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Dynamic Studies on Physiology and Biochemistry in American Ginseng Seed During Stratification -Part III. POD Activity, Contents of DNA and RNA, Isozymes of POD and ES-

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Abstract : Dynamic parameters of biochemistry including the POD (peroxidase) activity, contents of DNA and RNA, isozymes of POD and ES (esterase) in American seng (*Panax quinquefolium* L.) seed are reported in the present paper. The dynamic changes of POD activity proved that the PAS (physiological afterripening stage) is a stage in which some substances are prepared for seed germination. The POD activity correlated with ER (embryo ratio) significantly. DNA content changed little only within 0.0036~0.013 mg/m*l*, which did not correlate with ER. RNA content changed from 0.1539 to 1.0313 mg/m*l* and correlated significantly with RE during all of the embryo afterripening. None of the POD isozyme band was obtained in ESGS (embryo slowly growth stage), but five bands in ERGS (embryo rapidly growth stage) and six bands in PAS. Four bands of ES isozymes were obtained in ESGS, but six bands in ERGS, particularly, the content of ES isozymes increased in PAS. All of these may provide some information for understanding the dormancy mechanisms of American seng seed.

Key words : *Panax quinquefolium* L, seed, biochemistry, dormancy, stratification, afterripening.

Introduction

American seng (*Panax quinquefolium* L.) originated from North American has been recorded in China since 1757 and planted in China since 1976.[1] As we know, American seng is propagated by seed and the seed is with dormancy property.[2] Up to now, only little is known about the dormancy mechanisms of American seng seed.[3,4] Although some endogenous germination inhibitors,[5,6] exo- and endogenous germination promoters[7~12] and hastening methods for germination[13] were studied, no more paper was found in the research of physiology and biochemistry during American seng seed stratification. For understanding the dormancy mechanisms, the dynamic parameters of embryo ratio (ER), dry weight ratio (DWR) and respiration rate (RR) were investigated in Part I[14] and the contents of soluble carbohydrate (SC), crude fat (CF), fatty acid (FA) and soluble protein (SP) were explored in Part II (will be published soon on *Korean J. Ginseng Sci.*) of this series papers. Continuously, the information about POD activity, contents of DNA and RNA, isozyems of POD and ES is studied in the present paper.

Materials and Methods

1. Seeds

All of the seeds used in our experiments were harvested from four-year-old American seng plants in middle September, 1992 on Huafu Ginseng Farm of Jilin Agricultural University,

Changchun, China. The fruit (berries) were hand-harvested and mechanically depulped. The seeds were washed with water and dried in shade. Weight of 1000 seeds was 61.25±0.07 g, water content of seed was 43.34±0.21% and seed vitality by TTC method[15] was 96%.

2. Seed Stratification

The fresh seeds were mixed with mortar sand (1 vol seed/4 vol sand with about 10% moisture) and then stratified in an incubator successively at 20±1℃ (0~80 days), 13±1℃ (81~180 days) and 3±1℃ (181~260 days).

3. Peroxidase (POD) Activity

The endosperm (1 g) with embryo was homogenized with a little water, fixed to 100 m*l* and centrifuged (4000 rpm, 5 min). Then, the supernatant fluid (1 m*l*) was mixed with 3 m*l* reaction solution [0.2 M phosphate buffer (pH 6.0) 50 m*l*, hydrogen peroxide 0.028 m*l* and guaiacol 0.019 m*l*] and the time was recorded immediately. Five minutes later, optical density (OD) was measured at 470 nm with "722" Optical densitometer and the 0.1 M phosphate buffer (pH 6.0) was used as 0 point control. POD activity was expressed by ΔOD 470 $min^{-1}g^{-1}$ (Fresh weight).

4. DNA and RNA Contents

The extract of the nucleic acid was obtained with the routine methods.[16] And then, the OD of the extract was measured at 260 nm and 280 nm with a "751" Optical densitometer (Beijing Analytic Instrument Plant, Beijing, China) and the heated 0.5 M perchloric acid was used as 0 point control.

Total nucleic acid (mg/m*l*) = 0.0629 A 260 - 0.036 A 280

The standard curve was made by OD and standard DNA concentration from 0.2 to 20 μg/m*l*.

The extract (1 m*l*) was mixed with 2 m*l* solution of DPA (diphenylamine) and ethanol (20 m*l* DPA : 0.1 m*l* ethanol). After 24 hrs under the room temperature, the OD of DNA was measured at 600 nm with "722" Optical densitometer and the DNA content could be obtained according to the standard curve. Therefore, the RNA content could be got by using the content of total nucleic acid to minus DNA content.

