Chronological changes in plant hormone and sugar contents in cv. Ao-Shuang autumn flowering tree peony

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Abstract

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Successive secondary flowering is critical for tree peony industry. Varying the levels of hormones and sugars are reported to influence plant flowering. This study analyses quantitative changes in the levels of endogenous hormones [indole-3-acetic acid (IAA), abscisic acid (ABA) and gibberellic acid (GA_3)] and carbohydrates (sucrose, reducing sugar and starch) in the buds of cv. Ao-Shuang tree peony during autumn and spring flowering seasons. The study shows different levels of hormones (ABA, IAA and GA_3) and carbohydrates (sucrose, reducing sugar and starch) in spring (SFB) and autumn (AFB) flowering buds. Not only is there increase in IAA, GA_3, sucrose and reducing sugar, but also decrease in ABA and starch during AFB developmental stages. This probably contributes to induced flowering in AFB. Compared with SFB, IAA could be a vital AFB flowering hormone because it peaks at three critical bud developmental stages of bud swelling, shoot elongation and flower bud opening. Whereas sucrose and reducing sugar contents increase in AFB, that of starch decreases. SFB shows similar trends for sucrose, reducing sugar and starch. The findings suggest that cv. Ao-Shuang tree peony blooms in autumn probably due to lack of dormancy, a phenomenon induced by low ABA. Thus flowering of tree peonies in SFB and AFB could be regulated by different combinations of hormonal and sugar signals.

Keywords: autumn/spring flowering; plant hormone; ornamental plant; *Paeonia suffruticosa*; sugar

Tree peony (*Paeonia suffruticosa* Andr.) is native to China and is a magnificent, beautiful and attractive ornamental plant (Wister 1995). Flowering is a critical process in the development and life-cycle of most plants. The time of flowering largely determines the socio-economic and cultural values of plants. In China and Japan, tree peony cultivation is a traditional flower industry practice. Hence regulating the time of flowering of tree peony is critical for potted and cut flower supply to the markets during the New Year and the Spring Festival periods (Cheng et al. 2001). Research on the physiology, growth and development of the plant could enhance the quality and rate of flower production.

Tree peonies require a cold period prior to shoot, bud and flower development. The flower buds grow on perennial crowns in late summer, followed by shoot senescence and bud dormancy. Bud development only resumes after exposure to a cold period in winter. Chilling is normally required for dormancy release before tree peonies flower in spring, autumn or even winter under forced conditions (Aoki, Yoshino 1989). However, cv. Ao-Shuang (Fig. 1) a more recent cultivar of tree peony released by

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Fig. 1. The cv. Ao-Shuang autumn flowering tree peony (*P. suffruticosa*) taken at the Jiufeng Site of Beijing Forestry University Tree Peony Collection Center, China on October 2, 2010

Cheng, Zhao (2008), does not follow this phenological routine. It instead blossoms in autumn without cold vernalization. This provides a unique opportunity to explore the relationship between coldness and dormancy via comparative studies on physiological factors such as hormones and nutrients. This could pave the way to regulate the time of flowering in tree peonies that suits commercial, cultural and market conditions.

Peonies, like other flowering plants, require hormones for growth and normal maintenance of physiological and biochemical processes. Plant hormones are critical plant developmental elements that regulate countless plant processes, including root and flower development, and cell division and elongation (Liu et al. 2008; Pallardy 2008). However, the effects of hormones change with changes in environmental conditions and seasons of the year (Koshita et al. 1999). For instance, IAA, GA and ABA are known to delay, enhance and inhibit summer bud sprouting in Citrus, respectively (Altman, Goren 1972). Recent studies also show that cytokinin profiles in different plant organs change with seasonal change in *Abies nordmanniana*. For instance, cytokinin levels in apical buds are lowest in mid-June and highest in late summer (Rasmussen et al. 2009). Furthermore, inhibitory effects of exogenous IAA and ABA, along with endogenous IAA are reported in *Pharbitis nil*; in which exogenous GA induces flowering (Wijayanti et al. 1997).

