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Full Length Article

Do Stored Reserves and Endogenous Hormones in Overwintering Twigs Determine Flower Bud Differentiation of Summer Blooming Plant – *Styrax tonkinensis*?

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Abstract

Styrax tonkinensis (Pierre) Craib ex Hartwich is one kind of important tree species with a combination of oil, medicine and ornamental values. With the aim of understanding the effects of stored reserves and endogenous hormones on flower bud differentiation, thirty 4a *S. tonkinensis* trees were used for experimental analysis. The overwintering twigs in two consecutive years were collected to measure the content of stored reserves and endogenous hormones to build the relationship with the flower bud differentiation of *S. tonkinensis.* The results showed that soluble sugar and soluble protein could help flowering, while starch was not conducive to the flowering. ABA contributed to flowering, however, GA could not promote flowering. IAA had no significant effect on flowering. The number of flowers in two consecutive years had a significant positive correlation with the content of soluble sugar, soluble protein and ABA, and had a negative correlation with the content of starch, GA and IAA. Our experimental results could provide a basis for controlling flowering and further study on *S. tonkinensis*. © 2019 Friends Science Publishers

Keywords: Flower bud differentiation; Stored reserves; Endogenous hormones; Flowering regulation

Introduction

Flower bud differentiation refers to the transformation from leaf buds physiology to flower buds physiology, which also indicates the transformation from vegetative growth to reproductive growth. Therefore, mastering its rules is of great significance to improve the quantity of flowers and the quality of seeds. It is particularly important to understand the changes in physiological indexes such as nutrients, hormone contents and enzyme activities during flower bud differentiation. Meristems would develop into flowers when receiving evolutionary or environmental signals (David, 2009). Flower bud formation in Jojoba (*Simmondsia chinensis*) is inhibited and flowering is delayed under water stress condition (Benzioni *et al.*, 1992). On the other hand, 'Tahiti' lime trees (*Citrus latifolia*) have more flowers under severe water stress condition (Southwick and Davenport, 1986). Some studies have shown that GAs inhibited the flower bud initiation of fruit trees (Prang *et al.*, 1998), while ABA promoted the flower bud differentiation (Zhu *et al.*, 2015). It is thought that the impact of GAs on flower bud differentiation is related to the species and varieties. GA_3 and GA_7 could inhibit flowering by inhibiting the flower bud initiation of cherry (*Prunus avium*) and apple (*Malus pumila*). The inhibitory effect of GA_3 may be caused by stimulation of IAA biosynthesis at the signal-generating site, while GA_4 could be involved in the promotion of flowering (Oliviera and Browning, 1993; McArtney and Li, 1998).

Styrax tonkinensis (Pierre) Craib ex Hartwich is widely distributed in subtropical areas including Vietnam, Laos and Southern China (Zhang *et al.*, 2018). It has become one kind of ornamental tree species with values in oil, medicine and timber. Leaves in *S. tonkinensis* emerge in early March. Leaves appear red-green at first and then turn into yellow-green. *S. tonkinensis* blooms in late April and quickly enters the blooming stage with white flowers. The flowers are in strings with light fragrance and can be used as medicine to relieve pain. Xu and Yu (2015) measured the aromatic constituents of *S. tonkinensis* flowers and found that terpenoids were rich in varieties and contents. In order to discover the value of flowers of *S. tonkinensis*, it is necessary to study the factors that influence its flower bud initiation. The mixed buds formed in previous year grow into new shoots and young leaves in the early March of next year. The inflorescence primordia inside the leaf will develop into inflorescences. Flowers are differentiated in middle and late March and the morphological differentiation is completed before May.

Some species bloom first and then sprout leaves. Stored reserves in twigs in previous year will impact the

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flowering in next year. Therefore, fertilization in previous summer and autumn to supplement nutrients in twigs can promote flowering. Nitrogen application on *P. cerasus* was carried out by Lindhard and Hansen (1997) between May and October in 1993 and 1994, respectively and then they estimated the shoot growth, number of flowers and fruit set in 1995. They found that early summer nitrogen application could produce more flowers and fruits, while later nitrogen application could not. How about *S. tonkinensis* that sprouts leaves in spring and blooms in summer, is not yet known. We predict that soluble sugar, soluble protein and ABA can contribute to flowering. Thus, our research focused on exploring, which stored reserve in overwintering twigs was favorable to blooming in summer and evaluating their potential for flowering. Results we obtained can help producers make management strategies in advance.