5. POD Isozymes

Three seeds without seed coat were homogenized with 1.5 m*l* reaction solution (16% saccharose, 2% VC and 0.25% mercaptoethanol). After centrifugation at 15000 rpm, 15 min, 20~30 μ*l* supernatant was applied for GE (gel electrophoresis) with a sample applicator. DYY-III Electrophoresis apparatus (Beijing 61 Instrument Plant, Beijing, China) was used with 20 mA, 150~500 V for 4~6 hrs.

Separating gel : T = 10%, C = 2%, Tris = 36.6 g, TEMED = 0.6 μ*l*/m*l* gel solution, Acr = 28 g, Bis = 0.74 g, APS = 0.14% and distilled water = 0.125 m*l*/m*l* gel solution.

Enriching gel : T = 3%, C = 2%, Tris = 5.98 g, TEMED = 1.5 μ*l*/m*l* gel solution, Acr = 10 g, Bis = 2.5 g, hepatoflavin=4 mg and distilled water = 0.5 m*l*/m*l* gel solution.

Where

T = (Acr + Bis)/100 m*l* × 100%

C = Bis/(Acr + Bis) × 100%

Acr = Acrylamide

Bis = Methylene-bis-acrylamide

Tris = Tris-hydroxy-methyl-aminomethane

TEMED = Tetramethyl-ethylene diamine

APS = Ammonium persulfate

Staining reagent : VC 70.4 mg, BD mother liquor [2 g BD (benzidine), 18 m*l* glacial acetic acid] 20 m*l*, 0.6% H_2O_2 20 m*l* and distilled water 60 m*l*.

Rf = (De × Lb) / (Dd × La)

Where

Rf = Rate of flow

De = Moving distance of enzyme band

Dd = Moving distance of dye

Lb = Gel length before dyeing

La = Gel length after decolorizing

6. ES Isozymes

Staining reagent : Fast blue-RR salt (100 mg) and 3 m*l* acetone were fixed to 100 m*l* with buffer (10 m*l* 0.2 M Na_2HPO_4+50 m*l* 0.2 M NaH_2PO_4, and then mixed with 30 mg α-NAA (naphthalene acetic acid) with 1 m*l* acetone and 60 mg β-NAA with 1 m*l* acetone. Others were exactly the same

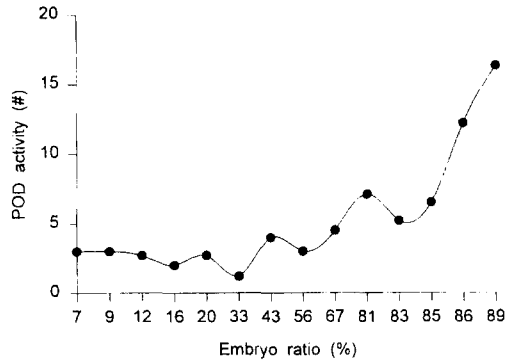

Fig. 1. Dynamic changes of POD activity with ER. (#) = (10^{-2} OD 470/min g). Embryo ratio and POD activity were tested once every 20 days.

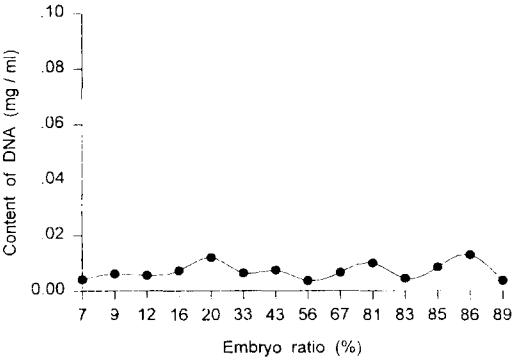

Fig. 2. Dynamic changes of DNA content with ER. Embryo ratio and content of DNA were tested once every 20 days.

as mentioned for POD isozymes.[17]

The parameters of POD activity, DNA and RNA contents, POD and ES isozymes were tested once every 20 days during the seed stratification and all statistical analyses were carried out by using the SYS program (SAU, Liaoning, China).

Results and Discussion

1. Dynamic Changes of POD Activity

POD activity showed a increasing tendency with fluctuation during the embryo afterripening. Particularly, after ER arriving to 85.10%, POD activity showed a straightly increasing tendency and reached the highest point before seed germination (Fig. 1).

