) until the end of chilling treatment (600 CH), and an abrupt change to free-state water during the afterward heating accumulation period was observed (except for SS of bud base). Previous studies in peach suggested a correlation between the level of bound water and cold hardiness (Erez et al., 1998) through the activation of dehydrin, a hydrophilic protein (Faust et al., 1997). The difference in T_2 values between treated and NC buds at the end of chilling accumulation (600 CH) is probably resulted from reduction on percentage of normal primordia (Fig. 28B) occurred during cold deprivation in buds grown under mild winter conditions (SS and CS). Differences in T_2 signal intensity with time (Fig. 29) as a consequence of environmental temperature variation were detected in buds of plants grown for only one season under SS, whereas response under CS tended to occur slowly and was specific to some portion, 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. 30) showed a low water content in the bud scales

under SS and CS, and a high water content in specific portions of the floral primordia (600 CH) and bud base (4000 GDH) under CS. As discussed in previous chapter, a possible loss of functionality in vascular connections between the bud base and primordia under mild winter conditions resulted in progression of floral primordia abortion (Fig. 28B). From this irregular distribution of water, which resulted in high water mobility in specific portions of bud base, thereafter caused the inflorescence duplication (Fig. 28D) as a consequence of consumption of reserve substances.

Scales, which are modified leaves responsible for enclosing and protecting buds of perennial plants, had high T_2 (Fig. 29) and PD (Fig. 30) values in the NC buds during all dormancy stages of our experiment. In contrast, the low water mobility (T_2) and low water content (PD) detected in the scales of plants exposed to cold deprived conditions before chilling might have been related to an increase on sensibility of the floral primordia to external temperature oscillations. Photographs comparing buds of plants grown under NC and mild winter conditions (SS) showed a clear difference in conditions of bud scales (Fig. 32). Yooyongwech et al. (2008b) reported that oscillation temperature conditions accelerate water movement in peach buds, but promoted an irregular bud growth.

Low water mobility (low T_2) and low relative water content (PD) detected in scales in the MR images might have been related to increased sensibility of floral primordia to temperature oscillations and/or reduced protection from freezing during winter. Such abnormalities on bud scales were observed after one month of cold deprivation (December) in samples collected from trees grown under SS. Under CS samples collected at same date showed more accentuated reduction on signal intensity in bud scales compared to SS, indicating an effect of consecutive seasons of mild winter on reduction of water mobility and water content in this organ.

Buds under NC had all of floral primordia developed normally, only a single inflorescence was observed during the dormant period, and all primordia sprouted at flowering. However, plants grown under mild winter conditions developed a second inflorescence, resulting in more than 8 floral primordia per bud in average (data not shown).

Chapter 6

General discussion

6.1. Temperature and dormancy progression

From 2005 to 2009, temperatures under field conditions of Tsukuba, Japan, varied among seasons during period of dormancy stage (November to March). 2005-2006 had the lowest weekly minimum temperature during all period. The subsequent 2006-2007 season showed the highest average temperature in greater part of time, whereas 2007-2008 showed an abrupt decrease from middle of January and a rapid increase at the end of next month. 2008-2009 season had a great fluctuation on average temperature, but it was relatively warm (Fig. 13).

By submitting potted plants to natural start of chilling accumulation (Treatment 1), approximately two months were required in average to accumulate 600 CH during the four studied seasons. On the contrary, 36 and 32 days in average were necessary to accumulate same amount of chill when its accumulation started on December or January, respectively. Such differences occurred because of differences on field temperature: January has the lowest temperature of the season, and more hourly temperatures below 7.2 °C resulted in a rapid accumulation of chilling amount.

The amount of 600 CH, corresponding to 80% of theoretical chilling requirement of ‘Housui’ and ‘Kousui’ Japanese pear (Nishimoto et al., 1995), was possibly sufficient to release the endodormancy stage in all treatments and all seasons of study. In this study it was adopted the same amount of CH observed in Southern Brazil, where the Japanese pear production is faced with problems of dormancy progression and occurrence of floral bud abortion.

Other two mathematical models were used in this experiment: DVR model (Fig. 16) and CU model or Saitama method (Fig. 17). According to Sugiura and Honjo (1997b), endodormancy stage is considered to be released when DVI values reach 1.0, but we found several treatments where values below 1.0 were sufficient to release the endodormancy stage. As lateral buds of all treatments and all seasons released from dormancy under conditions of mild winter of this experiment, the best mathematical method to determine progression of endodormancy stage might be the CU model, proposed by Asano and Okuno (1990). However, both DVI and CU models for Japanese pear is not frequently used to calculate the chilling accumulation during dormancy of this fruit in Brazil. The chilling accumulation curves showed more clear difference among seasons, whereas CU and DVI curves were resembled (see Appendix 1).

