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EFFECTS OF COLD STRATIFICATION ON THE ENDOGENOUS HORMONE, DORMANCY AND GERMINATION OF *CORNUS WALTERI* WANGER SEEDS

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Abstract

The most critical phase in plant life is the germination period, which is influenced by both intrinsic and environmental factors. Assessment of cold stratification on several endogenous hormone, IAA, abscisic acid (ABA), gibberellin $GA_{1/3}$ ($GA_{1/3}$), zeatin-riboside (ZRs) and isopen-tenyl adenine (iPAs), and germination of *Cornus walteri* Wanger. seeds was done. Relationship between endogenous hormone and seed germination and mechanism of seed dormancy of *C. walteri* were also analysed. The results showed that the significant fluctuatation of both IAA and iPAs content was fond during cold stratification period, while the variation of ZRs was little, that of both ABA and GAs content increased with old stratification days. Effects of cold stratification on both GR and GP were significant ($p < 0.05$), which play an important role in releaving of seed germination and improving seed germination. The GR and GP were significantly negatively correlated with the contents of ABA and $GA_{1/3}$, and positively correlated with the following iPAs, ZRs/ABA, iPAs/ABA.

Introduction

Cornus walteri Wanger. (Cornaceae) is a deciduous tree which mainly grows in the valley areas and hills of Korea and China (Lee *et al.* 2017). In Chinese traditional medicine, fruits and leaves of *C. walteri* have been widely utilized for the alleviation of skin inflammatory symptoms and the treatment of glycosuria (Kim *et al*. 2011, Kim *et al*. 2013, Park *et al.* 2016). Moreover, *C. walteri* is a woody oil tree, with very high oil contents in fruits (Paul and Don 2005), so the application and cultivation of this species has a rapid expansion in China recently. Tradditionly the breeding method of *C. walteri* is sowing, but the germination rate is very low without treatment before hand, which is usually a serious hurdle for production of the desired quantity of seedlings in nurseries (Kang *et al.* 2012). An understanding of seed dormancy mechanisms can be helpful in optimizing the distribution of seed germination in time or space (Nasreen *et al.* 2002). According to research there are some factors controlling seed germination and dormancy, including plant hormones, which are produced by both plant and soil bacteria, and interactions between plant hormones and plant genes affect seed germination (Miransari and Smith 2014). Research showed that hormones are the primary factors to cause seed dormancy, and ABA is the endogenous inhibitor of seed dormancy, and both GA and IAA are the corresponding germinating promoters (Carr 2009). Some scholars have also suggested that seed dormancy and germination depend on the balance between endogenous growth inhibitors and growth hormone agents (Wang *et al.* 2007). The studies regarding seed dormancy on *C. walteri* previously have been reported (Paul and Don 2005, Svendsen *et al.* 2007, Karen, *et al*. 2014), but relative mechanism has not yet been determined. Hence, further investigations on understanding possible responses, adaptations and physiological mechanisms of *C. walteri* to limit seeds germination is required.

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Materials and Methods

Fruits of *Cornus walteri*, were collected from Zijinshan National Forestry Park in Nanjing, China (lat 32°03'53" N, long 118°49'29" E), in November, 2016. The fruits were rubbed, freed from area sarcocarp, decorticated, fetched the kernel, washed in advance, and thus pure stone fruits (including endocarp, seed coat, endosperm, cotyledon, etc. were obtained. The shape of seeds was spherical, flesh pink, diameter 4.31 - 5.18 mm, the 100-grains weight was 4.33 - 4.43 g. The experiment was carried out in a laboratory belonging to Forestry Academy of Jiangsu (JAF), Nanjing China, lat 31°51'41" N, long 118°46'41" E). The seeds were conducted the cold stratification (wet sands culturing, temperature 0 - 5°C) treatment from December, 2016, the ratio of seeds and sands was 1 : 3, water content was 45%. The sands were fresh river sands which were treated with high temperature sterilization. From beginning, November, 2016, the experiments were performed in a completely randomized sampling in the middle of each month, with three replications. The whole sampling times were 7, experiment period lasted for 180 days. In every sampling, seeds were sieved, washed in cold water and dried on absorbent paper, for acquiring pure samples. One part of seeds used was for germination test, the other part for hormone determination.