The regression between POD activity and ER was:

$$Y = 9.6177 - 1.1475X + 5.362 \times 10^{-2}X^2 - 9.289 \times 10^{-4}X^3 + 5.377 \times 10^{-6}X^4 \quad (R^2 = 0.8636)$$

$$r = 0.9293 > r_{0.01} = 0.8610$$

The changes of POD activity correlated significantly with ER. This indicated that the POD activity increased with the embryo growth. The dynamic changes of POD activity in American seng seed during embryo afterripening were just similar to that in apple seed[18], Korean pine seed[19] and Oriental ginseng seed.[20] It was reported[21] that POD could activate PPP (pentose phosphate pathway) which has the function for relieving seed dormancy. PPP provided not only NADP (nicotinamide ademine dinucleotide phosphate) but also many kinds of intermediate products which could be used as the materials for the biosynthesis of embryo. In our experiments, the strong activity of POD occurred just in PAS, *i.e.* in the rapidly increasing stage of embryo weight.[14] We may consider that PAS is a stage in which some essential substances are prepared for American seng seed to break dormancy and promote germination.

2. Dynamic Changes of DNA Content

DNA content changed little only within 0.0036~0.013 mg/ml (Fig. 2).

The regression between DNA content and ER was:

$$Y = -6.365 \times 10^{-4} + 1.884 \times 10^{-3}X - 7.426 \times 10^{-5}X^2 + 1.08 \times 10^{-6}X^3 - 5.272 \times 10^{-9}X^4 \quad (R^2 = 0.3272)$$

$$r = 0.5720 < r_{0.05} = 0.7860$$

The DNA content did not correlated with ER. In generally speaking, the DNA content in cell is relatively steady.[22] The change tendency of DNA content in American seng seed during embryo afterripening was similar to that in Oriental ginseng seed.[23] The changes of DNA content related to the cytokinesis.[21] In the MAS (morphological afterripening stage), on one hand, the DNA content increased gradually with the cytokinesis of embryo cells; On the other hand, a great amount

of endosperm cells were consumed, the DNA in endosperm cells were decomposed as well. The both sides mentioned above made a dynamic equilibrium of DNA content occur during MAS. Therefore, in the PAS, ER increased very slowly but DWR increased rapidly. This indicated that the quantity of cytokinesis decreased but only the volume of embryo cells increased. So that, the DNA content was still not changed much.

3. Dynamic Changes of RNA Content

The statistics result showed that RNA content correlated significantly with ER. The regression equation was:

$$Y = -0.9276 + 0.1023X - 2.638 \times 10^{-3}X^2 - 1.859 \times 10^{-5}X^3 \quad (R^2 = 0.6012)$$

$$r = 0.7754 > r_{0.05} = 0.7260$$

As can be seen from Fig. 3, the dynamic changes of RNA content could be divided as four phases as following:

(1) The 1st phase

The RNA content was not changed much when ER was from 7.31 to 8.50%. At the beginning of the seed stratification, the substances in American seng seed had not been transformed yet. Particularly, the inhibitory influence originated from pulp had still not been eliminated, the biosynthesis of RNA was inhibited.

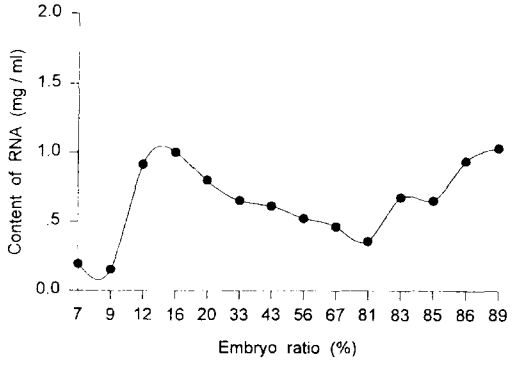

Fig. 3. Dynamic changes of RNA content with ER. 7.31~8.50% = The 1st phase (RNA content from 0.1959~0.1539 mg/m*l*); 8.50~16.20% = The 2nd phase (RNA content from 0.1539~1.0027 mg/m*l*) : 16.20~80.98% = The 3rd phase (RNA content from 1.0027~0.36 mg/m*l*); 80.98~88.50% = The 4th phase (RNA content from 0.36~1.0313 mg/m*l*). Embryo ratio and content of RNA were tested once every 20 days.

(2) The 2nd phase

The RNA content increased from 0.1539 to 1.0027 mg/m*l* when ER was from 8.50 to 16.20%. At the same time, the respiration rate and embryo dry weight increased as well. The increasing RNA content may promote the biosynthesis of some enzymes which may carry out morphological formation and respiration in the early stage of MAS.

(3) The 3rd phase

When ER was from 16.20 to 80.98%, the RNA content showed a decreasing tendency. This was just in the late stage of MAS. The morphological afterripening of embryo nearly finished. Therefore, the degrading speed of RNA in endosperm may be higher than the synthesizing speed of RNA in embryo. This may make the RNA content decrease in this stage.