After accumulate 600 CH, average of 20 to 25 days in a heated greenhouse were necessary to accumulate sufficient heating to release the ecodormancy stage and flowering in all treatments. A correlation between duration of chilling and heating accumulation was observed in this experiment: during warm seasons, longer chilling accumulation was followed by shorter heating accumulation, in comparison with cold seasons (Table 4). This inverse relationship was observed in previous studies in peaches (Citadin et al., 2001). A possible effect of high temperatures during warm winter seasons under field conditions needs to be considered, because it provokes increase of temperatures inside the greenhouse, where only the minimum temperature was controlled.

By keeping potted plants under cold deprived conditions before exposure to chilling temperatures (Treatments 2 and 3), an abrupt decrease of temperature occurred when they were transferred to field. Such situation did not occur under natural condition, even in mild winter climates of subtropics, but it was possible to detect the effect of

both delayed chilling accumulation and severity of chilling temperatures on dormancy progression. Variations on temperature pattern, as described above, may have resulted in different degrees of adaptation for cold temperatures (hardening), and consequently in abnormalities on dormancy progression.

Saure (1985) suggested that depth of dormancy under mild winter conditions might be more superficial when compared to plants growing under sufficient chilling accumulation conditions. It was observed that a few consecutive days of relatively high temperature during the chilling accumulation period in Southern Brazil can promote the dormancy release (flowering) in 'Housui' Japanese pear.

During the successive seasons of mild winter conditions, changes on dormancy induction and/or depth of dormancy may have occurred as a consequence of chilling accumulation below the requirement. Previous studies conducted in Japanese pear grown during several seasons under continuous high temperature (Rakngan et al., 1996) demonstrated phenological changes regarding of endogenous metabolism, which became progressively worse with time. It can be hypothesized that 'Housui' Japanese pear showed high sensibility and a rapid response to air temperature fluctuations, which resulted in adaptations of their physiological functions to conditions of mild winter.

We suggested a hypothetical scheme of dormancy progression of this experiment (Fig. 35). The CH accumulated were the same for all treatments in all seasons (600 CH), but the CU and DVI data indicated the highest amount of chill accumulated during the Treatment 1 and the lowest in Treatment 3, resulting in a more profound dormancy.

6.2. Floral primordia abortion and inflorescence duplication

Lateral mixed buds of current year shoots obtained from potted plants were observed during dormancy progression, and an arbitrary scale was adopted to determine the severity of floral primordia abortion. Among treatments, natural accumulation of chill (Treatment 1) resulted in the highest percentage of normal primordia, especially until middle of heating accumulation (4000 GDH). On the contrary, by keeping plants under cold deprivation before exposure to chilling temperatures resulted in the lowest percentage of normal primordia (Fig. 19). High incidence of primordia abortion in these treatments might be related to cold acclimatization or cold hardening, when an abrupt decrease of temperature occurred when they were transferred to field, resulting in plants less resistant to variations of extreme environmental conditions (chilling temperatures).

Morphological abnormalities on floral primordia were detected earlier and in higher levels than previous season until the third season (2007-2008). In the forth season, initial phases of cold deprivation and chilling accumulations presented higher percentage of normal primordia compared to previous seasons. These results indicate that ‘Housui’ became more adapted to delayed or mild winters after three seasons.

The most important Japanese pear cultivar in Japan, ‘Kousui’, showed lower incidence of floral primordia abortion in Brazil compared to ‘Housui’. However, production area of ‘Kousui’ in Brazil is not increasing because of high susceptibility to shoot blight and other diseases, small fruit size, among other factors. For comparison, ‘Kousui’ potted plants with same age were submitted to same temperature treatments, and the incidence and severity of floral primordia abortion were observed in this experiment (see Appendix 2). Even under artificial mild winter conditions, results showed lower percentage of floral primordia abortion in ‘Kousui’ when compared to ‘Housui’ in all treatments and seasons. It is possible that floral bud of ‘Kousui’ had

lower sensibility to external temperature fluctuations, which resulted in lower incidence of primordia abortion.

Under natural condition (field), ‘Housui’ showed green scales and primordia, a single inflorescence, and flowers with similar pedicel length (Fig. 23). By submitting plants to low chilling accumulation, however, we found abnormalities in buds at different levels: floral primordia abortion in different levels, buds completely necrosed, and buds with multiple inflorescences (Fig. 20). At flowering, plants growing under mild winter conditions showed differences on number of opened flowers, variations on pedicel length, and also flowers at different stages of development (Fig. 22).

