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Viability and Germination Percentage Analysis of *Platanthera bifolia* Seeds at Different Degrees of Maturity

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Abstract

Platanthera bifolia (L.) Rich. (Lesser Butterfly Orchid) is considered difficult to propagate by seeds due to complex environmental requirements, while the number of studies that consider seed age as the experimental variable is insufficient.

The effectiveness of determining the viability of seeds of different ages remains uncertain, as significant discrepancies between staining and germination data are observed for several orchid species.

This study compares three approaches for determining the quality of orchid seeds: asymbiotic germination, vital TTC staining and detection of seeds with embryos.

The aim of the study was: 1) to identify the morphological differences between age-diverse *P. bifolia* seeds; 2) to evaluate the effects of *P. bifolia* seed age and modified nutritious media (1/4 MS and Malmgren) on achieving high germination rates and seedling growth; 3) to determine the relationship between three different assessments of *P. bifolia* seed viability: vital staining using TTC, detection of seeds with embryos, and asymbiotic seed germination.

Assessed by TTC staining, 68.4% of 30 DAA *P. bifolia* seeds were colored, while 41 DAA and 20 DAA seeds exhibited decreased viability – 51.6% and 36.2%, respectively. The percentages of full 30 DAA and 41 DAA seeds with embryos were higher than their viability values in the TTC test, as well as germination frequencies *in vitro*.

The variation in germinability of *P. bifolia* seeds on two different media formulations was marked: 49.1% of 30 DAA *P. bifolia* seeds developed into seedlings on modified Malmgren (mM) medium and 41.3% on 1/4mMS. Moreover, 30 DAA seeds were developed to advanced protocorm stages 3-6 in the mM medium, which was due to the coconut water organic additive. During the same 6-month incubation period, 41 DAA seeds were only able to develop through developmental stages 3 or 4, with no seedlings at the stages 5-6, regardless of the medium used. On the basis of results obtained in the studies of *P. bifolia* seed micromorphology and coat ultrastructure, the most favorable 'harvesting window' for successful *in vitro* seed germination was determined to be the post-anthesis 30 day interval.

Keywords: Orchidaceae, seed viability, germination test, orchid immature seeds

1. Introduction

Platanthera Rich. is one of the most species-rich genera among the orchids of the temperate zone, including 144 accepted species (The World Checklist of Vascular Plants). High morphological plasticity and fast evolution among genus representatives (Gamarra et al., 2008; Efimov, 2011) led to their wide-amplitude ecological characteristics contributing to dispersal in the northern hemisphere, with diversification centers in East Asia and North America. Genus *Platanthera* in the flora of Russia comprises 15 species, several subspecies and varieties (Efimov, 2020). The current trend of decreasing *P. bifolia* populations has led to a shift to a more protected status than 'Least Concerned' owing to habitat degradation (Rankou, 2011). In the Mediterranean countries, the tuber of the species is widely used for salep production (Teoh, 2016), while the leaves are known as a remedy against rheumatism and as an antineuralgic agent in non-traditional medicine (Calevo et al., 2020) owing to the high content of phenolic compounds and flavonoids, such as quercetin and kaempferol in the leaves of individuals from both the disturbed habitats and from natural sites, as detected by Maleva et al. (2021).

Two leaves of oval shape appear at inflorescence base, which is spike-like. There are from 10 to 25 white flowers in the inflorescence, opening not simultaneously but from the base to the top. One flower is open within a few days during the flowering period (Boberg et al., 2014), which occurs between May and July, both in Northern Europe (Esposito et al., 2018) and in central Russia (Vachrameeva et al., 2014), while in Siberia it usually starts 1-2 weeks later. The fruit of *P. bifolia* is a capsule that opens with six long slits, yielding a mean of 4251 seeds, as recorded by Kirillova and Kirillov (2017) from *P. bifolia* capsules gathered in Komi Republic, Russia. Tiny dusty seeds become mature after 2 months of development, and in nature, they may germinate following capsule dehiscence in autumn, but further development of the protocorms occurs after 1-2 winters (Vachrameeva et al., 2014).

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In vitro culture, which provides vast opportunities for studying the growth and development of orchid seeds, and for obtaining viable seedlings, is intended to be used to reduce overharvesting of orchids from natural populations (Jolman et al., 2022).