(4) The 4th phase

In PAS (ER from 80.98 to 88.50%), RNA content increased from 0.36 to 1.0313 mg/m*l* with the increase of RR, POD activity and DWR. The increasing of RNA content may promote the formation of a great amount of hydrolytic enzymes in endosperm and synthetases in embryo which may participate in the transferring process of substances from endosperm to embryo. The increasing phenomenon of RNA content during PAS occurred not only in American seng seed but also in *Fraxinus excelsior* seed[24] and Oriental ginseng seed.[23]

4. Dynamic Changes of POD Isozymes

No POD band was found during ESGS (ER from 7.31 to 20.48%) (see Fig. 4 A). There may be two reasons. The first reason was that the contents of some phenolic substances was high at the beginning of stratification (Three kinds of phenolic substances were identified from American seng seed by the present authors and that will be published soon). It was reported that the phenolic substances could disturb the expression of POD activity.[25] The second reason could be that the temperature in ESGS was high (20±1℃)

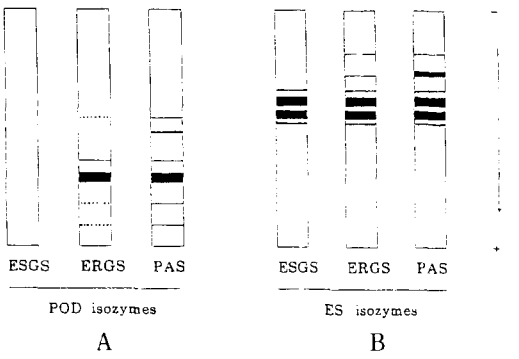

Fig. 4. Dynamic changes of POD and ES isozymes during seed stratification. ESGS = 0~80 days (20±1℃), ER from 7.31~20.48%; ERGS = 81~180 days (13±1℃), ER from 20.48~80.98%; PAS = 181~260 days (3±1℃), ER from 80.98~88.50%. POD and ES isozymes were tested once every 20 days.

and the bands of some isozymes could not be obtained under high temperature.[26]

When ER was from 32.67 to 66.97% (ERGS), five bands of POD isozymes could be obtained. Band 3 (Rf = 0.68) and band 4 (Rf = 0.62) formed the main band area where the bands were wide and with deep color. While, the band 1 (Rf = 0.90), band 2 (Rf = 0.77) and band 6 (Rf = 0.51) formed the secondary band area where the bands were narrow and not clear. The temperature in this stage was 13±1℃ and the low temperature may induce the kinds and contents of POD isozymes increase. While on the other hand, some phenolic substances may be disintegrated and their concentrations may be decreased. As a result, the bands of POD isozymes increased evidently.

In PAS (ER : 80.90~88.50%) with the temperature of 3±1℃, the bands of POD isozymes increased from five to six. Band 3 and 4 in main band area were not changed but band 1, 2 and 6 became more clear than before. In addition, the band 5 (Rf = 0.55) occurred. The zymogram change pattern of POD isozymes in American seng seed during stratification was similar to that in Oriental ginseng seed.[27]

5. Dynamic Changes of ES Isozymes

As shown in Fig. 4 B, the band 1, 2, 3 and 4 (Rf = 0.52, 0.49, 0.48 and 0.45, repectively) formed the main band area and existed from the beginning until to the end during all of the seed stratification. However, band 5 and 6 (Rf = 0.38 and 0.33) formed the secondary band area and occurred from ERGS.

There were four bands of ES isozymes in ESGS (20±1℃) but six bands in ERGS. We should pay attention to that the band 5 and 6 occurred only when the temperature decreased to 13±1℃. This makes us understand that the low temperature in ERGS may be advantageous to the kind increase of ES isozymes which promoted fat metabolism and made CF content decrease (see Fig. 2 in Part II) and FA content accumulate (Fig. 3 in Part II). Furthermore, the abundant intermediate products produced in fat metabolism made embryo grow rapidly in ERGS.[14]

After into PAS (3±1℃), the bands of ES isozymes were still six but band 5 widened evidently and the color deepened as well. This indicated that the content increase of ES isozymes which made CF content decrease continuously (Fig. 2 in Part II). The intermediate products in fat metabolism were transferred into embryo and used as both the respiratory ground substances and the materials for histological formation. Finally, the embryo got into the rapidly increasing stage of embryo weight[14] with the FA content decreasing (Fig. 3 in Part II), RR increasing[14], POD activity increasing and RNA content increasing to prepare enough substances and energy for breaking dormancy and promoting the germination of the American seng seed.

Conclusions

From these experiments one can conclude that ① POD activity showed an increasing tendency with fluctuation and the highest peak occurred before seed germination; ② DNA content changed little and did not correlate with ER; ③ The dynamic changes of RNA content could be divided as four phases and correlated significantly with that of ER; ④ Bands of POD isozymes were

0, 5 and 6 in ESGS, ERGS and PAS, respectively and ⑤ The kinds and contents of ES isozymes increased with the temperature decreased during the stratification of American seng seed.

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