6.3. Carbohydrate and water dynamics under mild winter conditions

Late start of chilling accumulation reduced fluctuations variations in carbohydrate concentrations between initial and final phase of dormancy (Fig. 24). Plants growing under mild winter conditions for several years gave higher concentrations of carbohydrates during the chilling accumulation period, except for sucrose, concentration of which was similar to, or lower than, that in the first season (2005-06). Low variations in carbohydrate concentration may have been related to the simultaneous absorption and consumption of carbohydrates for respiration and maintenance. A possible involvement of energy metabolism, such as ATP, ADP or NTP, need to be consider. Previous studies showed that under cold deprived conditions during dormancy resulted in low levels of nucleotides, which can be related to the incapacity of floral primordia to utilize available carbohydrates (Bonhomme et al., 2000a, b). It is possible that reductions on metabolic processes or synthesis of molecules, such as nucleotides, which provides energy needed to growth, are causes of floral primordia abortion. Bonhomme et al. (2005) found loss of functionality in vascular connections between the bud base of peach and primordia during the processes of dormancy release, resulting in a block of metabolic substances. It is possible that similar phenomenon occurred in mixed buds of Japanese pear grown under mild winter conditions; thereafter the duplication of inflorescence could be occurred due to consumption of reserve substances.

MRI measurements showed that scales had a high intensity of T_2 and PD signals in buds growing under NC during all dormancy stages of this experiment (Figs. 29, 30). In contrast, plants grown under low chilling accumulation conditions had similar T_2 values in floral primordia, bud base, and whole bud, although we observed increasing floral primordia abortion and inflorescence duplication in treated plants at the beginning

of cold deprivation (December). Differences in T_2 values of floral primordia between treated and control buds during February were probably resulted from the low percentage of normal primordia in buds grown under mild winter conditions (Fig. 28). Progression of floral primordia abortion and inflorescence duplication caused an irregular distribution of water, resulting in high water mobility and content in few healthy primordia present and some portions of bud base. Low water mobility (T_2) and relative water content (PD) in bud scales might have been related to increased sensibility of floral primordia to external temperature oscillations and/or reducing on protection from freezing temperatures during winter. Along with changes on water dynamics, some translocated substances such as carbohydrates, which were cited as affecting dormancy level and cold hardening in peaches (Durner and Gianfagna, 1991) might be involved on floral primordia abortion.

6.4. Possible sequence of events and methods to avoid the effect of mild winter conditions

Our findings suggested that dormancy progression in 'Housui' Japanese pear is highly affected by mild winter conditions. A possible sequence of events in lateral mixed buds of 'Housui' Japanese pear plants grown under mild winter conditions, such as observed in Brazil, New Zealand, and Thailand, were suggested according to Fig. 36:

1. mild winter conditions cause reductions on water mobility (T_2) and relative water content (PD) in bud scales;
2. an increase of sensibility to external temperature fluctuations occurs in floral primordia;
3. alterations on water dynamics results in partitioning in specific portions of buds;
4. a possible incapacity to utilize available carbohydrates (low variations on their concentrations);
5. reduction of cold resistance (hardening level);
6. an unavailability (possible block) of water and other functional carbohydrates in the floral primordia resulted in their necrosis (abortion)
7. possible reallocation of water and other functional carbohydrates, which might be initially translocated to the floral primordia, for development of new inflorescences (probably from a leaf primordia) in mixed buds during dormancy progression (Fig. 37);
8. at flowering, buds had irregular number of flowers, which developed at different stages, and flowers with variations in pedicel length

Possible techniques, to reduce the effect of mild winters on dormancy progression and consequently reduction of "floral primordia abortion" incidence on

‘Housui’ Japanese pear can be adopted by growers:

1. reorientation of shoots to the horizontal position: to accelerate the floral bud formation by changing some plant hormone activities, resulting in a possible increase of sink capacity and thereby stimulate bud growth (Ito et al, 2002, 2004);
2. increase on percentage of old shoots with spur buds: ‘Housui’ showed high levels of bud loss on young wood of old trees in our experiment and other reports (Klinac and Geddes, 1995, Petri et al., 2002);
3. reduction on soil temperature during dormancy stage: previous studies reported the influence of root temperature on dormancy progression (Young et al., 1987; O’Hare, 2004);
4. improve the soil water management: it was observed lower incidence of floral primordia abortion in ‘Housui’ Japanese pear grown in soil with high moisture content under mild winter conditions of Southern Brazil;
5. determination of dormancy progression: test of CU model (Saitama method) under mild winter conditions;

More detailed studies will be needed in order to find methods to avoid the occurrence floral primordia abortion of Japanese pear grown under mild winter conditions:

1. studies focusing water management: as water dynamics seems to be important and closely related to floral primordia abortion and other physiological disturbs, further studies on moisture content in soil under mild winter conditions of Southern Brazil are needed;
2. a comparative study between mixed buds of current season’s shoot and spur buds of old shoot: in order to observe any physiological differences between these two

kinds of buds which contain floral primordia;

3. a deeper study on carbohydrate metabolism and their functions: in order to observe availability in different portions inside the mixed bud, including some enzymatic activity;
4. create an appropriate model for determination of dormancy progression: based on CU model (Saitama method), in order to propose a mathematical model adapted to mild winter conditions;
5. studies on morphology of mixed buds: especially on causes of modification from leaf primordia to a new inflorescence;
6. find a cultivar adapted to mild winter conditions: genotypic and phenotypic characteristics, including new cultivation techniques, need to be considered under such environmental conditions.

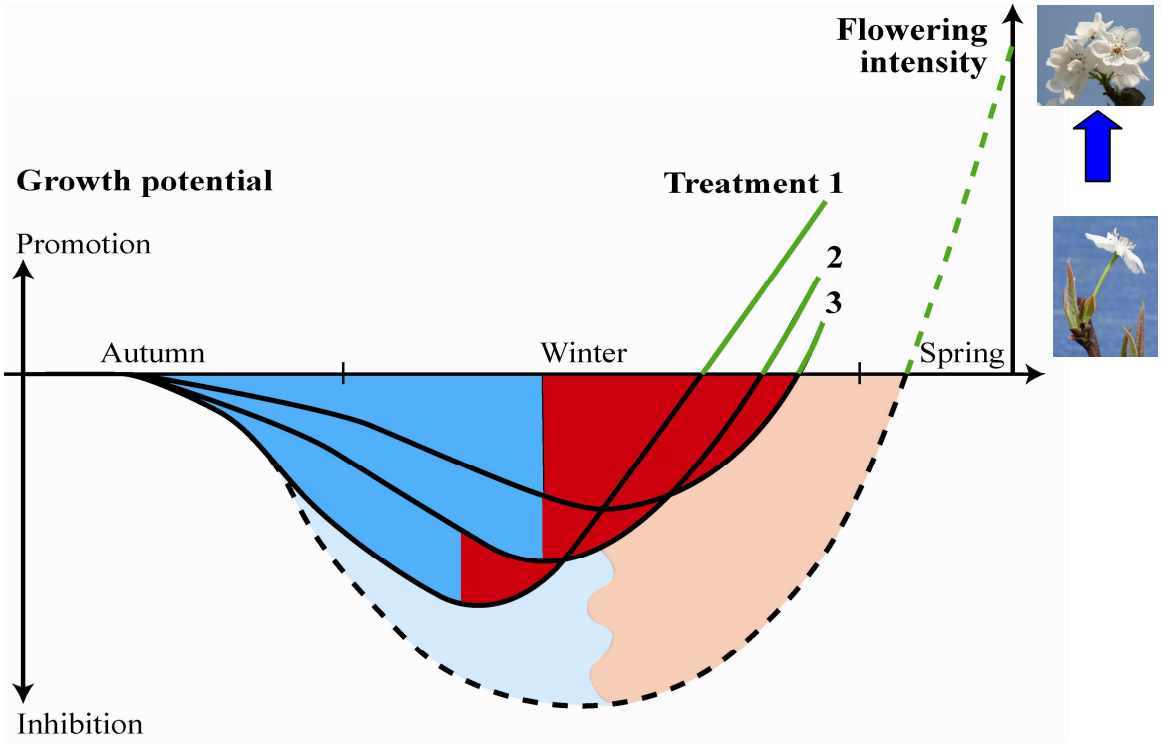

Fig. 35. Hypothetical scheme of dormancy progression of 'Housui' Japanese pear growing under mild winter conditions (low chilling accumulation). Numbers 1, 2, and 3 indicates the possible depth of dormancy of Treatment 1, Treatment 2, and Treatment 3, respectively. Green lines indicate flowering status of each treatment. Dotted line represented the depth of dormancy and flowering intensity when grown under sufficient chilling accumulation conditions. Blue color represent chilling accumulation, whereas red color indicate heating accumulation