Plant growth and development are also influenced by nutrients such as sucrose, glucose and fructose; which constitute the main assimilates of most plants (Katovich et al. 1998; Pallardy 2008). Sucrose is the main carbohydrate transported through the plant, and breakdown of sucrose is a vital source of energy and carbon skeletons needed in the synthesis of amino acids, lipids and metabolites (Pallardy 2008). Changes in sugar levels are also associated with flowering, and alterations of soluble sugar contents regulate plant growth processes. Studies show that carbohydrates regulate dormancy status, and that sucrose and glucose not only inhibit bud growth in *Euphorbia esula* (Chao et al. 2006), but also influence shoot emergence, flower bud formation and flowering in *Lythrum salicaria* (Katovich et al. 1998).

Therefore, understanding hormonal changes associated with floral bud development could be used to rationally transform tree peony flower industry. So far, not much research has been documented on the driving factors of autumn flowering in tree peonies (Zhang 2004; Jiang et al. 2007). In fact, there is hardly any literature documentation of the effects of changes in endogenous hormones on the flowering behaviour of tree peonies. Even less documented are the variations in endogenous hormones and sugars in spring and autumn flowering tree peonies. The objective of this study was to determine the dynamics of endogenous hormones and sugars in bud developmental stages of spring and autumn cv. Ao-Shuang flowering tree peonies. The findings of this study could deepen existing insights into the physiological processes that lead to autumn flowering in tree peonies. The acquired new knowledge will improve and better adapt the production practices of tree peony flowers to cultural and market conditions.

MATERIALS AND METHODS

Sample collection

The study was conducted at the Jiufeng Site of Beijing Forestry University Tree Peony Collection Center, China for two consecutive years (2009 and 2010). A total of 20, 5-year-old cv. Ao-Shuang (*P. suffruticosa*) plants with similar growth vigour in each season were selected for sample collection. Samples were collected from late February to mid-May, and

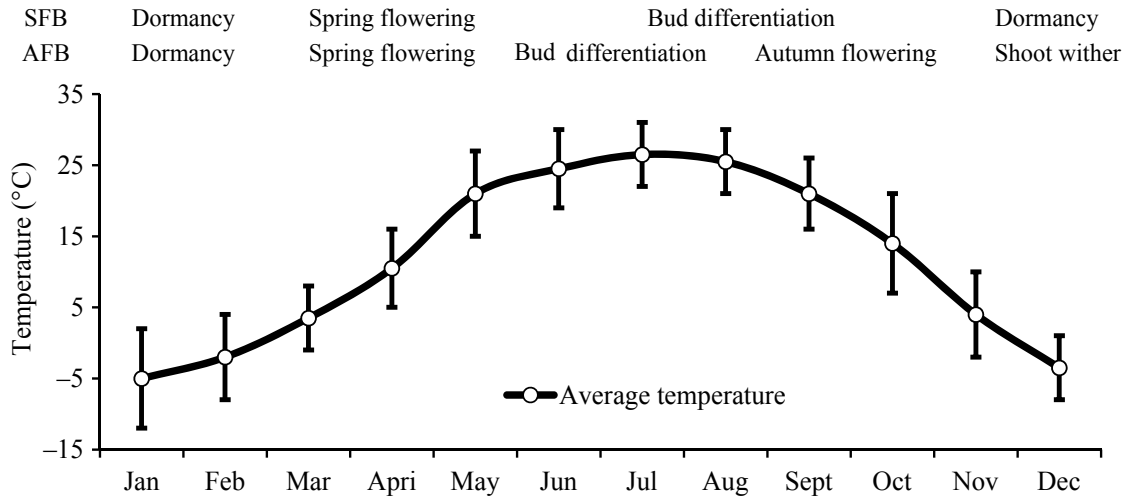

Fig. 2. Life-cycles of spring (SFB) and autumn (AFB) flowering cv. Ao-Shuang tree peonies. The bottom plot depicts the average monthly temperature for 2009–2010, and the error bars on the plot denote the minimum and maximum average monthly temperatures (source: Beijing Global Historical Climatology Network, Version 1)

then from mid-August to late September in 2009 and 2010; corresponding to the spring and summer seasons in Beijing (Fig. 2).