The reducing of some samples was performed by quartering, then acquiring white test material (0.5 - 1.0 g), which contains embryo, endosperm and seeds skin, by scraping pericarp. After liquid nitrogen freezing, they were grinded by adding 4 ml 80% chilled methanol distillation, then shifted to 10 ml test tube, left for 4 hrs at 4°C before 15 min centrifuge separates of 3500 r/min and the resulting supernatant liquid was directly injected to test tube after centrifuge. The 1 ml extract was added to sediment, mixed them, re-extracting 1 hr at 4°C, merging supernatant and recorded volume after centrifuging. Endogenous hormone was measured experimentally by ELSA (Du *et al*. 2012, Park *et al*. 2016).

Seed germination test was completed in an artificial climate box (LRH-250-GS I, Guangzhou, China). Seeds were sown in vessel with wet sand incubator, 100-grain every vessel, covering sands for 2 - 3 cm, water was sprinkled even to ensure sand bed level and wet. Each treatment was replicated three times. Temperature was set in box at 25 - 30°C. Seeds were observed every day, germination potentiality (GP) and germination rate (GR) were recorded (USDA Forester Bureau, 1984).

The average of three measures was taken as a single value, the average value of all three replications for each treatment was compared for statistical analysis (One-Way ANOVA). The ANOVA was conducted using SAS 9.4 software (SAS Institute inc.), treatment means were compared by using LSD test at 99 and 95%, confidence interval to estimate their significance under different treatment time. The results were represented by their means ± standard deviation (SD).

Results and Discussion

Variation traits of five endogenous hormone content in seeds were studied (Fig. 1). The cold stratification significantly affected IAA, ABA, GAs and iPAs content in seeds ($p < 0.05$), while non-significant effect was observed on ZRs ($p > 0.05$). IAA content in untreated seeds was the highest (81.861 ng/g), but after cold stratification that declined despite some flutuation. The maximum IAA content (65.751 ng/g) was achieved at the 120th day after treatment while the minimum (31.141 ng/g) was attained at the 150th day. So, the IAA content contributing traits responded to the time of the cold stratification.

Different with the former, variation of ABA and $GA_{1/3}$ content was very significant ($p < 0.05$), which showed a gradual decline with the increase of cold stratification days (Fig. 1B, C), specially variation of ABA. Before the cold stratification, ABA content in seeds was 329.406 ng/g, but it

reached minimum 235.933 (ng/g) at the 150th day, which was about 0.716-fold compared with the value before cold stratification. $GA_{1/3}$ increased slowly during initial experiment stage (Fig. 1C) and showed a peak value (5.734 ng/g) at the 30th day, but the variation began to reverse since then, this tendency continued to end.

Cytokinins (CTK) are a class of plant-specific hormones that play a central role during the cell cycle and influence numerous developmental programs (Brault *et al*. 1999, Werner *et al*. 2001, Lovatt *et al*. 2006). Variation of ZRs in seeds was very little (Fig. 1D), which closed to one straight line, the cold stratification posed non-significant effect on ZRs, however slight reduction observed with the increasing treatment ($p > 0.05$). So, the effects of the cold stratification on ZRs was limited. On the contrary, the change of iPAs was significant with the cold stratification ($p < 0.05$; Fig. 1E), but the curve showed M-shaped curve, namely underwent two rises and two drops, which were recorded at the 30, 90, 120 and 180th day, respectively. Two peak values were 5.937 and 7.124 ng/g, respectively, while two valley values were 3.553 and 4.557 ng/g.