In the wild, most *Platanthera* species are considered mycorrhizal generalists (Betechtina et al., 2013; Vogt-Schilb et al., 2020). For this reason, the symbiotic protocols were utilized for the *in vitro* germination of rare *Platanthera* native to North America, including *Platanthera praeclara* Sheviak and M.L. Bowles (Sharma et al., 2003*), P. leucophaea* (Nutt.) Lindl (Poff et al., 2016).

Low seed germination of *Platanthera* species and slow growth of seedlings in asymbiotic culture reported by Nadarajan et al. (2011) appear to be partly due to the lack of specific nutrients in the medium, required to replace mycorrhizal associations (Rasmussen, 1995). Developing *in vitro* germination strategies is becoming increasingly attractive for horticultural practice, involving autochthonous genetic resources of terrestrial orchid species (Zale et al., 2022).

It is known that immature seeds, if inoculated before dormancy occurs, germinate faster and in more significant proportion than seeds from mature fruits (Kendon et al., 2017). The success of growing seeds from green pods is thoroughly explained by the increased metabolic activity of the seed that reaches the phase of physiological maturity (Dalziell and Tomlinson, 2017). However, incomplete information on seed quality and optimal timing of *in vitro* seed sowing often resulted in inconsistent *in vitro* developing data protocols for *P. bifolia*: it was shown in the study of Tonecki and Dobrzynski (2008) that immature *P. bifolia* seeds germinated better than mature ones, while other authors indicated similar germination ability in both mature and immature seeds of the species (Kulikov and Phillipov, 1998).

Thus, it is important to determine the specific stage of seed development before inoculation, especially when green pod culture is a choice for the micropropagation of terrestrial orchid species. Understanding the factors influencing seed viability can facilitate the interpretation of seed germination results for producing the most appropriate germination protocol for *P. bifolia.*

Testing the viability of seeds by TTC is considered to be a simple but not quite reliable method for determining the optimal seed age inoculation for successful germination *in vitro* (Pradhan et al., 2022). The correlation between seed germination and viability assessed by TTC staining often varied among species (Dowling and Jusaitis, 2012; Metsare et al., 2015) and even within a species (Lemay et al., 2015).

The viability assessed by utilizing the tetrazolium test should be modified for mature seeds because hard-coated seeds require different testa pretreatment for better stain penetration. Scarification with hypochlorite solutions prior to vital staining was reported to increase TTC test effectiveness (Kauth et al., 2008; Custidio et al., 2016), in cases when impermeability is due to the presence of suberin, a waxy substance found on the testa of orchid seeds (Barsberg et al., 2013). However, long-term treatments by NaOCl significantly constrain the viability of the embryos of experimental seeds, damaged by bleaching during a surge in the permeability of the seed coat, which was confirmed by Van Waes and Deberg (1986).

In order to find the appropriate physiological age of seeds for sowing and successful TTC test, a comparison of the ultrastructure of age-diverse seeds may be useful to understand the mechanisms of seed dormancy.

The aims of this study were: (1) to provide morphometric data on *P. bifolia* seed and embryo characteristics along with the seed coat microstructure alterations during maturation; (2) to evaluate germination of age-diverse *P. bifolia* seeds on two different media; (3) to determine the relationship between three different assessments of *P. bifolia* seed viability: vital staining using TTC, detection of seeds with embryos, and asymbiotic seed germination.

2. Material and methods

2.1. Seed source

The studied population is located in the Novosibirsk Region, Russia, and includes approximately 45 individuals, which were found in scattered groups or single plants spread out in the birch forest surrounded by agricultural landscape. The taxonomic identity of the species was confirmed at the I.M. Krasnoborov Herbarium of the Central Siberian Botanical Garden, where a voucher specimen has been placed (NS0049018).

The time of *P. bifolia* pod development was scored when 10 generative specimens were marked during anthesis to recognize them when fruiting. The open-pollinated seeds were harvested in July 2020 from the capsules of the middle parts of inflorescences of the same plants 3 times at the selected intervals: 20 d, 30 d and 41 d after anthesis (DAA), determining their age by taking the date of flower anthesis as a starting point. The seeds were stored for no more than 1 day in the dark at 4 °C prior to inoculation.

After surface sterilization, the seeds from each capsule of definite age were divided into three samples consisting of at least 150 seeds, and three replicates were made to determine three characteristics of seed viability: 1. Number of seeds with embryos; 2. The mean embryo stainability; 3. Germination in the *in vitro* culture.