For autumn sampling, leaf deletion was manually done in mid-August. This was followed by (gibberellic acid) GA_3 application at 500 mg/l per dose. Using paintbrush, GA_3 was applied on developed buds (as a growth promoting agent for uniform bud growth) five days after leaf deletion. Spring plants were then allowed to go through winter cold for smooth and uniform bud growth. Next, bud samples were collected before (BD) and after (AD) defoliation and then after bud sprouting; targeting bud developmental stages I–VIII as described by Cheng et al. (2001). These stages include bud swelling (S1), bud sprouting (S2), shoot emergence (S3), shoot elongation (S4), leaflet extension (S5), flower bud enlargement (S6), flower bud opening (S7) and flower emergence (S8), in that order.

Sample buds were harvested using sterilized knives and then rinsed in distilled water for surface contamination. The collected samples were placed in ice box, conveyed to the laboratory, dipped in liquid nitrogen (N_2), and stored at –80°C until preparations were completed for the hormone and sugar analyses.

Extraction, purification and analysis of ABA, IAA and GA_3

With slight modifications, the endogenous hormone extraction was based on the procedure described by Chen et al. (1991). The levels of each of the endogenous (abscisic acid) ABA, (indole-3-acetic acid) IAA and GA_3 hormones were determined using 0.5 g of fresh buds. The buds were ground in 10 ml of 80% cold methanol with copper to a complete homogenate, and then transferred into test tubes. About 20 mg of polyvinylpolypyrrolidone (PVP) antioxidant was added to the homogenate, thoroughly mixed on the shaker for 10 min, and then incubated overnight at 4°C. The supernatant was transferred into 10 ml test tube the next morning and centrifuged at 6,000 rpm for 20 min. The residue was re-extracted in 2 ml of cold methanol for 12 h and centrifuged again at 6,000 rpm for 20 min, before finally discarding the dust. The combined extracts, after adding 2–3 drops of NH_3, were evaporated to aqueous phase at 35°C to 40°C using rotary evaporator. Thereafter, the aqueous phase was dissolved in distilled water, and the solution adjusted with 1N HCl to pH 2.5–3.0. It was then extracted three times in equal volumes of ethyl acetate. The combined ethyl acetate fraction was again condensed to dryness. The residue was dissolved in 80% aqueous methanol, purified in C_{18} column and the elute evaporated to dryness. The residue was collected, dissolved in methanol and dried under N_2 gas. The purified extracts were dissolved in 50% methanol, filtered through 45 μm membrane and subjected to high performance liquid chromatography (HPLC). No internal standard application and hormone analysis were done on computer-aided Agilent HP 1100 (Agilent Technologies Inc., Santa Clara, CA, USA) equipped

with vacuum degasser, auto-sampler, quandary pump, thermostat column compartment and diode array detector. The conditions of the HPLC were as follow: ZORBAX RX-C_8 column (250 × 4.6 mm); mobile phase (3% methanol and 97% 0.1M acetic acid) for IAA, GA_3 and ABA determination after filtration through 0.45 μm filter membrane; detection wavelength of different hormones (IAA = 280 nm, ABA = 260 nm, GA_3 = 210 nm); and sample quantity of 10 μl was auto-injected at a flow rate of 1 ml/min. Hormones were quantified by comparing peak areas of the samples with standard samples (Sigma Chemical Co., St. Louis, USA).

Soluble sugar extraction and analysis

After 1.0 g of fresh bud material was ground in 20 ml of distilled water, the powder was extracted in water-bath at 80°C for 30 min. The suspension was centrifuged for 10 min at 6,000 rpm. The supernatant was used to determine sucrose and reducing sugar, and the pellet to determine starch. The reducing sugar was colorimetrically determined using dinitrosalicylic acid. The sucrose and starch were determined (using anthrone reagent with glucose as the standard) by the colorimetric anthrone method as modified for non-reducing sugar determination (Xue, Xia 1985). The absorbance was then determined using spectrophotometer (TU-1901, Purkinje General Instrument Co., Beijing, China).