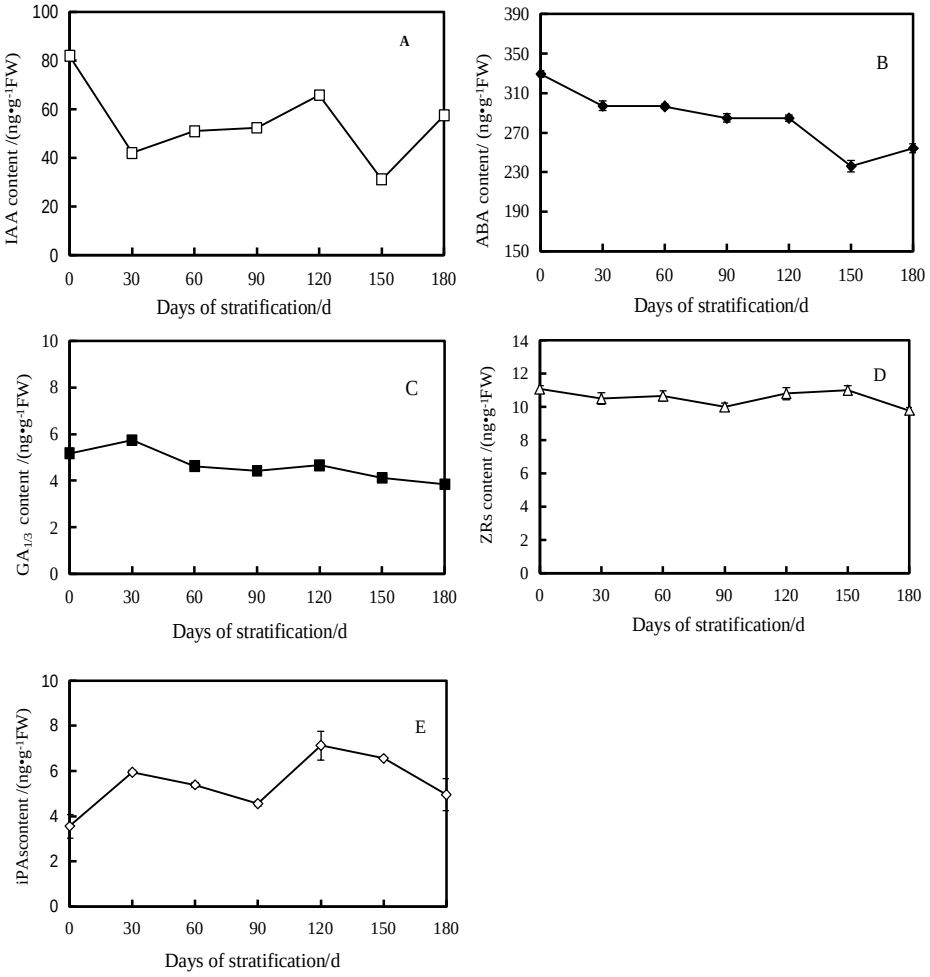

Fig. 1. Variation of endogenous hormone content in seeds: A, IAA content; B, ABA content; C, $GA_{1/3}$ content; D, ZRs content and E, iPAs content.

From Fig. 2 it is apparent that the ratio of IAA/ABA was mainly similar to IAA (Fig. 2A). That of IAA/ABA was the biggest (0.248) at the beginning of cold stratification, then descended and showed a low value (0.141). The IAA/ABA usually was raised from 30th to 120th day, but it dropped to a low dot at 150th day (0.132). Relatively, the $GA_{1/3}$/ABA was 0.015 initially, that rapidly increased with the time extention, but the ratio was steady at later period (Fig. 2B). The variation of ZRs/ABA was smooth and steady in earlier stage, but it raised slightly in later stage (Fig. 2C). The change of iPAs/ABA was similar to the iPA (Fig. 2D).