2.2. Seed-quality testing: embryo presence and biochemical viability

For counting *P. bifolia* seeds with embryos under a stereomicroscope, no additional treatments were required since the cell wall of all tested seeds was quite transparent, and the embryo was clearly visible. The mean percentage of seeds containing well-developed embryos was counted by dividing the number of seeds with embryos by the total number of seeds analyzed × 100 for 10 capsules. Seeds from the same capsule were then used to assess biochemical viability provided by the implementation of the protocols developed by Van Waes and Deberg (1986) and Custidio et al. (2016), ordinarily used to detect the activity of dehydrogenase enzymes as the characteristic of living tissues. The application of this method was carried out by staining *P. bifolia* seeds with 2, 3, 5-triphenyltetrazolium chloride (TTC). For staining, 20 DAA and 30 DAA seeds incubated in 0.5% (w/v) TTC (Sigma, T8877) solution at 30 °C in the dark for 24 hours were then treated twice with distilled water to remove the excess

stain. The viability of 41 DAA *P. bifolia* seeds was assessed using the modified version of the TTC test proposed by Custidio et al. (2016): the seeds were first soaked in 1% (w/v) $Ca(OCl)_2$ for 2 min, then washed three times thoroughly with distilled water and incubated in 0.5% TTC solution for 24 h at 30 °C in the dark. After discarding the TTC solution with distilled water, the seeds were examined under the stereomicroscope Stereo Discovery V 12, Carl Zeiss, Germany. As a result, the embryos appearing to stain different red were scored as viable, while the seeds with pale or unstained embryos were considered unviable. The number of embryos stained by TTC was analyzed for 10 capsules of definite age from three replicates and counted as the mean percentage of viable seeds by dividing the number of stained embryos by the total number of seeds × 100.

2.3. In vitro culture

Intact capsules were surface disinfected under laminar flow, and the seeds were sown on two culture media with different compositions: 1) modified quarter-reduced Murashige and Skoog (1962) medium (1/4 mMS) and Malmgren (1996) medium (mM). The media were solidified with 0.6% Bacto® agar (PanReac®, Barcelona, Spain) and modified similarly by the addition of 1.2% sucrose and 0.1% activated charcoal. Additionally, Malmgren medium was supplemented by an organic additive – 100 ml l^{-1}, coconut water (CW), extracted from green coconut. Before autoclaving at 121 °C and 101 kPa for 20 minutes, the pH of the culture media was adjusted to 5.6.

At least, 150 seeds of definite age were placed on the surface of 200-ml jars and incubated on mM or 1/4 mMS media under continuous darkness for 3 months at 23±2 °C. Then, all germinated cultures were exposed to a photosynthetic photon flux density of 40 µmol $m^{-1}s^{-1}$, provided by cool white fluorescent lamps (Phillips, Poland) under the same temperature, 23±2 °C, during the next 3 months. After inoculation, seed germination, protocorm formation and seedling development were monitored and scored after 3 and 6 months.

2.4. Seed morphometric assay, definition of embryo developmental stages and germination scoring

Morphological measurements of seeds isolated from *P. bifolia* capsules of different ages were performed using the following two equations according to Arditti et al. (1979): seed volume, $SV = 2\ [(W/2)^2 \times L/2 \times \pi/3]$, where W is seed width; L is seed length; embryo volume, $EV = 4/3\pi \times EL/2 \times (EW/2)^2$, where EL is the length of the embryo and EW is the width of the embryo. The percentage of air space in the seed was determined using the formula: (SV – EV)/SV × 100.

The evaluations of embryo developmental stages were provided at 3 and 6 months after sowing as follows: observed under stereomicroscopy, germinating seeds were scored on a modified scale of 0 – 7 according to growth stage values reported for *in vitro* studies of other terrestrial orchids (Stewart and Zettler, 2002).

The seeds were considered germinated when the embryo emerging from the testa approximately doubled in size (Stage 2), when imbibed dead seeds became well distinguished from viable seeds. When counting the germination percentages, seeds without an embryo or with an undeveloped embryo were excluded. The initial (G_1) percentage of germination was assessed as the sum of the percentages of seeds, which developed to the advanced stages recorded after 3 months in relation to the initial percentage of seeds with embryos × 100. Germination (G_2) was scored after 6 months from sowing as the total percentage of germinated seeds and developing protocorms in relation to the initial percentage of seeds with embryos × 100. Final germination was determined as the mean germination percentages scored between the two media after 6 months of seed culture.

