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EFFECTS OF STRATIFICATION AT DIFFERENT TEMPERATURES ON THE EMBRYO DIFFERENTIATION AND DEVELOPMENT OF *PARIS POLYPHYLLA* VAR. *YUNNANENSIS* SEEDS

YU YE, SHU-YING CHEN, LI WANG*, YAN-QIONG YANG AND CHANG-LING ZHAO

College of Agronomy and Biotechnology, Yunnan Agricultural University, Kunming 650201, China
** Corresponding author E-mail: wangli5820840@yahoo.com.cn*

Abstract

Effects of stratifications at 6 temperatures on the embryo differentiation and development of *Paris polyphylla* var. *yunnanensis* seeds were studied by using photomicrography based on paraffin section. The results indicated that there were striking differences among the differentiating and developing processes of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified under different temperatures. The stratifications alternating between 15ºC and 20ºC promoted the embryo differentiation and development best, next are the stratifications at 15ºC and 20ºC, the stratification at 10ºC did not display obvious effects and the stratifications at 5ºC and 25ºC showed inhibiting effects to some extent. Additionally, the embryos differentiated and developed slowly under natural conditions. This research could provide a basis for the dormancy-breaking of *Paris polyphylla* var. *yunnanensis* seeds.

Introduction

As a rare and valuable genuine medical material in Yunnan Province of China, *Paris polyphylla* var. *yunnanensis* (Franch.) Hand.-Mazz is also popularly named "Chonglou yizhijian", "Dujiaolian", "Qiye yizhihua", etc., (Li, 1988). Holding many pharmacological activities e.g., hemostasis, anti-tumor, analgesia, immunoloregulation and so on (Yuan *et al.*, 2003), *P. polyphylla* var. *yunnanensis* is the key raw material of many Chinese patent medicines e.g., "Yunnan baiyao" and "Gongxuening" etc., (Ji *et al.*, 2001). With the rapid development of the industry of Chinese medicinal materials, the requirement of *P. polyphylla* var. *yunnanensis* produced by the manufacturers is increasing 20% per year (Zhang *et al*., 2004), which leads to the severe decrease and deficiency of the wild resources of *P. polyphylla* var. *yunnanensis* and greatly hampers the yields of the pharmaceutical manufacturers and the quality of the products (Li & Yang, 2005).

However, the propagation of *P. polyphylla* var. *yunnanensis* had become a severe problem. Naturally, *P. polyphylla* var. *yunnanensis* reproduces mainly by stem tubers or seeds (Li, 1988). Now in practice, *P. polyphylla* var. *yunnanensis* is chiefly reproduced to seedlings by using wild stem tubers (Zhang *et al*., 2004), which is bound to occupy large quantity of tubers and intensifies the consuming contradiction between the cultivation and medicine-producing of the tubers. Instead, holding higher propagation coefficients, seedling-reproducing based on seeds has been an economical and pragmatic method which suits to be broadly used in the large scale production of seedlings. Nevertheless, since 1980s, *P. polyphylla* var. *yunnanensis* seeds were proved to germinate difficultly because of their deep dormancy and the treatments of different temperatures had been confirmed to be useful to break the seed dormancy. Li (1986a) observed, being sowed under the natural conditions, *P. polyphylla* var. *yunnanensis* seeds germinated after two winters and one summer, being accompanied by the low germination ratio and the death of a large quantity of seeds in the dormancy process, indicating that *P. polyphylla* var. *yunnanensis* seeds were typical seeds with "secondary dormancy". Li (1986b) further used "two heterotherm method" to break the dormancy of *P. polyphylla* var. *yunnanensis* seeds. Yang *et al.*, (2003) thought the incomplete differentiating and developing embryos, hard endosperms and after-ripening embryonic axes made *P. polyphylla* var. *yunnanensis* seeds possess the characteristics of double dormancy. Yuan *et al.*, (2003) shortened the germination time of *P. polyphylla* var. *yunnanensis* seeds by treating the seeds with two low temperatures alternating two high temperatures and at 18~20ºC, the roots germinated most early, the root-germinating ratio was the highest and the radicles grew most rapidly. Zhou *et al.*, (2003) confirmed *P. polyphylla* var. *yunnanensis* seeds remained dormant for 18 months or longer in natural environments, the most effective stratification scheme was an interval of 14 days at 4°C and 14 days at 22°C. Chen *et al.*, (2007) found, when *P. polyphylla* var. *yunnanensis* seeds were harvested, there was only one undifferentiated, round tiny embryo in every seed and the development and germination of the embryos were closely related to temperature. Huang *et al*., (2008) confirmed the embryos of *P. polyphylla* var. *yunnanensis* seeds developed slowly, stayed at the proembryo stage and did not proliferate any longer at about 120 d after fertilization; the fact that the embryos stagnated at the globular stage during development indicated that the seeds held the property of morphological after ripening. Taken together, although how to efficiently break the dormancy of *P. polyphylla* var. *yunnanensis* seeds is very important for the industrial propagation of *P. polyphylla* var. *yunnanensis*, for the stratification of *P. polyphylla* var. *yunnanensis* seeds, a detailed characterization of the temperatures to benefit the dormancy-breaking of the seeds is urgently needed.