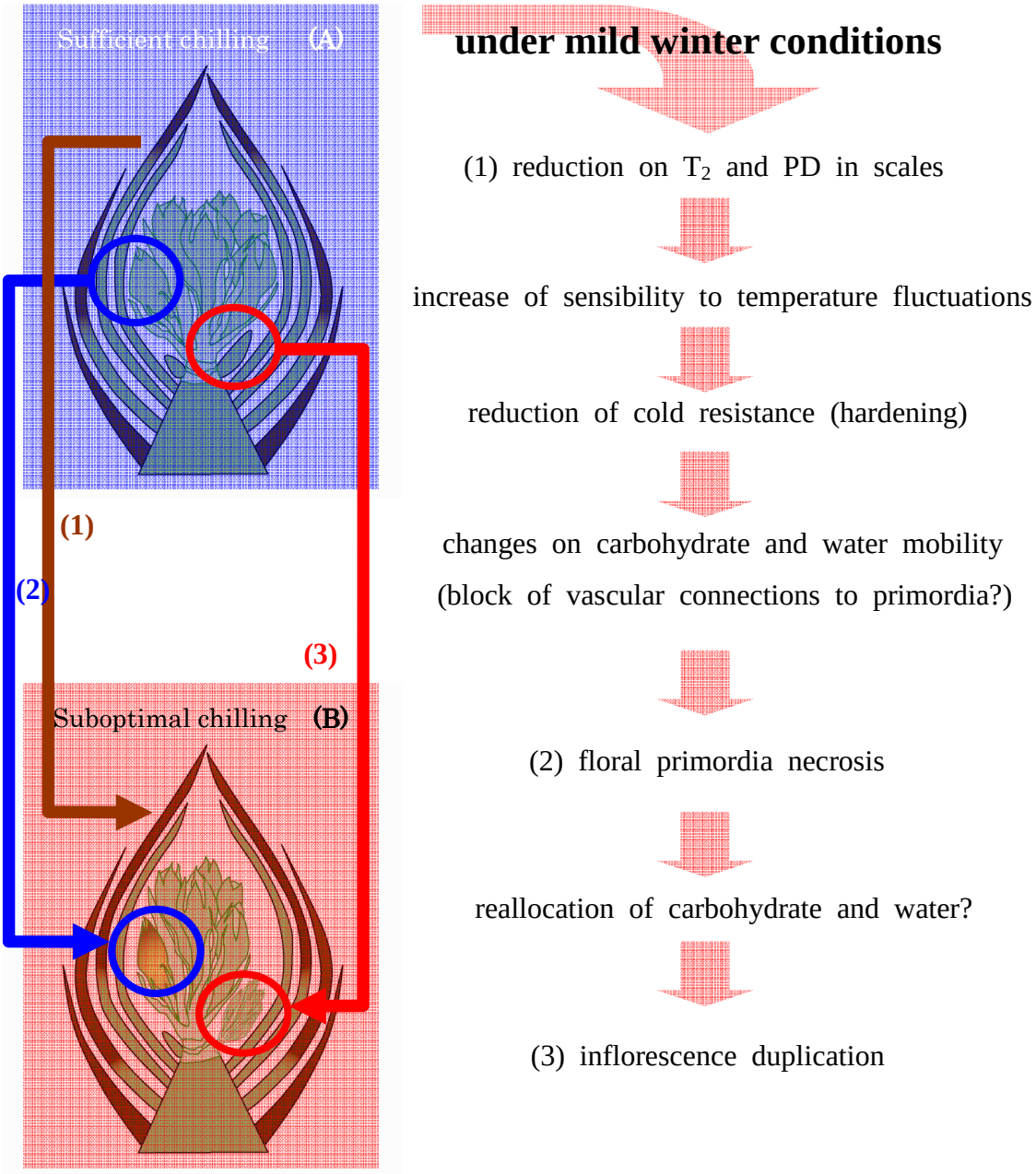

Fig. 36. Possible changes on mixed buds of 'Housui' Japanese pear during dormancy progression. Buds growing under sufficient chilling accumulation (A) have a well-developed group for primordia, one or two leaf primordia in the lower part, and green scales in overlapped portions. Under mild winter conditions (B), scales tend to dry (brown line, 1), one or several floral primordia show some abnormalities (blue circle, 2), some changes on initial inflorescence occur, and leaf primordia transform into a new inflorescence with floral primordia (red circle, 3).