Statistical analysis

All statistical analyses were done using the SPSS (Statistical Package for Social Scientists, version 16.0) software (IBM Corporation, New York, USA). The means of the target hormones and carbohydrates were used in a one-way ANOVA analysis to determine the level of significance at $p < 0.01$.

RESULTS AND DISCUSSION

Hormonal effect on bud growth, development and flowering

The study shows a significant variation in the levels of endogenous ABA in spring and autumn flowering buds of cv. Ao-Shuang tree peony from before defoliation (BD) to the S3 growth stages. At the initial bud developmental stage, there is a higher level of ABA in spring flowering buds (SFB) than in autumn flowering buds (AFB) (Fig. 3a). The difference at the initial growth stage is statistically significant at $p < 0.01$ when spring (Feb.–Mar.) temperature is lowest and autumn (Aug.–Sept.) temperature is highest. At the mid-to-late growth stage, however, the difference in ABA content between SFB and AFB is insignificant. The temperature difference at this stage is also insignificant. This suggests that bud physiological conditions are very much influenced by temperature regimes.

In general, hormonal effect on plant growth and development changes not only with hormone type and plant species, but also with seasonal and environmental conditions. Recent studies suggest that cytokinin profiles in *Abies nordmanniana* change with season (Rasmussen et al. 2009). Hence the high ABA content at the initial growth stage in SFB is not entirely surprising. This is because temperature levels are lowest at this stage; during which time SFB is either dormant or in transition from endo-dormancy to eco-dormancy. This condition is reported to be closely associated with high ABA content in plants (Altman, Goren 1972; Rinne et al. 1994). At a similar growth stage, ABA level in AFB is low. This implies that AFB may not experience dormancy at the time when SFB is either dormant or emerging from winter dormancy. With warmer temperatures in April to May (Figs. 2, 3a), ABA decreases (Barros, Neill 1988) to a level similar to that in AFB. This further suggests a degree of link between temperature and ABA level, which could influence dormancy release, bud sprout and tree peony re-growth.

The observed low level of ABA in AFB in this study probably contributes to inducing autumn flowering in cv. Ao-Shuang autumn flowering tree peonies. Our result is consistent with the works of Altman and Goren (1972) and Wijayanti et al. (1997); who noted inhibitory ABA effects on bud sprouting and flowering in citrus and *Pharbitis nil*, respectively. It, however, disagrees with the findings of Harada et al. (1971) and Nakayama and Hashimoto (1973); they reported promotion effects of ABA on black iris and *Pharbitis nil*. The discrepancy could be due to the differences in plant species and environmental/soil conditions.

High levels of IAA are noted at shoot extension (S3) and flower blooming (S8) stages in SFB. In AFB, however, IAA levels are high at bud swelling (S1), shoot elongation (S4) and flower bud open-