2.5. Statistical analysis and data representation

To compare viability assessed by TTC staining and by counting the mean proportion of seeds containing an embryo, seeds were selected from the same capsules of three different ages in three replicates; at least 150 *P. bifolia* seeds were used in each treatment. The mean percentage of seeds without an embryo was measured as a ratio of empty/sowed seeds (×100). Seed viability estimation was made by dividing the number of stained embryos by the total number of embryos counted for seed lots belonging to different age (×100). The result of the *in vitro* germination test was presented as a percentage of the number of germinated seeds to the total number of seeds with an embryo (×100). The percentage of protocorms at a certain developmental stage was scored by dividing the number of seeds that had developed to that stage by the total number of seeds with an embryo (×100).

Germination percentage was recorded as the total percentage of embryo development at Stages 2–6 after 3 and 6 months. The data on the frequency (%) of the developmental protocorm stages, along with all quantitative data, were submitted to the Shapiro-Wilk normality test, and an analysis of variance (ANOVA) in STATISTICA 8 software (StatSoft Inc., Tulsa, OK) was made. Student's t-test was used to determine the statistical difference between the mean morphometric values obtained for the three seed lots, while germination and viability data were transformed to normalize the distribution, but untransformed means were presented as the mean ± standard error (SE). A factorial experiment with a completely randomized design was performed to investigate the effect on germination percentage of two groups of variables and their interaction in a 3 × 2 factorial scheme (three seed ages and two culture media). The differences between means were evaluated with post hoc comparisons using Duncan's Multiple Range Test (DMRT) at a significance level of $p<0.05$. The Pearson's correlation was used to determine the relationship between the three indicators of seed viability.

3. Results

3.1. Embryo development and morphological changes during seed maturation. TTC staining

The morphological features of *P. bifolia* seeds of different ages along with the ultrastructure of seed coat were investigated. During the development of *P. bifolia* seeds, changes in the size of the capsules and morphological parameters of seeds and embryos have been established. Capsules representing three stages of seed development were predominantly green, but the seed color varied: immature seeds isolated from 20 and 30 DAA

capsules were white, while the color of 41 DAA seeds tended to be creamy white (Fig.1).

Figure 1. The appearance of immature seeds in longitudinally cut *Platanthera bifolia* capsules of different ages (20 DAA, 30 DAA and 41 DAA), scale bar represents 0.2 cm.

Depending on the degree of maturity, differences in the length and width of capsules of *P. bifolia* were observed (Table 1).

Table 1. Characteristics of *Platanthera bifolia* capsules of different age

The age of maturity	Length of seed capsule, mm	Width of seed capsule, mm
20 DAA	10.5±1.18	3.1±0.33
30 DAA	13.8±2.04	3.8±0.41
41 DAA	15.1±1.89	4.0±0.56

Data represent mean ± standard errors. The length and width of capsules are shown as the average of 10 capsules.

The development of the capsules continued until 41 DAA, but then from 30 to 41 days it slowed down.

The study provides evidence of significant variability between *P. bifolia* seeds of different degrees of maturity at the micro-morphometric level (Table 2), especially between 20 DAA seeds and more mature seeds, while between 30 DAA and 41 DAA slight numerical differences in seed and embryo size were recorded (Table 2).

Table 2. The morphometric characteristics of *Platanthera bifolia* seeds

Seed lots according to their age	Seed dimensions, mm			Embryo dimensions, mm			Air space, %
	Length	Width	Seed volume	Length	Width	Embryo volume	
	M±SE		Volume of seed, $\times 10^{-3}$ mm^3	M±SE		Volume of embryo, $\times 10^{-3}$ mm^3	AS= SV-EV / SV ×100
1) 20 days after anthesis	0.56 ± 0.05 b	0.13 ± 0.01 b	2.47 b	0.12 ± 0.01 b	0.09 ± 0.01 b	0.50 c	79.64 a
2) 30 days after anthesis	0.71 ± 0.03 a	0.15 ± 0.01 a	4.19 a	0.14 ± 0.01 a	0.11 ± 0.01 a	0.88 a	78.79 ab
3) 41 days after anthesis	0.75 ± 0.07 a	0.14 ±0.01 b	3.85 ab	0.15 ± 0.03 a	0.12 ± 0.01 a	1.13 b	70.59 b

Note: AS – indicates what part of the empty air space in the seed is occupied, SV and EV – represent volumes of the seed and embryo, respectively. Experimental values represent mean ± standard error (SE); the values in each column followed by the same letter are not significantly different, as determined by Duncan's post hoc test ($P < 0.05$)

The embryo occupies only a minor proportion of the volume of 20 DAA seeds (20.4%), but its volume substantially increases during maturation. Hence, the size of the embryos of 41 DAA seeds was the largest and occupied 29.4% of the seed volume, and the embryo of 30 DAA seeds – 21.2% of the seed volume, while the values of the air space differed significantly between 20 DAA and 41 DAA seeds (Table 2).