In this research, for the first time, six temperatures were designed to stratify the *P. polyphylla* var. *yunnanensis* seeds with the aim to explore the effects of the stratifications at different temperatures on the embryo differentiation and development of the seeds, and the results could provide a reference for the dormancy-breaking of the seeds.

Materials and Methods

Plant material: *P. polyphylla* var. *yunnanensis* seeds were collected from the Heqing County of Dali Eparchy of Yunnan Province of China.

Stratifications at different temperatures on the seeds: Fifteen aliquots with 100 grains per aliquot of the seeds were selected randomly. In stratification, 6 temperatures, i.e., 5ºC, 10ºC, 15ºC, 20ºC, 15ºC alternating with 20ºC, and 25ºC, were designed and the alternate treatment was performed day and night, i.e., 12 h of 15ºC alternating with next 12 h of 20ºC. After being mixed with matrix, the seeds were placed in a climatic cabinate where the relative humidity was maintained at 60%. As a control (CK), an aliquot of the seeds was sowed under the natural temperature conditions in the Germplasm Resource Garden of Chinese Medicinal Materials of Yunnan Agricultural University. All treatments were triplicated.

Paraffin sectioning and microphotograph of the embryos: The paraffin sections of the embryos of the stratified seeds were made according to the standard procedure. Observation

and photograph-taking of the paraffin sections were performed on a Nikon E200 microscope once every 30 days and lasted four months, all photos were analyzed by using software Motic Digiclass. The lengths of the embryo and endosperm were determined by using the vernier caliper in the microscope, the embryo-emerging ratio was calculated (=Embryo length/ Endosperm length) and the correspondent statistical analysis was accomplished by using software DPS.

Results

Proembryo of *P. polyphylla* var. *yunnanensis* seeds: When *P. polyphylla* var. *yunnanensis* seeds dispersed from parent plants, there was only one undifferentiated, round embryo, i.e., proembryo, in a seed (Fig. 1). The embryo length and embryo-emerging ratio of the proembryo were 0.25±0.03 mm and 5.65±0.76% respectively, showing that the embryo was only a clump of undifferentiated cells and the seeds did hold the property of the morphological after ripening of embryos, which is in good agreement with the results reported by Huang *et al*., (2008).