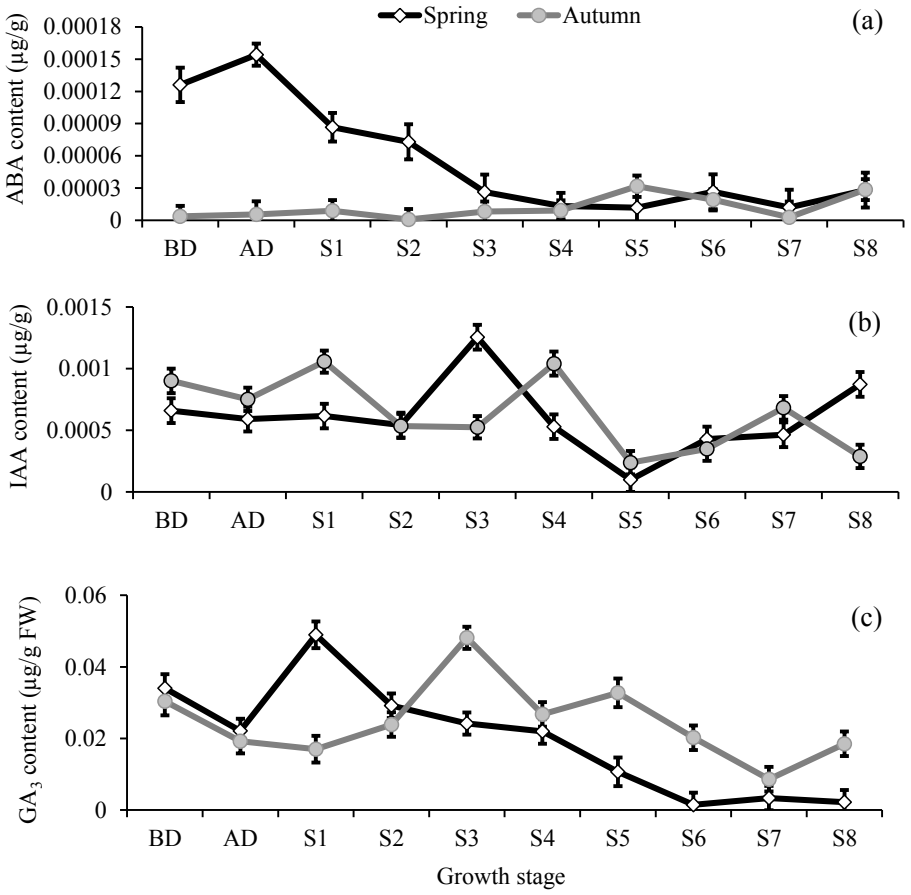

Fig. 3. Changes in ABA (a), IAA (b) and GA_3 (c) contents of spring and autumn flowering cv. Ao-Shuang tree peony. On the abscissa, BD and AD denote fresh-weight contents of the hormones before and after defoliation, respectively. S1–S8 denote the growth stages from bud swelling to blossoming (Cheng et al. 2001)

The values are the means of three replicate extractions for each stage of bud development in 2009–2010 cropping seasons. Error bars denote the ± standard deviation

ing (S7) stages (Fig. 3b). Interestingly, the tri-peak levels of IAA in AFB coincide with the stages with relatively low IAA levels in SFB. This suggests that environmental conditions such as temperature and seasonal variations influence endogenous IAA levels. In AFB, endogenous IAA production could be related with flower bud formation, bud growth and anthesis. It is observed in citrus (Koshita et al. 1999; Koshita, Takahara 2004) and chestnut (Liu et al. 2008) that endogenous IAA significantly increases during bud outgrowth.

High IAA levels are observed in AFB at S1, S4 and S7 stages, the stages with obvious bud-growth-related physiological metabolism (also see Pallardy 2008). This suggests that IAA could be a critical element that induces and promotes growth and development of AFB. For SFB, IAA peaks only at S3 and S8 stages of the cv. Ao-Shuang tree peony development. The peak level of IAA at the S8 stage could be due to the continuing growth of SFB plants after anthesis; which is needed for seed development and next-generation bud differentiation. As IAA level is normally associated with nutrient attraction (Koutinas et al. 2010), high IAA level could be due to a high nutrient demand at the S8 stage of bud development. On the contrary, shoots of cv. Ao-Shuang AFB plants completely wither after blooming; which prepares the way for winter vernalization. The low IAA level in AFB at S8 could therefore be due to low nutrient demand at this stage.