Within 20 DAA capsules, many differentiating ovules associated with placental tissue have been observed (Fig. 2A). Single seeds, free of placental tissue, differed from more mature seeds in the shape of their apical region, which was more rounded than truncated (Fig. 2 B). Most of the 20 DAA seeds have an embryo at a pre-globular stage and a suspensor extending beyond the seed, which is eliminated at the later stages of *P. bifolia* embryo development. It is well distinguished that the testa cells of 20 and 30 DAA seeds are more translucent than in 41 DAA seeds (Fig. 2 A, E, C).

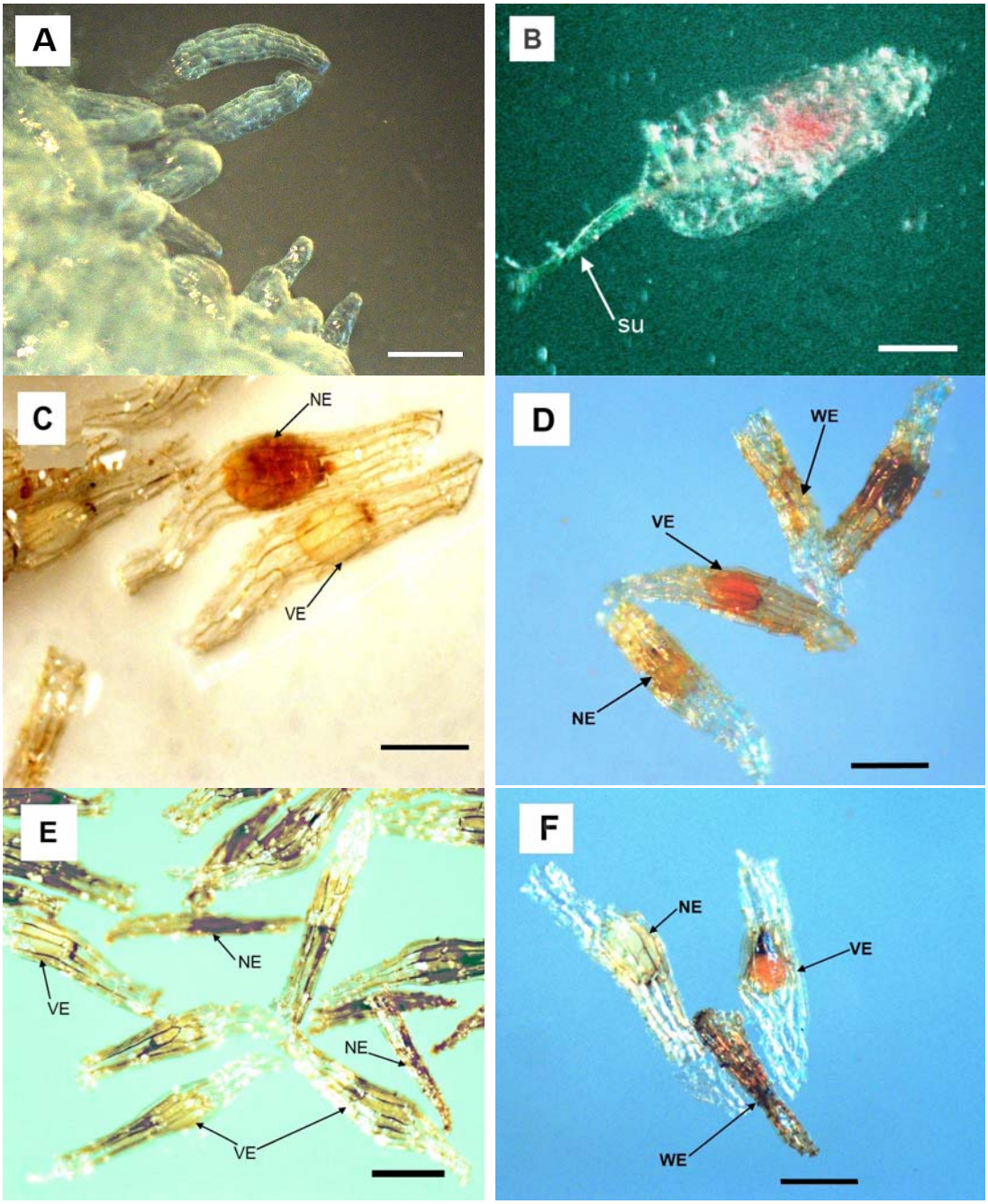


Figure 2. The morphological variability of *Platanthera bifolia* seeds of different ages (A – 20 DAA intact seeds; B – 20 DAA seed after TTC staining, su – suspensor; C – 30 DAA intact seeds; D – 30 DAA seeds after TTC staining; E – 41 DAA intact seeds; F – 41 DAA seeds bleached by 2-minutes treatment with 1% $Ca(OCl)_2$ before TTC staining. Seeds without embryos are marked as WE, viable embryos are identified as VE, and non-viable as NE, respectively. Bar = 250 µm (A), 125 µm (B), 150 µm (C), 200 µm (E, D, F).

As a result of TTC staining, the majority of 30 DAA *P. bifolia* seeds examined microscopically turned out to be viable (68.4%), while the viability of 20 DAA seeds was lower – 36.2%. For seeds collected 41 days after flowering, staining of the embryos