Materials and Methods

Experimental Materials

Experiments were performed in planting base of Jiangsu Guoxing Biotechnology Co. Ltd., located in Luhe district, Nanjing (32°54'N, 118°84'E) in 2016 and 2017. *S. tonkinensis* seeds were obtained from Jishui, Jiangxi Province and sown in 2012, then they grew under natural conditions.

Treatments

At the end of February 2016, a total of thirty trees with similar height, which grew well with no pest attacks, were selected and tagged. Before sprouting leaves, collected three similar annual twigs from lower part of each tree in the south direction on 22nd February, 2016 and 27th February, 2017. Twigs were taken back to the laboratory and stored in ultralow temperature freezer with -70°C for determining the contents of non-structural carbohydrates, soluble protein and endogenous hormones (GA, IAA and ABA). On 10th May, 2016 and 4th May, 2017, the number of flowers per tree was counted.

Determination Methods

Twigs were ground after drying at 70°C for 72 h. There were three replicates in each sample. A 0.2 g of samples in each replicate was ground and diluted to 10 mL. After two times of 30-min extraction with boiling water, dilute them to 25 mL. Add 0.2 mL of extracting solution, 1.8 mL of distilled water, 0.5 mL of anthrone ethyl acetate and 5 mL of 98% concentrated sulfuric acid respectively. Calculate soluble sugar content according to the method described by Li (2006). Transfer the residues from extracting soluble sugar into test tubes and dilute them to 10 mL. Place the tubes in boiling water for fully extraction for 15 min and add 2 mL of 9.2 mol/L perchloric acid, then extract it for another 15 min. The measurement steps were the same as above.

A 0.2 g of samples was ground and diluted to 5 mL. After centrifugation at 8000 r/min for 15 min at 4°C, add 1 mL of extraction and 5 mL of Coomassie brilliant blue G-250 to measure soluble protein content according to Li (2006) and Bradford (1976).

Fresh sample (0.3 g) was extracted with 80% cold methanol at 4°C for 4 h. After purifying, vacuuming and drying, solve filtering samples with little PBS. Add standard substance, samples and antibody, take the enzyme with peridium out and wash it. Enzyme-linked immunosorbent assay (ELISA) to measure GA, IAA and ABA content according to Koshita *et al.* (1999) and Weiler *et al.* (1981).

Statistics Analysis

Values were expressed as mean ± SD for three replicates in each group. Statistics were processed by Excel (Office 2013 Pro Plus, Microsoft Corporation, USA) and SPSS 22.0 (IBM, USA). Associations between stored reserves and endogenous hormones in overwintering twigs and the number of flowers of *S. tonkinensis* were determined using Pearson's correlation analysis. The significance of correlation between the samples at the level of 0.05 and 0.01 was established.

Results

Changes in the Number of Flowers

The number of *S. tonkinensis* flowers was presented in Fig. 1. We could find that the number of flowers for most sample trees appeared an increasing trend. Therefore, the number of flowers in 2017 was higher than that in 2016 (20248 and 8274 respectively). Most flowers appeared around 25,000 in 2016 and more than 35,000 in 2017. Several trees had almost no flowers within two years and tree number increased in 2017. The variation range of the number of flowers in 2017 was bigger than that in 2016.

Changes between Stored Reserves and Number of Flowers

Soluble sugar content: The content of soluble sugar in overwintering twigs of *S. tonkinensis* was shown in Fig. 2. Soluble sugar content was obviously higher in 2017 than that in 2016. The highest content in 2017 was more than 50 mg/g FW. While soluble sugar content in 2016 was highest to 40 mg/g FW. In Table 1, we found that the number of flowers had a positive correlation with the soluble sugar content in overwintering twigs (p<0.01), which indicated that more flowers were produced with a rise in soluble sugar content. The correlation coefficients were 0.786 in 2016 and 0.599 in 2017. In 2016, very rare flowers were seen when the soluble sugar content in overwintering twigs of *S. tonkinensis* was less than 25 mg/g FW. When the soluble sugar content reached 28 mg/g FW or higher, over ten thousand flowers appeared. In 2017, flower production was near zero when the soluble sugar content was less than 20