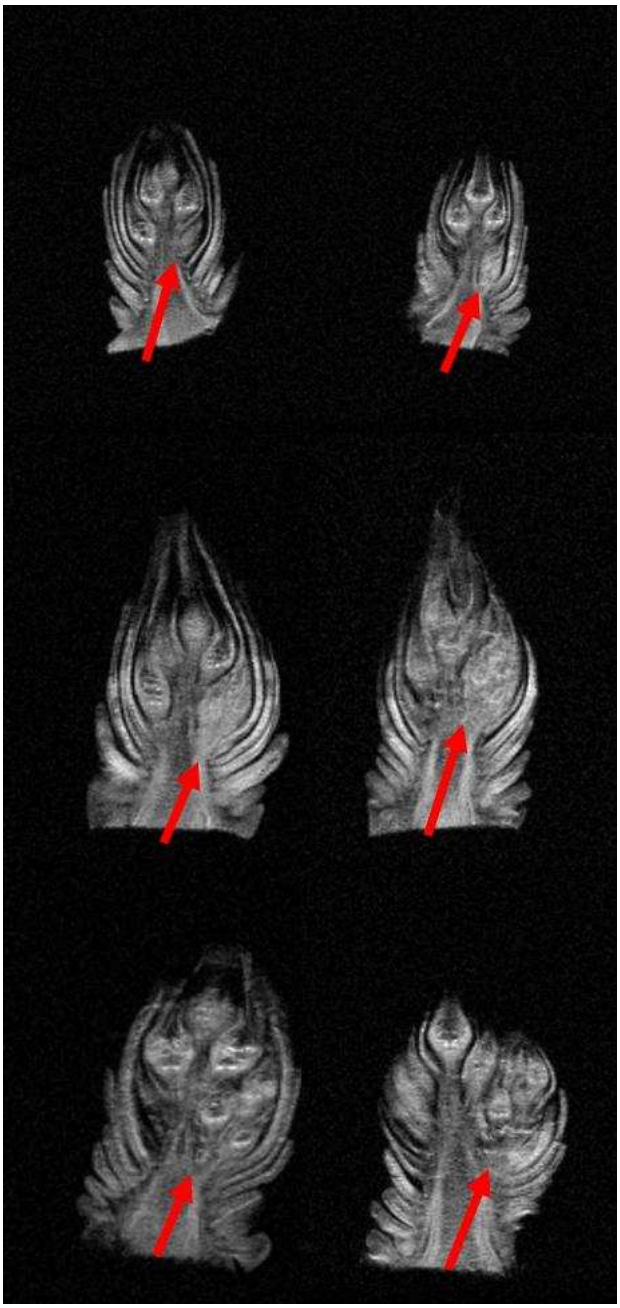

Fig. 37. Morphological images obtained by MRI showing a possible sequence for development of a new inflorescence in mixed buds of ‘Housui’ Japanese pear grown under mild winter conditions (from left to right, and from top to bottom).

Summary

Brazil has only a small area of pear production and national consumption depends largely (near 90%) on imports, provided by Argentina, a member of the free trade region of South America called Mercosur. It was observed a potential market in Brazil for high quality pear fruits, such as 'Housui' Japanese pear.

In temperate-zone deciduous fruit trees, accumulation of chilling temperatures and subsequent heating accumulations are necessary to release endodormancy and ecodormancy stages, respectively. However, under mild winter conditions, chilling accumulation do not satisfy the requirement, resulting in an abnormal progression of dormancy, and consequently problems on bud break and flowering can be observed. In Southern Brazil, where is located the Japanese pear production area, climate conditions during autumn and winter seasons are very variable with great temperature fluctuations. The average of accumulated hours below 7.2 ^{o}C during the last 45 years in this region was 600 chilling hours (CH).

Under mild winter conditions, abnormalities during dormancy progression and its release were observed in Japanese pear trees growing under mild winter conditions, such as in Brazil, New Zealand, Thailand, and under forcing cultivation (greenhouse) of Japan. In some locations in Southern Brazil, over 60% "flower bud abortion" was found in 2001 (Petri et al., 2002) and more than 90% in 1999, resulting in low numbers of flowers at budburst and consequently low production. It has been suggested that ecophysiological (temperature fluctuations), nutritional (carbohydrates), and morphological factors (flower development) were closely related to such abnormalities.

The main objective of our study was to analyze the effect of artificial mild winter conditions on dormancy progression and "floral primordia abortion" occurrence

in ‘Housui’ Japanese pear potted plants. The amount of 600 chilling hours, frequently observed in conditions of warm winters (such as in Japanese pear production area of Southern Brazil) and considered below the requirement for studied cultivar, was submitted at different times and for several consecutive seasons. Different mathematical models were used to estimate the dormancy progression: CH, chilling units (CU model), and developmental rate index (DVR model).

During the experiment, it was detected temperature fluctuations, resulting in different velocities of chilling accumulation. 2005-2006 season was the coldest season, which resulted in a rapid accumulation of chill, whereas 2006-2007 and 2008-2009 were relative warm.