was difficult due to the dense inner covering surrounding the embryo (Fig. 2 E). For this reason, the viability of more mature *P. bifolia* seeds was determined after they were bleached for 2 min with 1% $Ca(OCl)_2$ before testing (Fig. 2 F), and as a result, 51.6% of 41 DAA seeds were assessed as viable.

3.2. Embryo presence assessment

P. bifolia embryos are generally ellipsoidal and located at the center of the seed (Fig. 2 A, C). Since an embryo is quite visible through the testa of *P. bifolia* immature seeds under light microscopy, the proportion of seeds with full embryos was easily estimated for the seeds of different ages. The mean proportion of seeds containing an embryo increased from 31.0 ± 2.4% among 20 DAA seeds to 71.8 ± 3.6% among 30 DAA seeds and 75.5 ± 5.4% among 41 DAA seeds. Between 20 DAA seeds and the more mature seeds, the means differed significantly (ANOVA: $F_{(2.33)}$ =

160.4, p = 0.00). Thus, during normal *P. bifolia* seed development, only a small proportion of empty seeds appears to occur among 41 DAA seeds near the maturation age.

3.3. Testa cells ornamentation during maturation

Seed ornamentation was determined by means of scanning electron microscopy (SEM) with a Hitachi TM-1000 microscope (Japan). As seeds matured, cell wall thickening occurred: spindle-shaped 41 DAA seeds have oblique parallel thickenings of the testa anticlinal walls.