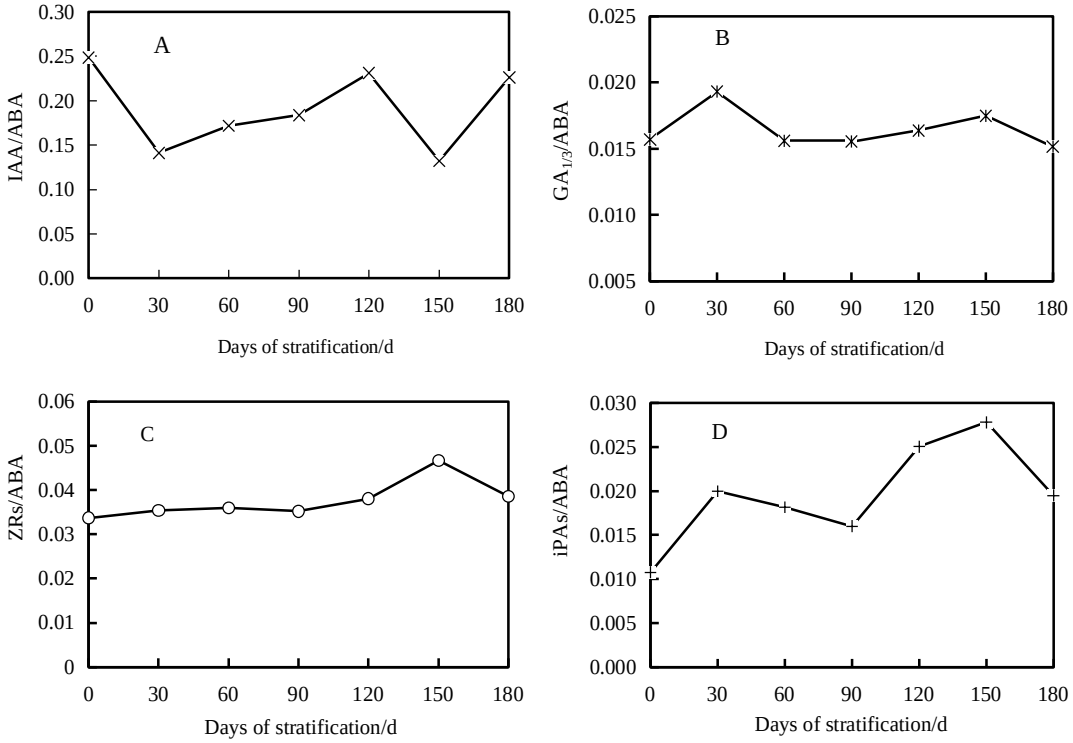

Fig. 2. Variation of IAA/ABA, $GA_{1/3}$/ABA, ZRs/ABA, iPAs/ABA in seeds during cold stratification.

Leubner and Nambara (2003) reported that ABA involved the abduction and maintained seeds dormancy to more species. ABA, which is a main factor to control seeds dormancy, played an important role in embryo development, the synthesis of protein and RNA (Han *et al.* 2002, Nambara and Marion-Poll 2003, Kermode 2005). Fu *et al.* (2009) reported that ABA can reduce the water absorbing capacity and osmotic potential of embryo, hinder extending of radicle, control the seeds germination. Above mentioned results indicated that ABA in *C. walteri* Wanger. seeds always reduced with the cold stratification, which was quite similar to the findings on *Cistanche deserticola* (Chen *et al.* 2009), *Davidia involucrate* (Lei *et al.* 2009) and *Cyclocarya paliurus* (Yang and Fang 2008). So, the variation of ABA in seeds might be the activating signal anterior to seeds germination, which is a regular physiological response to keep seeds inclusion, and plays a key role to relieve seeds dormancy.

About the function of IAA to control seeds dormancy, different scientists have different opinions. Khan (1975) thought that the effect on seeds dormancy of IAA was obvious, GA_3 could receive seeds dormancy by inhibiting ABA, increasing IAA; Kochankov (1998) reported that IAA

involved in the regulation of dormancy. Yang and Fang (2008) suggested that the IAA content in seeds of *Cyclocarya paliurus* increased during cold stratification, which was thought to be beneficial to seeds germination. On the other hand, some research showed that IAA is disadvantage to seeds germination; Gao *et al.* (1998) reported that the increase of IAA in seeds resulted from the growth of embryo, and it was not highly correlated to seeds dormancy and germination. The present study indicated that the effects of IAA on seeds dormancy of *C. walteri* Wanger. were beyond doubt, but the effects were not significant ($p < 0.05$), and further investigation is needed.

Plant hormones play central roles in the ability of plants to adapt to changing environments, by mediating growth, development, nutrient allocation, and source/sink transitions (Zvi and Eduardo 2011). The effect of hormone on plant physiological activity was not isolate, was restrict and promoted each other (Khan 1975, Horibe *et al.* 2010). Recent evidence indicated that plant hormones are involved in multiple processes, crosstalk between the different plant hormones results in synergetic or antagonic interactions that play crucial roles in response of plants to abiotic stress (Zvi and Eduardo 2011).

Wareing and Saunders (2003) suggested that $GA_{1/3}$ affected positively the receiving of seeds dormancy and germination by ABA and environment factors. This view has also been confirmed in the studies of *Lactuca sativa* (Gonai *et al*. 2004), *Styrax japonicus* (Wang *et al*. 2010), *Fraxinus mandshurica* (Ling 1986) , *Ilex purpurea* (Wang *et al*. 2010, Zhang *et al*. 2010) and so on. Bewley (1997) reported that $GA_{1/3}$ did not involve the adjustment and control of the seeds dormancy but promoted and maintained seeds germination by antagonising ABA. In the study, there was negatively correlation between $GA_{1/3}$ and ABA contents in seeds (Table 2), in spite of correlation was not significant ($p > 0.05$), such results manifested that there was certain antagonism function between ABA and $GA_{1/3}$.