Accumulation of 600 h below 7.2 °C (CH), corresponding to 80% of theoretical chilling requirement for ‘Housui’ Japanese pear (Nishimoto et al., 1995), brought about for endodormancy release, since bud burst (flowering and leafing) was observed in all treatments of all seasons. An inverse correlation between duration of chilling and heating accumulation was observed. The chilling unit (CU) model, proposed by Asano and Okuno (1990), might be adapted for mild winter conditions, since all treatments of all studied seasons reached the chilling requirement (512 CU). However, such condition of low chilling accumulation promoted floral primordia abortion, which increased with delaying to initiate the chilling accumulation (cold deprivation condition). ‘Housui’ plants growing under two months of cold deprivation before chilling (Treatment 3) had the highest incidence and severity of floral primordia abortion. Several seasons of mild winter conditions anticipated the occurrence of the disorder.

Cumulative effect of previous season of mild winter condition could be observed until the third season, when floral primordia abortion occurrences were anticipated with progression of such conditions. Under mild winter conditions, it was observed floral

primordia abortion in different grades, desiccation of bud scales, development of second inflorescence, and completely necrosed buds, flowers with different pedicel length, and development of flowers at different stages. On the contrary, plants growing under natural condition of Tsukuba, Japan, showed green scales at the “green tip” stage (dormancy release) and uniform flowering.

Carbohydrate concentrations were low and stable under low chilling accumulation conditions, as a possible consequence of incapacity of floral primordia to utilize available carbohydrates. Low levels of mobile water (T_2) and relative water content (proton density) in bud scales of buds grown under mild winter conditions might be affect the sensibility of floral primordia to external temperature oscillations and/or reducing on protection from freezing temperatures (hardening level) during winter. MRI images showed high water mobility and content in specific portions of bud base. Such phenomenon might cause a partial or complete necrosis of floral primordia as a consequence of an unavailability of water and other functional carbohydrates, which were possibly reallocated for development of new inflorescences. Incidence of floral primordia abortion (low percentage of normal primordia) and inflorescence duplication need to be considered as affecting water absorption potential. We can suggest that ‘Housui’ is highly sensitive to air temperature fluctuations during dormancy stage.