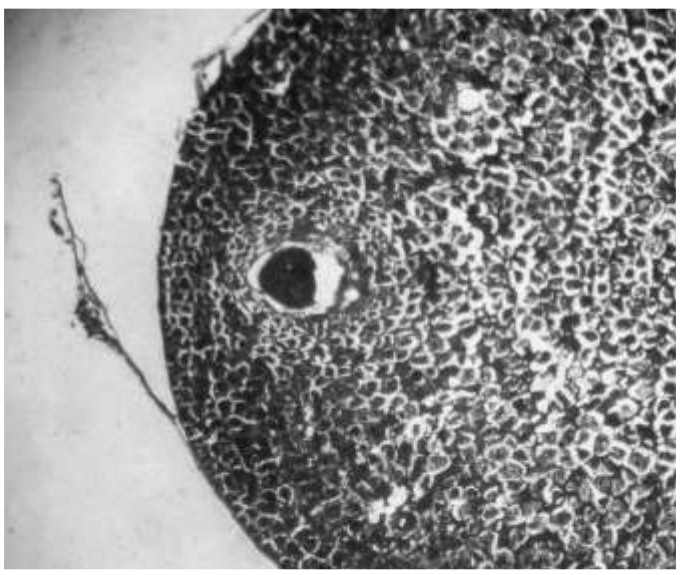

Fig. 1. Proembryo of *P. polyphylla* var. *yunnanensis* seed.

Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 30 days: After 30 days of the stratifications, the embryos became somewhat longer than the proembryos. However, there were obvious differences among the embryo lengths and the embryo-emerging ratios resulting from different stratifying temperatures (Fig. 2A~G and Table 1). The seeds stratified at 15ºC alternating with 20ºC differentiated and developed most rapidly, being evidenced by the entry into the later stage of globular embryo whose length was 1.06 mm and increased 0.57 mm than the CK (Fig. 2A~D and Table 1). The seeds stratified at 20ºC had the slower speed of differentiation and development, being indicated by the formation of globular embryo whose length was 0.77 mm and increased 0.28 mm than the CK (Fig. 2E and Table 1). The seeds stratified at 15ºC began to differentiate and develop and just entered into the early stage of globular embryo whose length was 0.62 mm and increased 0.13 mm than the CK (Fig. 2C and Table 1). On the whole, the increases of the embryos of the seeds stratified at above three temperature conditions with respect to the CK all reached the extremely significant level. However, the stratifications at 5ºC and 25ºC showed inhibiting effects on the embryo development because the embryos differentiated and developed more slowly than the CK, and the embryo length was 0.67 and 0.80 fold of the CK respectively, reaching the extremely significant level (Table 1). In fact, there were no apparent changes for the sizes and shapes of the embryos of the seeds stratified at 5ºC and 25ºC, indicating that the embryos still stayed at the proembryo stage (Fig. 2A, F).

Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 60 days: 60 days of the stratifications at different temperatures made the embryo lengths and the embryo-emerging ratios of *P. polyphylla* var. *yunnanensis* seeds increase manifestly. In particular, the embryos of the seeds stratified at 15ºC alternating with 20ºC, 15ºC and 20ºC grew greatly, reaching the extremely significant level (Fig. 3 A~G and Table 2). For the seeds stratified at 15ºC alternating with 20ºC, the radicles had broken through the episperms, the embryo length was 2.48 mm, increased 1.82 mm than the CK, and the embryo-emerging ratio reached 50.86%. Additionally, the radicles and cotyledons differentiated and developed obviously, being evidenced by the visible pericycles and cylindrical embryos (Fig. 3D and Table 2). For the seeds stratified at 20ºC, the embryos differentiated and developed comparatively quickly, the radicles grew strikingly, the embryo length was 2.09 mm, ascended 1.42 mm than the CK, and the embryo-emerging ratio was 41.63%. Besides, the radicles were about to break through the episperms. The cotyledons were approximately as long as half of the embryo, ruts appeared at the embryo bases, and plumules began to form, indicating that the cotyledons had differentiated and developed clearly (Fig. 3E and Table 2). For the seeds stratified at 15ºC, the obviously developed radicles, epicotyls, hypocotyls and cotyledons could be easily found on the embryos. The embryo length was 1.52 mm, raised 0.85 mm than the CK, and the embryo-emerging ratio was only 30.42%. The comparatively long suspensors formed too. But the embryos were still buried deeply in the endosperms (Fig. 3C and Table 2). Thus, it is evident that the development of all the organs of the embryos has begun when the radicles do not break through the episperms, and with the elongation of the embryonic axes, the organs further develop and consummate. Moreover, the embryos of the seeds stratified at 10ºC just started to differentiate and develop, entering into the early stage of globular embryo (Fig. 3B). Except the increase of the cell number and the enlargement of the embryo volume to some extent, there were not other manifest changes in the embryos of the seeds stratified at 5ºC and 25ºC, and the growing speed of the embryos was much slower than the CK (Fig. 3A, F). As a result, temperature is one of the important factors which break the dormancy of *P. polyphylla* var. *yunnanensis* seeds and promote the development of the embryos of the seeds.

Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 90 days: The embryo-emerging ratios of the *P. polyphylla* var. *yunnanensis* seeds stratified at 15ºC alternating with 20ºC, 15ºC and 20ºC were 57.65ºC, 86.87 ºC and 124.81ºC respectively and all of the radicles had broken through the episperms. However, in contrast to the CK, the embryos of the *P. polyphylla* var. *yunnanensis* seeds stratified at 5ºC, 10ºC and 25ºC commenced differentiating and developing tardily, being indicated by the embryos which were deeply buried in the endosperms (Fig. 4 and Table 3). The embryo lengths of the seeds stratified at 15ºC alternating with 20ºC reached 6.28 mm, approximating 7 fold of the CK. The radicles had broken through the episperms and grown 2~3 mm outward, and the cotyledons completely formed (Fig. 4E and Table 3). The radicles of the seeds stratified at 20ºC broke

through the episperms just now, and shrank to become pieces of thick stemlets where the obvious bud-like enations, i.e., the developing terminal buds, could be found. The miniature forms of cotyledons appeared and were deeply buried in the endosperms. In total, the differentiating and developing speed of the embryos was much quicker than the CK, reaching the extremely significant level (Fig. 4E). The well differentiated and developed radicles of the seeds stratified at 15ºC also broke through the episperms and displayed manifest shrinking tendency, being followed by the quickly grown hypocotyls. The embryo length was 2.74 mm, increased 1.86 mm than the CK, reaching the extremely significant level. Compared with the CK, the embryo-emerging ratio had reached 57.65%, showing that the embryos developed much rapidly (Fig. 4C and Table 3). The embryos of the seeds stratified at 10ºC entered the later stage of globular embryo. The basal and apical cells of the embryos differentiated to turn into conic embryos, and the embryo length was 1.29 mm, increased 0.41 mm than the CK, reaching the extremely significant level (Fig. 4B and Table 3). Finally, the embryos of the seeds stratified at 5ºC, 25ºC began to differentiate, being evidenced only by the increase of the cell number and the enlargement of the embryo volume and not by any obviously differentiated organs. The embryo lengths were 0.79 and 0.81 fold of the CK respectively, and the differences did not reach the significant level (Fig. 4A, F and Table 3).

Table 1. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 30 d.

Stratifying temperature (ºC)	Embryo length (mm)	Embryo-emerging ratio (%)	α=0.05	α=0.01
5	0.34 ± 0.04	6.63 ± 1.42	e	E
10	0.53 ± 0.03	11.44 ± 0.82	d	D
15	0.62 ± 0.03	12.45 ± 2.66	c	C
20	0.77 ± 0.03	15.23 ± 4.63	b	B
15/20	1.06 ± 0.03	20.82 ± 5.41	a	A
25	0.37 ± 0.021	7.81 ± 0.88	e	E
CK	0.49 ± 0.02	9.84 ± 2.42	d	D