Although the peak stages are different, the trends in GA_3 are largely similar for SFB and AFB. While GA_3 peaks at S1 stage in SFB, it peaks at S3 stage in AFB (Fig. 3c). For all the other developmental stages, the trend in bud GA_3 for SFB is similar to that for AFB. It is, however, slightly higher in AFB than in SFB. Generally, GA_3 increases as bud sprout (S1) progresses in SFB. This suggests that early spring

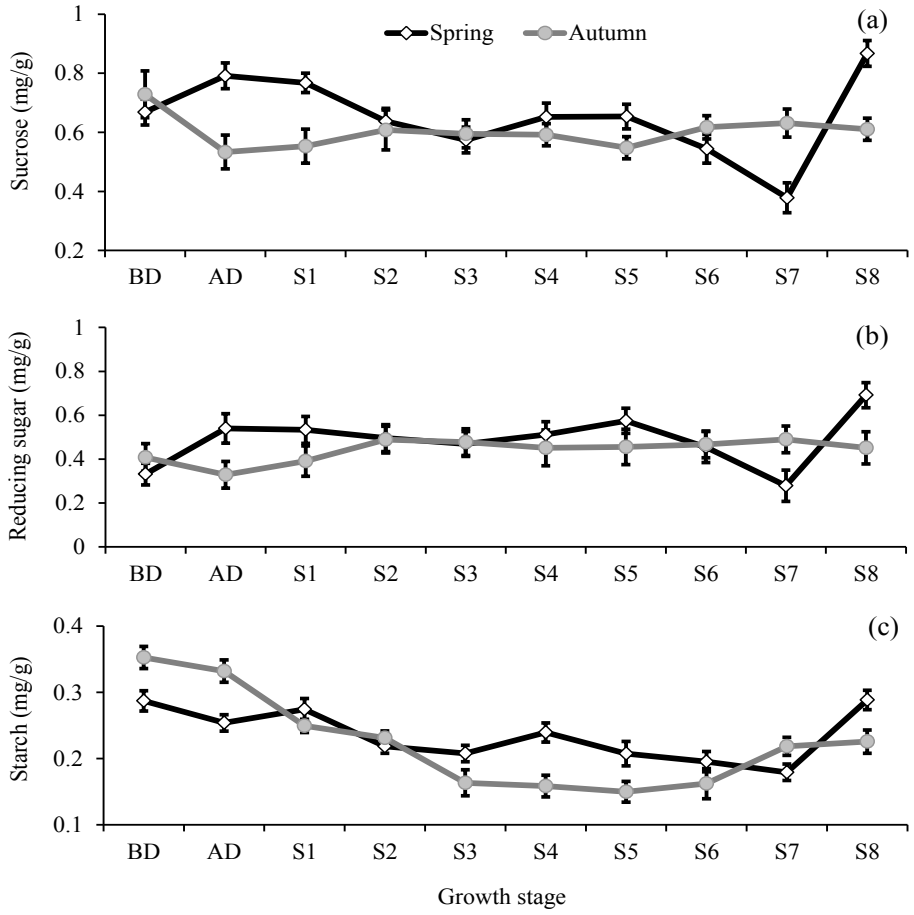

Fig. 4. Changes in fresh-weight contents of sucrose (a), reducing sugar (b) and starch (c) in spring and autumn flowering cv. Ao-Shuang tree peony. On the abscissa, BD and AD denote the contents of the sugars before and after defoliation, respectively. S1–S8 denote the growth stages from bud swelling to blossoming (Cheng et al. 2001)

The values are the means of three replicate extractions for each stage of bud development in 2009 and 2010 cropping seasons. Error bars denote the ± standard deviation

temperatures could induce high GA_3 production, which promotes dormancy release. The high GA_3 level in AFB also coincides with the stages of full shoot and flower bud emergence. It further suggests that high GA_3 induces flower bud formation in AFB.

Although earlier studies suggest GA_3 has an inhibitory (Koshita et al. 1999; An et al. 2008) or no (Garner, Armitage 1996) effect on flower bud formation, our study shows that GA_3 could be related with flower bud formation. Several other studies have also attributed flowering to high GA_3 levels (Cheng et al. 2005; Liu et al. 2008). In fact, GA_3 is generally involved in promoting bud sprouting in herbaceous peonies (Cheng et al. 2005, 2009) and in substituting chilling in dormancy release (Cheng et al. 2005). It is noted in this study that GA_3 functions more or less as a growth promoter, rather than a dormancy-release agent in AFB. This is because the stages at which high GA_3 levels occur are characterized by full bud breakout, rapid shoot development, high gibberellin production, and enhanced mitosis (Pallardy 2008). No high GA_3 levels are noted at dormancy release stage (S1), which is the onset of tree peony re-growth (Cheng et al. 2009). This could also explain the lack of dormancy in cv. Ao-Shuang autumn flowering tree peony plants.