Table 1: Correlation analysis between soluble sugar, starch and soluble protein content in overwintering twigs and the number of flowers of *S. tonkinensis*

Year		Soluble sugar	Starch	Soluble protein
2016	Content (mg.g^{-1})	28.489±7.940	68.691±6.854	6.100±1.123
	Correlation coefficient	0.786**	-0.280	0.550**
2017	Content (mg.g^{-1})	38.767±10.110	58.492±10.156	6.436±1.182
	Correlation coefficient	0.599**	-0.346	0.572**

Data in the table was the average of three replicates ± SD; ** indicated that there was a significant correlation at the level of 0.01; * indicated correlation at the level of 0.05; the same below

Table 2: Correlation analysis between endogenous hormones contents in overwintering twigs and the number of flowers of *S. tonkinensis*

Year		ABA	GA	IAA
2016	Content (ng.g^{-1})	83.267±13.376	12.788±3.549	53.303±13.695
	Correlation coefficient	0.646**	-0.459*	-0.305
2017	Content (ng.g^{-1})	98.050±14.112	8.328±1.329	52.419±11.562
	Correlation coefficient	0.555**	-0.497**	-0.054

mg/g FW, and more flower buds failed to develop into flowers normally. There were 15,000 to 30,000 flowers produced when the soluble sugar content was between 30 and 50 mg/g FW.

Starch content: Fig. 3 showed the content of starch in overwintering twigs of *S. tonkinensis* in two years. A negative correlation was found between the number of flowers and the starch content in overwintering twigs (p>0.05). Overall, the starch content in 2017 was lower than that noted in 2016. The correlation coefficients in 2016 and in 2017 were -0.280 and -0.346, respectively. In 2016, the starch content in overwintering twigs of *S. tonkinensis* was concentrated from 65 mg/g FW to 75 mg/g FW and some trees had very rare flowers, while others reached more than 15,000 flowers. When the content reached 75 mg/g FW or higher, a small number of flowers were appeared. In 2017, while the starch content was mostly between 50 mg/g FW and 70 mg/g FW, 10,000 and 30,000 flowers could be found.

Soluble protein content: Fig. 4 was the soluble protein content in overwintering twigs of *S. tonkinensis*. The highest contents were about 8.5 mg/g FW in both years and the lowest contents were close to each other. In 2017, the content of soluble protein was slight higher than that in 2016. There was a positive correlation between the number of flowers of 30 trees and the soluble protein content (p<0.01, Table 1), with the correlation coefficients of 0.550 in 2016 and 0.572 in 2017. The flower buds of *S. tonkinensis* could not develop into flowers when the soluble protein content was between 4.2 and 5.0 mg/g FW. However, when the soluble protein was between 5.1 and 6.9 mg/g FW, the corresponding number of flowers was 5,000 to 15,000 in 2016 and 10,000 to 25,000 in 2017. The content of soluble protein of a few trees could reach up to 7.5 mg/g FW or higher with 22,000 to 25,000 flowers in 2016 and 30,000 to 35,000 flowers in 2017.

It is evident from the data that the soluble sugar content in overwintering twigs in 2017 was about 36.08% higher than that in 2016 with a wider range of variation, which was relatively unstable (Table 1). The starch content was 68.691 and 58.492 mg/g FW in 2016 and 2017, respectively. The starch content in 2017 was about 17.43% lower than that in 2016. The soluble protein content was similar in these two years with a slightly increase in 2017 than that in 2016. From the effects of soluble sugar, starch and soluble protein content in overwintering twigs on the number of flowers of *S. tonkinensis*, the increasing of soluble sugar and soluble protein was conducive to flower formation and the influence of soluble sugar was greater than that of soluble protein. Few starch content could promote the number of flowers in a non-significant way.

Changes in Endogenous Hormones and Number of Flowers

ABA content: As given in Fig. 5, an increase in the content of ABA could be seen from 2016 to 2017, meanwhile more flowers were formed. The average content was nearly 100 ng/g FW in 2017, higher than 80 ng/g FW in 2016. The number of flowers correlated positively with the ABA content in overwintering twigs in these two years (p<0.01), indicating that the rising of ABA content could enhance the formation of flowers.