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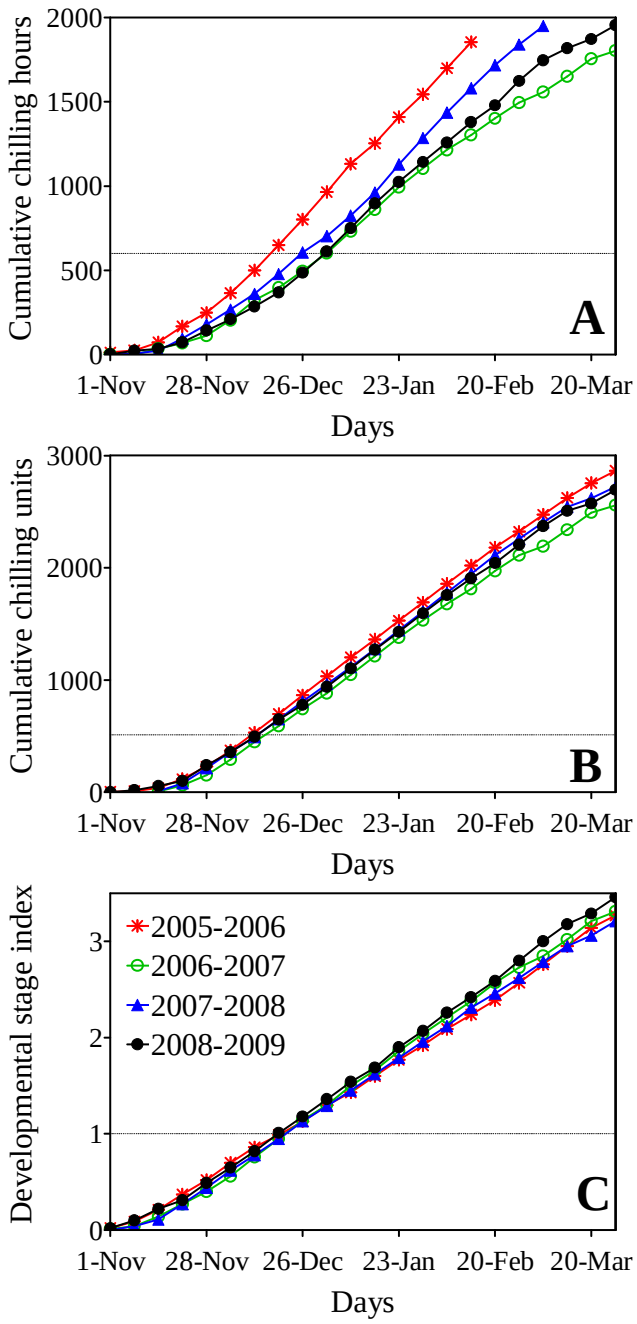
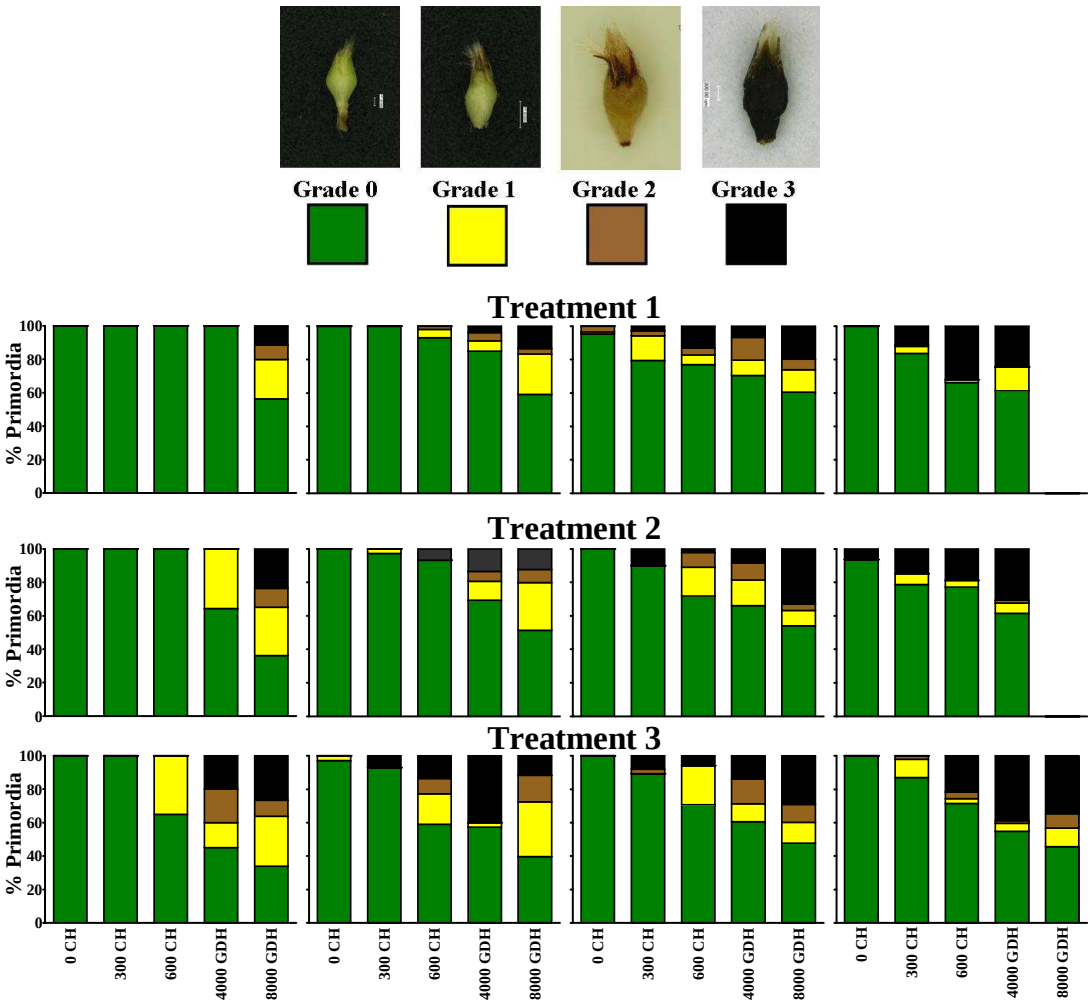
Appendix



Appendix 1. Field temperature data at the Agricultural and Forestry Research Center, University of Tsukuba, Japan (36° N, 140° E). Cumulative chilling hours (A), chilling units (B) and developmental stage index (B) were calculated from November until March during four seasons of study: from 2005-2006 to 2008-2009. Dotted lines indicate the theoretical amount of chilling necessary to release the endodormancy stage in floral buds of 'Housui' Japanese pear: 800 CH, 512 CU, and DVI=1.0.



Appendix 2. Incidence and severity of floral primordia necrosis in mixed buds of 'Kousui' Japanese pear grown with low chilling accumulation, during four consecutive seasons (2005-2006 until 2008-2009). Same group of plants were submitted to same temperature treatment. Chilling accumulation began at different times in each treatment (Fig. 11). Results are expressed as percentages of primordia. Scale adopted to evaluate the severity of floral primordia abortion: grade 0, normal primordia (green); grade 1, yellow primordia; grade 2, primordia with partial necrosis; grade 3, completely necrosed primordia (n = 10).