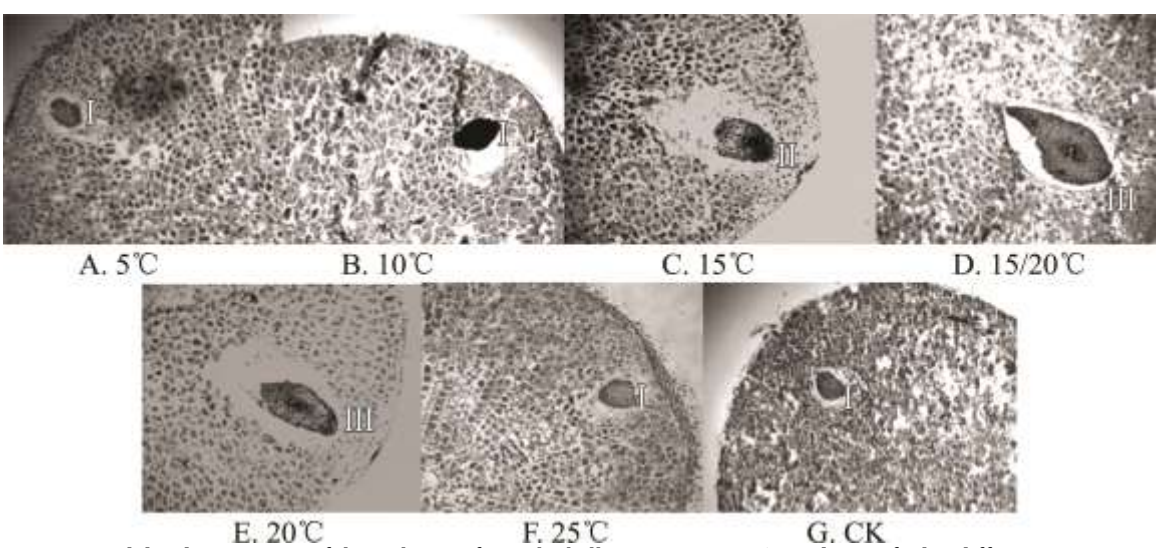

Fig. 2. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 30 d. I. The embryo began to differentiate. II. Early stage of globular embryo. III. Latter stage of globular embryo.

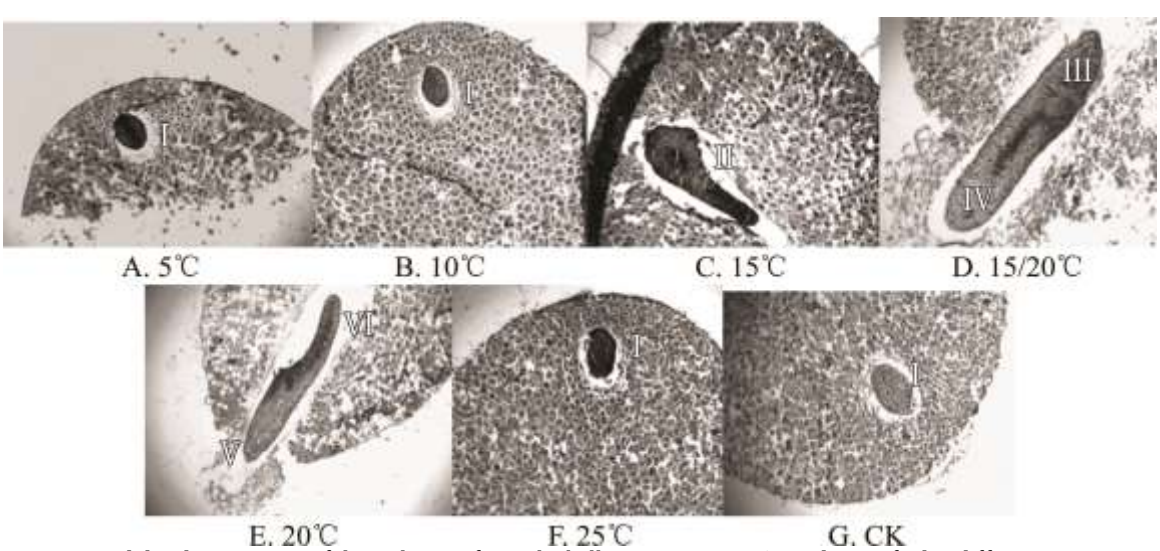

Fig. 3. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 60 d. I. Embryo began to differentiate. II. The cells at the embryo base divided to form the conic radicle. III. The apical cells of the embryo started to grow (the cotyledon will form). IV. The radicle was about to break through the episperm. V. The radicle broke through the episperm. VI. The apical cells of the embryo started to grow, the epicotyl elongated manifestly.