The correlation coefficients were 0.646 in 2016 and 0.555 in 2017. When the ABA content was less than 70 ng/g FW, the number of flowers was extremely small. Compared with the number of flowers in 2016, it was higher in 2017 with higher ABA content. In 2016, when the number of flowers was less than 5,000 or between 10,000 and 20,000, the corresponding ABA content was under 80 ng/g FW or between 80 and 100 ng/g FW. In 2017, for most trees, more flowers were formed with an increase in ABA content. When the ABA content was between 100 and 120 ng/g FW, the number of flowers was extremely large (between 20,000 and 350,000).

GA content: High GA content in 2016 and low GA content in 2017 was noted (Fig. 6). There existed a big difference between the contents of these two years. The content of GA in 2016 was absolutely higher than 10 ng/g FW, while that

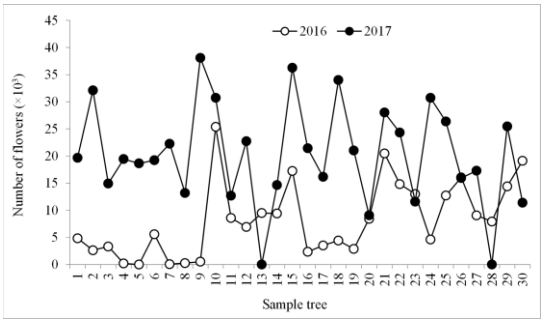

Fig. 1: The number of flowers of *S. tonkinensis* sample trees

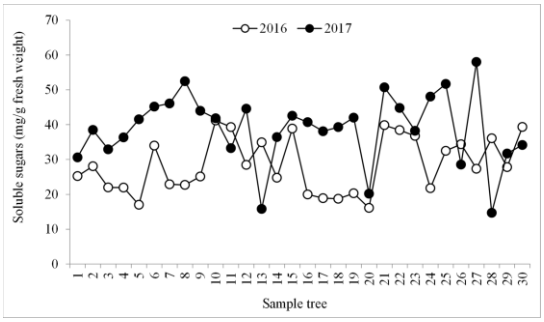

Fig. 2: Content of soluble sugar of *S. tonkinensis* sample trees

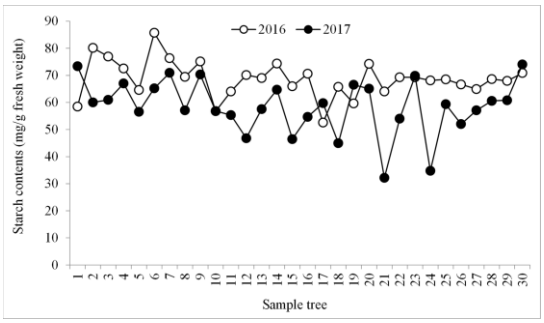

Fig. 3: Content of starch of *S. tonkinensis* sample trees

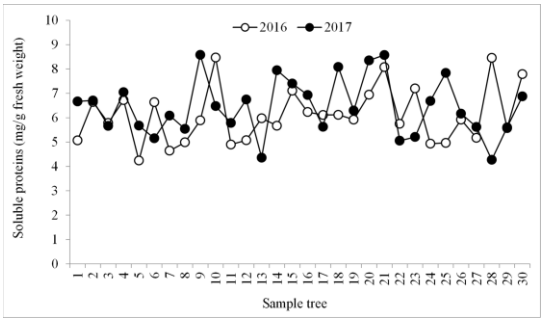

Fig. 4: Content of soluble protein of *S. tonkinensis* sample trees

content was almost lower than 10 ng/g FW in 2017. In Table 2, a negative correlation was found between GA content and the number of flowers (p<0.05 in 2016, p<0.01 in 2017). The correlation coefficients were -0.459 in 2016 and -0.497 in 2017. When the GA content was about 12 ng/g FW, the number of flowers was over 5,000 for most trees or under