Endogenous hormone interaction

Studies suggest that plant growth and development is influenced by the balance or opposing effects of hormones (Pallardy 2008). In this study, it is noted that IAA content in SFB and AFB is in the inverse trend for almost all the growth stages. In other words, high levels of IAA in AFB coincide with low levels of IAA in SFB and vice versa (Fig. 3b).

After stage S3, generally low levels of GA_3 are noted in SFB against high levels in AFB (Fig. 3c). Furthermore, low IAA level in SFB at S1 coincides with high level of GA_3 and low level of ABA but in AFB, high IAA level at S1 coincides with low levels of ABA and GA_3. Similarly, decreasing level of ABA coincides with increasing level of GA_3 in SFB at S1 stage (Fig. 3a, c). It then suggests that IAA, GA_3 and ABA may concurrently act at the same site, but with an opposing effects during dormancy release and flowering in cv. Ao-Shuang SFB and AFB plants. There is therefore an apparent functional relationship among these hormones. It is possible that higher IAA, GA_3 or lower ABA level induces autumn flowering in cv. Ao-Shuang tree peonies.

Effect of nutrient on bud growth, development and flowering

Differences exist in the trends of the carbohydrates in SFB and AFB plants for the various developmental stages (Fig. 4). At the initial growth stages (AD–S1), the concentrations of sucrose and reducing sugar (glucose and fructose) are higher in SFB than in AFB. But at bud sprouting (S2) and shoot emergence (S3), these differences almost disappear. In SFB, the levels of sucrose and reducing sugars increase at flower bud development stages S4 and S5, drop at flower bud opening stage S7, and then sharply rise again at blooming (S8) stage. On the other hand, the levels of sucrose and reducing sugars in AFB initially decrease, followed by a gradual increase at stages S2 and S3. Then after these stages, the levels of sucrose and reducing sugars relatively stabilize for the rest of the growth period in AFB (Fig. 4a, b).

For SFB, the trend in starch almost tracks like those of sucrose and reducing sugars. The only exception is for the initial stages of bud development, where low starch coincides with high sucrose and reducing sugars (Fig. 4c). For AFB, starch level initially increases, then decreases with the flourishing of new vegetative organs. This trend continues for the rest of the growth stages S3, S4 and S5, hitting the lowest point at leaflet extension stage S5 (Fig. 4c). At the initial stages of bud growth (BD to S3) there is a decline in starch levels in AFB indicating that starch could be highly utilized by autumn flowering buds at these stages. As observed in *Pinus sylvestris* (Fischer, Höll 1991), starch degradation could increase sugar levels that are much needed to satisfy the energy needs for bud sprout and shoot emergence.

There is no significant difference in the trends of sucrose, reducing sugar and starch across bud developmental stages in SFB (Fig. 4). This suggests that the plant requirements for these sugars are similar at flower bud developmental stages. In contrast, the levels of starch in AFB further decline at the early stages of vegetative growth (S3–S5) with a steady trend of reducing sugar. Such trend of reducing sugar to starch, coupled with the steady accumulation of sucrose and then low starch levels at active growth stages could favour autumn flowering in cv. Ao-Shuang AFB tree peonies.