Table 1. GR and GP of *C. walteri* seeds under different cold stratification.

Days of treatment/d	0	30	60	90	120	150	180
GR /%	0	5.66±2.12e	12.85±2.89d	25.43±3.13c	34.81±5.11b	36.81±5.11b	41.34±5.28a
GP/%	0	4.81±2.12e	10.23±3.34d	16.32±3.29c	23.23±4.32b	28.23±4.32b	36.13±4.89a

Data were the mean ± Sd. Different normal letters indicate significant difference by multiple comparisons between stratification treatment ($p < 0.05$).

Table 1 indicated that the effects of the cold stratification on both GR and GP were significant ($p < 0.05$), similar results were also reported on *Vaccinium myrtilloides* (Shafii *et al.* 2009, Jessica *et al.* 2017), *Pterocarya fraxinifolia* (Shafii *et al*. 2009), rose (Sandhu and Conev 2016) and so on. Seeds dormancy can also be controlled by environment and genetic factors (Syeda *et al*. 2002). Miransari and Smith (2014) reported that interactions between plant hormones and plant genes affect seed germination. Table 2 showed the correlation analysis between the endogenous hormone content, ratios and germination parameters of seeds after the cold stratification. The GR was significantly negatively correlated with $GA_{1/3}$ and ABA ($p < 0.05$), the GP was so. IAA, ZRs and $GA_{1/3}$/ABA were also negatively correlated with GR and GP, but not significant ($p > 0.05$). The iPAs, iPAs/ABA, ZRs/ABA and IAA/ABA were positively correlated with the GR and GP, despite of no significant ($p > 0.05$). Such results indicated common influence of different kinds of endogenous toseeds germination.

Table 2. The correlation matrix of five kinds of endogenous hormones and germination parameters of seeds.

Parameters	IAA	ABA	$GA_{1/3}$	ZRs	iPA	IAA/ ABA	$GA_{1/3}$/ ABA	ZRs/ ABA	iPA/ ABA	GR	GP
IAA	1.000	0.692	0.182	0.130	-0.538	0.940**	-0.554	-0.643	-0.661	-0.322	-0.331
ABA		1.000	-0.746	0.290	-0.532	0.421	-0.079	-0.875**	-0.781*	-0.870*	-0.892**
$GA_{1/3}$			1.000	0.390	-0.092	-0.105	0.604	-0.568	0.000	-0.850*	-0.867*
ZRs				1.000	0.208	-0.057	0.302	0.204	0.134	-0.388	-0.440
iPA					1.000	-0.422	0.503	0.597	0.935**	0.499	0.428
IAA/ABA						1.000	-0.668	-0.466	-0.483	0.004	0.006
$GA_{1/3}$/ABA							1.000	0.229	0.420	-0.242	-0.244
ZRs/ABA								1.000	0.832*	0.676	0.678
iPA/ABA									1.000	0.684	0.641
GR										1.000	0.979**
GP											1.000

*Correlation was significant at the 0.05 level (2-tailed), **correlation was significant at the 0.01 level (2-tailed).

Table 1 showed that both GP and GR of seeds cold stratified obviously rose, that of seed no stratification was almost zero. The difference of seeds germination which underwent the cold stratification for 120 and 150 days were not significant ($p > 0.05$), but both GR and GP increased obviously after 180 days of cold stratification, reaching 41.34 and 36.13%, respectively. Thus, the cold stratification may be an effective way to enhance seed germination. But the required time to relieve dormancy to different plants was different, such as *Taxus chinensis* var. *Mairei* about 1 year to finish physiological ripening (Tan 1991, Liu *et al.* 2011), above 60 days for *Punica granatum* (Zafer *et al.* 2007), 5 weeks for *Pterocarya fraxinifolia* (Shafii *et al.* 2009). For *C. walteri* seeds more than 150 days cold stratification is essential to maximize seed germination percentage. The present study may help to provide reference to sowing and cultivation of *C. walteri.*

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