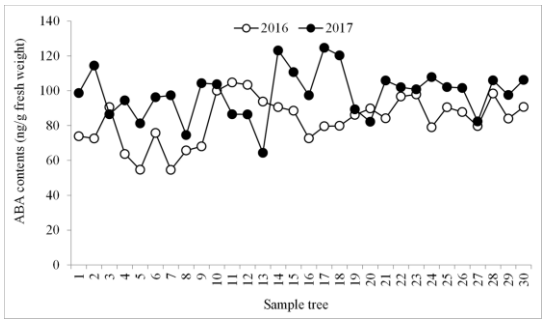

Fig. 5: Content of ABA of *S. tonkinensis* sample trees

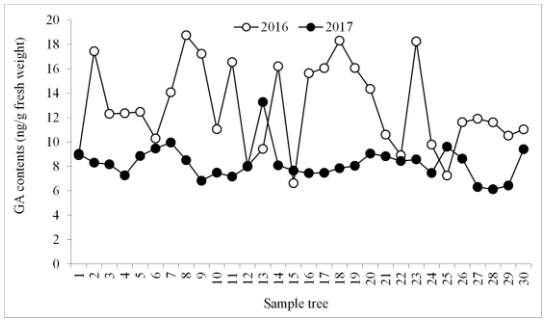

Fig. 6: Content of GA of *S. tonkinensis* sample trees

10,000 for some trees in 2016. In 2017, the GA content was between 6 ng/g FW and 10 ng/g FW. But the number of flowers was different even the content was similar.

IAA content: Fig. 7 shows IAA content in overwintering twigs of *S. tonkinensis*. In 2016 and 2017, the number of flowers correlated with the IAA content negatively with the correlation coefficients of -0.305 and -0.054 (p>0.05). In these two consecutive years, the IAA content was almost between 30 ng/g FW and 90 ng/g FW. The number of flowers was different under the similar content, indicating no obvious link between these two indicators.

Correlations between the number of flowers of *S. tonkinensis* and the contents of ABA, GA, and IAA in 2016 and 2017 were achieved (Table 2). It could be concluded that the ABA content in 2017 was about 17.75% greater than that in 2016. Narrow range of variation appeared in these two years and the GA content in 2017 was about 53.55% lower than that in 2016. The IAA content was similar in two consecutive years, but the range of variation was slightly more stable in 2017. To summarize, the number of flowers of these sample trees could increase with high ABA content and low GA content. The IAA content had no significant effect on the number of flowers of *S. tonkinensis*.

Discussion

Carbohydrates are essential nutrients in the growth of plants, which can provide energy. The processes of flower formation (Yu *et al.*, 2000), completion of flower organ differentiation (Clément *et al.*, 1996) and fruit setting

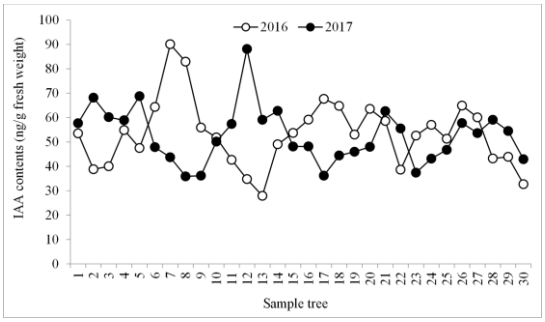

Fig. 7: Content of IAA of *S. tonkinensis* sample trees

(Iglesias *et al.*, 2003) all make use of carbohydrates. However, Ulger *et al.* (2004) pointed out that carbohydrates had no direct effect on the flowering of olive. Priestley (1977) argued that high levels of carbohydrates combined with appropriate concentrations of N in leaves could promote the flowering in olive. In our experiment, more flowers were appeared with high soluble sugar content in overwintering twigs in two consecutive years. Through correlation analysis, there was a significant positive correlation between the number of flowers and soluble sugar content, indicating that the photosynthesis in previous year of *S. tonkinensis* produced high content of soluble sugar, which could promote flowering. However, Stutte and Martin (1986) considered that soluble sugar had no effect on the flower formation of olive. The possible reason was that different tree species had different degrees of utilization of soluble sugar.