Despite the differences, the levels of sucrose and reducing sugar at S2 and S3 stages and that of starch at S2 stage of bud development are similar for both the spring and autumn flowering tree peonies (Fig. 4a, b, c). This could be due to the drastic increase in respiration as the plant breaks dormancy, leading to bud breakout and shoot emergence. During the early stages of shoot growth, there is a high rate of manufacturing of chlorophyll, structural components and protein, resulting into high catabolic activities that support the energy needs by the young shoots (Kozlowski 1992). The high rate of use of carbohydrate could override the available levels in both SFB and AFB at the S2 and S3 stages of bud development. It then suggests that carbohydrate hydrolysis is a critical factor for bud sprout and shoot enlargement. Furthermore, starch level in AFB decreases with the flourishing of new vegetative parts and bud growth. This phenomenon could be driven by the utilization of stored carbohydrate reserves to support shoot/flower bud development (Fig. 4c). This further suggests that sink activities could significantly determine starch reserve in flowering plants.

In Fig. 4, the levels of sucrose, reducing sugar and starch at blooming (S8) stage rise more rapidly in SFB than in AFB. This could be driven by the distinct fates of future developments in SFB and AFB. After flowering, SFB shoots continue to grow through summer and autumn to support seed development, fruit setting and differentiation of next-generation buds. AFB shoots, on the other hand, cease to grow and gradually wither during winter period. Accumulated carbohydrates in peonies could be needed in spring flowering plants to support new flower bud formation, which usually starts one month after full flowering (Barzilay et al. 2002).

In both seasons, sucrose apparently remains to be the main sugar, followed by reducing sugar and

then starch. This is largely consistent with the seasonal dynamics of carbohydrate observed in other perennial plant species (Kozlowski 1992; Katovich et al. 1998, Pallardy 2008), but differs from that of Liu et al. (2008); who reported that sugar is irrelevant in abnormal chestnut flowering. It could therefore be concluded that despite the seasonal variations, sucrose is the main source of carbohydrate in tree peonies. This could mainly come from the transformation of stored starch to provide energy and carbon skeleton for the synthesis of amino acids, lipids and metabolites needed for plant growth.

Tree peony dormancy and flower bud growth

Dormancy is a critical condition for regulating flowering in peony plants. Both tree and herbaceous peonies need a period of low temperature of ≈5.6°C (Aoki, Yoshino 1989) or GA_3 treatment to substitute for chilling (Cheng et al. 2005) for dormancy release. It is noted in this study that the interactions of hormones and sugars could regulate bud dormancy and growth in tree peonies. As noted in other plant species (Kozlowski 1992; Katovich et al. 1998), sucrose and reducing sugar also inhibit bud growth in SFB. Higher levels of these sugars are noted in SFB at AD–S1 stages. This coincides with dormancy or the transition from endo-dormancy to eco-dormancy. The reverse trend is noted in AFB, where low levels of sucrose and reducing sugar correspond with dormancy. Whereas low levels of starch are noted at stages (BD–AD) where SFB is in dormancy, high levels are noted in AFB. This also suggests that no dormancy occurs in AFB, as low starch and high sucrose levels are associated with dormancy (also see Katovich et al. 1998). Especially at the start of dormancy release (i.e., S1 stage), the trend in GA_3 and that in ABA and sucrose are generally inversely related.

The study also shows that sucrose possibly inhibits its GA_3 production during tree peony dormancy. In fact, sucrose is noted to inhibit GA_3 production in root buds of *Euphorbia esula* (Katovich et al. 1998). Because of the high levels of starch at dormancy release, the combined effects of sucrose/reducing sugar and GA_3 could regulate starch metabolism in tree peonies. This shows that carbohydrates could also regulate dormancy in cv. Ao-Shuang tree peony (Katovich et al. 1998; Chao et al. 2006).

CONCLUSION

Interaction between hormones and sugars appear to be involved in regulating flowering of tree peonies. The results of the study show that the levels of hormones in SFB are different from those in AFB. Thus the regulation of flowering in spring and autumn may be triggered by different combinations of hormonal and sugar signals. Right across the flower bud development stages, there is not only an increase in IAA, GA_3, sucrose and reducing sugar in AFB, but also a decrease in ABA and starch. Moreover, the quantitative changes in endogenous hormones and carbohydrates at flower bud developmental stages could be influenced by seasonal variations and environmental conditions such as temperature. The combination of these forces is the possible regulator of flowering in cv. Ao-Shuang tree peony plants.

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