Table 2. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 60 d.

Stratifying temperature (ºC)	Embryo length (mm)	Embryo-emerging ratio (%)	α=0.05	α=0.01
5	0.51 ± 0.02	10.21 ± 3.43	e	D
10	0.63 ± 0.03	12.66 ± 2.22	de	D
15	1.52 ± 0.02	30.42 ± 3.47	c	C
20	2.09 ± 0.20	41.63 ± 7.45	b	B
15/20	2.48 ± 0.07	50.86 ± 11.23	a	A
25	0.49 ± 0.02	9.83 ± 2.20	e	D
CK	0.67 ± 0.02	12.65 ± 1.82	d	D

Table 3. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 90 d.

Stratifying temperature (ºC)	Embryo length (mm)	Embryo-emerging ratio (%)	α=0.05	α=0.01
5	0.72 ± 0.03	14.22 ± 2.61	e	E
10	1.29 ± 0.05	26.84 ± 2.41	d	D
15	2.74 ± 0.16	57.65 ± 6.63	c	C
20	4.31 ± 0.10	86.87 ± 10.63	b	B
15/20	6.28 ± 0.20	124.81 ± 42.44	a	A
25	0.74 ± 0.04	14.65 ± 0.81	e	E
CK	0.88 ± 0.07	18.12 ± 1.43	e	E

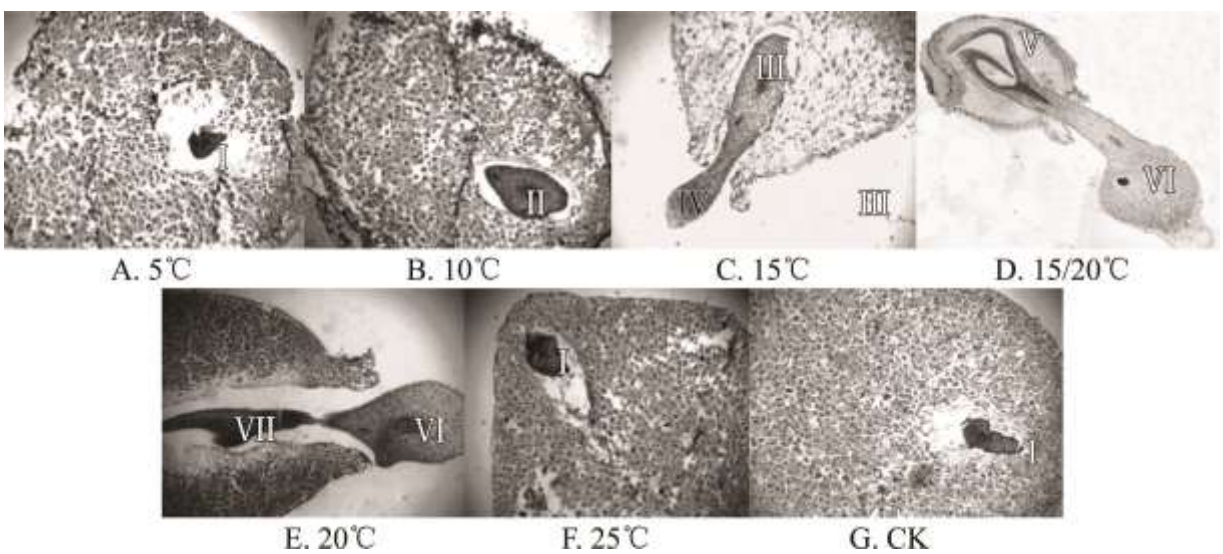

Fig. 4. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 90 d. I. Embryo began to differentiate. II. Latter stage of globular embryo. III. The apical cells of the embryo started to grow (the cotyledon will form). IV. The radicle had broken through the episperm. V. The miniature form of cotyledon had formed and was deeply buried in the endosperm. VI. Radicle. VII. The cotyledon had formed and was deeply buried in the endosperm.

Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 120 days: There were extremely significant differences among the differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 120 days (Fig. 5 and Table 4). The seeds stratified at 15ºC alternating with 20ºC differentiated and developed best. The embryo length was 13.06 mm, approximating 11 fold of the CK and reaching the extremely significant level. The cotyledons were about 3 mm long, the endosperms had disappeared totally, and the zigzag-shaped seedlings formed. The epicotyls and hypocotyls both elongated. The former elongated upward and pushed the cotyledons up to the matrix surface, forming the long leafstalks. The later elongated downward and penetrated deeply into the matrix (Fig. 5D and Table 4). The cotyledons of the seeds stratified at 20ºC had spread out completely, and the visible fork was because one cotyledon curled unilaterally. The endosperm cells surrounding the cotyledons had disappeared progressively, the pericycles and radicles became visible, but the cotyledons were still buried deeply in the endosperms. The embryo length was 9.16 mm, approximating 7 fold of the CK and reaching the extremely significant level (Fig. 5E and Table 4). The radicles of the seeds stratified at 15ºC had broken through the episperms, the miniature forms of the cotyledons appeared, ruts occurred on one side of the cotyledons. At this time, the embryo length was 5.53 mm, approximating 4 fold of the CK and reaching the extremely significant level (Fig. 5C and Table 4). As to the seeds stratified at 10ºC, the miniature forms of the radicles began to form, but the radicles did not yet broke through the episperms, ruts also occurred on the manifestly differentiated cotyledons, the plumules started to develop, and the pericycles and calyptra cells became visible. Above all, the embryo length was 2.34 mm, occupying about 4 fold of the CK and reaching the extremely significant level (Fig. 5B and Table 4). Conversely, the embryos of the seeds stratified at 5ºC and 25ºC began to differentiate, forming globular embryo. The embryo lengths were no more than 1.0 mm, and the differences between the treatments and the CK did not reach the significant level (Fig. 5A, F).