Flower organ development in woody plants was closely related to starch content (Jean and Lapointe, 2001; Ruiz *et al.*, 2001). The accumulation of starch acts as an energy source for plant flower bud initiation. Starch may be converted into soluble sugar to meet the need for metabolic energy during the process of flower bud differentiation. In our experiment, there was a negative correlation between the number of flowers and starch content in the overwintering twigs. The possible reason was that the starch produced by the photosynthesis of *S. tonkinensis* in previous year and in March and April of the next year was used for sprouting leaves, and the production of soluble sugar was used for flowering, which also increased the utilization efficiency of carbohydrates of *S. tonkinensis*. Emergence of leaves came after blooming in *Prunus* species. Thus, starch for flower development was stored in vegetative tissues because leaves did not photosynthesize (Rodrigo *et al.*, 2000).

Soluble protein is one of the most important energy source in plant flower bud initiation and the basis for flower organ morphogenesis (Zhang *et al.*, 2017). In 2016 and 2017, the number of flowers had a significant positive correlation with the soluble protein content in overwintering twigs of *S. tonkinensis*. It could be concluded that the increase of soluble protein content could help in flowering. Before the flowering of *Phalaenopsis aphrodite*, high content of soluble protein in roots, stems and leaves was essential, but the contents of soluble protein were different in each part (Wang *et al.*, 2007). This result was consistent with the study of nitrogen transport and soluble protein content in *Brassica napus* by Rossato *et al.* (2001).

The content of ABA in 2017 was higher than that noted in 2016, with more flowers respectively. Hence, we conclude that ABA could promote the flowering of *S. tonkinensis.* Studies by Koshita *et al.* (1999) showed that high content of ABA promoted the formation of *Satsuma mandarin* flower buds. However, ABA had a dual role in flowering of *Pharbitis nil* (Kulikowska-Gulewska *et al.*, 1998; Maeda *et al.*, 2000). Kinet (1993) regarded that the use of ABA could significantly stop the vegetative growth of plants, and the cessation of vegetative growth was the condition for the initiation of flower bud differentiation. Therefore, we believe that the effect of ABA on flower bud initiation was through the regulation of vegetative growth of plants.

Generally, GA is believed to inhibit flower bud growth of plants. Goldberg-Moeller *et al.* (2013) argued that GA treatment changed the expression of more than 2000 functional genes in citrus, among which the expression of 300 genes doubled at least. GA treatment also reduced the amount of mRNA of gene *FT* and *AP1* that induced apple flowering. The experimental results of Li *et al.* (2003) showed that GA_3 retarded the flower bud initiation of Bayberry by reducing IAAO and POD activity. However, GA contributed to the flowering of Cupressaceae and Taxodiaceae trees (Hashizume, 1962; Pharis and Morf, 1967). In a review article on gibberellin and cultivation measures to promote tree flowering, Pharis *et al.* (1987) pointed out GA promoted the flowering in 19 species of *Pinus* trees, and this promotion was accompanied by the branch growth. GA inhibited flowering in *S. tonkinensis* in this study. The content of GA in 2016 was higher than in 2017, but less flowers could be found in 2016. We assumed that the effect of GA on plant flowering might be related to its content and distribution in plants.

IAA may be an inhibitor of flower bud formation (Bernier, 1988). Bernier (1988) believed that the photoperiod induction of leaves was interfered by IAA. Effect of IAA in thin cell layers reduced the number of buds. Ethylene may mediate the effect of exogenous IAA. Seidlova and Khatoon (1976) also reached similar conclusion in a study on *Chenopodium rubrum*. Žárský *et al.* (1990) regarded that haploid tobacco had a higher flowering potency because of the low content of IAA. In our experiment, although the content of IAA was high, it had a little effect on flowering, which might be caused of different plant species.

Conclusion

High content of soluble sugar and soluble protein in overwintering twigs contributed to the flowering of *S. tonkinensis*. However, starch had no significant effect on flowering. High content of ABA promoted flowering,

while GA was contrary, and the effect of IAA was not obvious. Hence, we hypothesize that improving the contents of stored reserves and endogenous hormones in overwintering twigs is beneficial to the flowering of *S. tonkinensis* in the next year. Measures such as girdling, pruning, fertilizer and exogenous hormones application can be taken to realize it. We hope that our experimental results can provide guidance for regulating the flower bud differentiation and further study of *S. tonkinensis*.

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