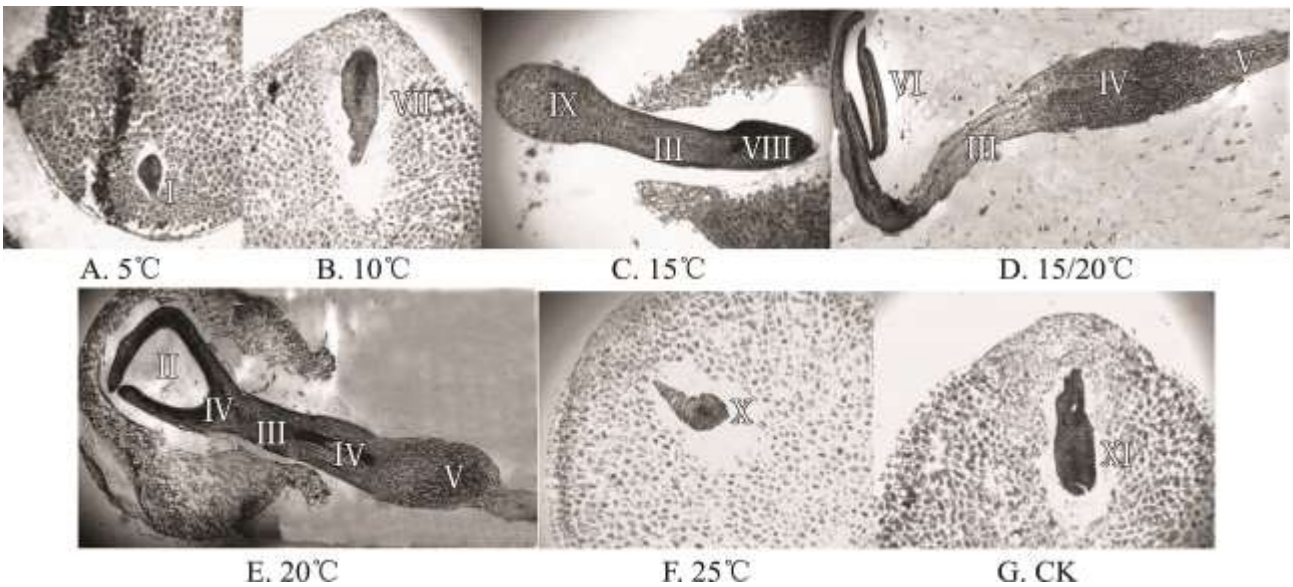

Fig. 5. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 120 d. I. Early stage of globular embryo. II. The cotyledon had formed and was deeply buried in the endosperm. III. Stemlet. IV. Rhizome. V. Radicle. VI. Cotyledon. VII. The cells at the embryo base divided to form the conic radicle. VIII. The miniature form of cotyledon formed. IX. The radicle had broken through the episperm. X. Latter stage of globular embryo. XI. The elliptic embryo formed.

Table 4. Differentiating and developing states of the embryos of *P. polyphylla* var. *yunnanensis* seeds stratified at different temperatures for 120 days.

Stratifying temperature (ºC)	Embryo length (mm)	Embryo-emerging ratio (%)	α=0.05	α=0.01
5	0.79 ± 0.03	15.85 ± 5.86	e	E
10	2.34 ± 0.15	46.49 ± 6.41	d	D
15	5.53 ± 0.30	111.82 ± 18.21	c	C
20	9.16 ± 0.21	181.82 ± 27.83	b	B
15/20	13.06 ± 0.61	263.46 ± 57.87	a	A
25	0.85 ± 0.05	17.21 ± 2.44	e	E
CK	1.21 ± 0.10	24.21 ± 1.26	e	E

Discussion

Holding only one cotyledon, *P. polyphylla* was defined as monocotyledon. Conversely, just like dicotyledons, *P. polyphylla* possesses the reticular veins and multiple carpels. So, the status of *P. polyphylla* in the monocotyledon system is pendulous all the time (Li, 1998). It was found in this study that the globular embryo of *P. polyphylla* var. *yunnanensis* seed did not produce two lateral enations. Instead, the cupular part of the embryo grew to form the cotyledon, and a rut appeared on one side of the embryo (Fig. 5C). Afterwards, the cupular part of the embryo curled, and was buried deeply in the endosperm (Fig. 5E). Consequently, the embryo development of *P. polyphylla* var. *yunnanensis* seed displays the typical features of those of monocotyledons (Liu, 2001). So, we proved again that *P. polyphylla* var. *yunnanensis* is monocotyledon, which is coincident with the results of Li (1998) and Liu (2001).

The morphological dormancy of seed embryo is very complicated, and the different parts of the embryo may display dormancy. Probably, the embryo dormancy is chiefly caused by the cotyledon, also by the fact that the growth of the radicle and the embryonal axis is slower than the cotyledon enlargement (Song *et al*., 2008). It was found in this study that, for the *P. polyphylla* var. *yunnanensis* seed, the radicle grew much quickly than the cotyledon and the embryonal axis, and the cotyledon differentiated and developed most slowly. In addition, during the developing process, the embryo formed the globular one when the embryo length reached about 0.7 mm, the rut appeared, the plumule formed, and the embryo developed into the organ-forming stage, but was immature when the embryo was about 2 mm long, the cotyledon development was accomplished and the embryo was mature when the embryo length exceeded 3 mm. Therefore, the dormancy of *P. polyphylla* var. *yunnanensis* seed belongs to the typical morphological one, and the incomplete development of the embryo is the main reason resulting in the longer dormancy of the seeds.

Acknowledgments

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