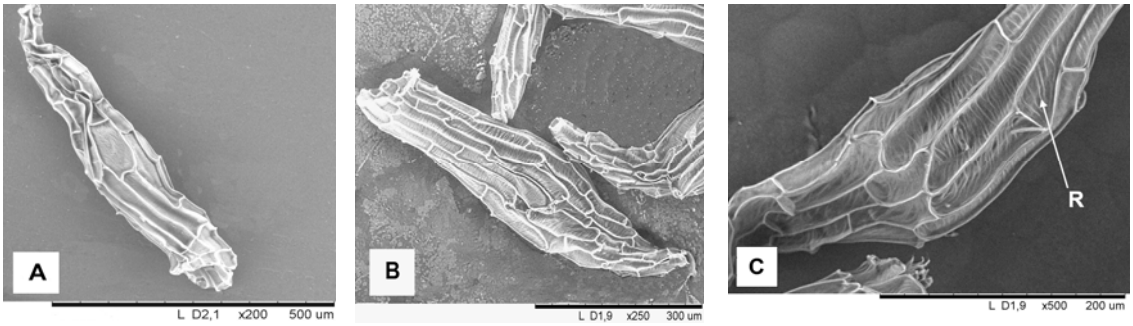


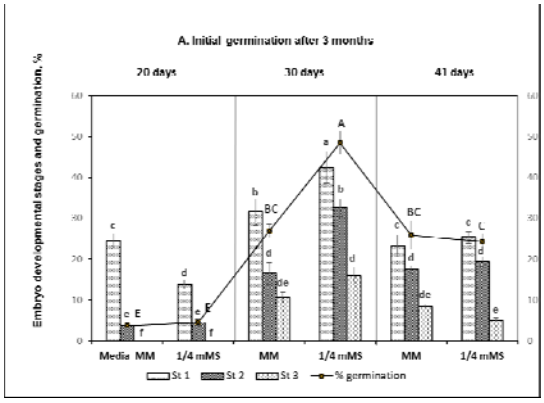
Figure 3. Scanning electron micrographs of A.) 30-day-old seeds. Bar=500 μM; B.) 41-day-old seeds. Bar=300 μM; C.) Elongated testa cells with thickened anticlinal walls and transversal ridges (indicated as R and by arrow) on the periclinal walls of 41-day-old seed are observed. Bar=200 μM.

Outermost layer of the seed coat thickened and compressed into a thin layer covering the embryo and transversal ridges, were revealed on the testa periclinal walls of 41 DAA seeds (Fig.3 C).

3.4. Germination in vitro. The effects of seed age and culture medium

Combinations of two basal media and three ages of capsule maturity were evaluated in the study to assess their effect on asymbiotic germination and subsequent protocorm development in *P. bifolia*. Our observations showed that germination percentage varied depending on seed maturity. Specifically, only a few 20 DAA seeds germinated (developed to Stage 2) both in the mM (3.7%) and 1/4 mMS (4.5%) media after three months, and no survivors were found after six months. Meanwhile, the highest frequency of germination (48.5%) was obtained after 3 months on 1/4 mMS medium with seeds harvested 30 d after anthesis, and a significantly lower percentage (26.9%) was recorded on mM medium (Fig. 4 A). The percentage of 41 DAA seeds, which developed to the embryo advanced stages, did not exceed 24.3% on 1/4 mMS and 25.8% on mM medium (Fig. 4 A).

By the end of the 6 month period, the number of germinated seeds and formed protocorms originating from of 30 DAA capsules was 49.1% on mM medium and 41.3% on 1/4 mMS medium, while during the same period, the recruitment from 41 DAA capsules was approximately the same as 3 months after sowing and included 25.9% germinated seeds and developed protocorms on mM and 21.6% on 1/4 mMS media. Moreover, 30 DAA seeds yielded the greatest percentage of seedlings at stages 5-6 (12.9% on mM and 12.1% on 1/4 mMS media), while 41 DAA seeds were capable of evolving only through 3-4 development stages, but no seedlings reaching stages 5-6 could be found. New germinates were not observed after incubation extended for 6 months, regardless of culture medium or seed age.



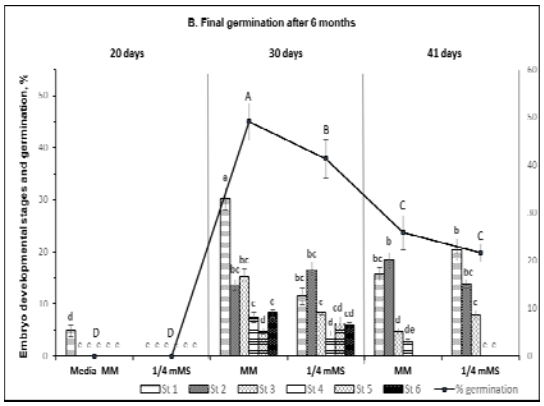


Figure 4 (A, B). Effects of culture medium and age (20-, 30- and 41 DAA) of *Platanthera bifolia* seeds on frequencies of embryo developmental stages and germination assessed after 3 (A) and 6 (B) months. The broken line in the graphs represents the final germination percentages, recorded as the total percentage of Stages 2–6 of embryo development. Vertical bars represent mean ± S.E. Different letters indicate significant differences identified by post hoc comparisons using Duncan's test (P< 0.05).

Germination results obtained after 6 months were significantly different ($p < 0.001$) between 30 DAA and 41 DAA seeds, regardless of the culture medium used. Although 30 DAA seedling growth was poorer in mM medium evaluated for the first 3 months, this medium composition was more favorable for subsequent seedling development than 1/4 mM medium (Fig. 4 A, B). A statistically significant interaction ($p < 0.05$) was found between medium composition and seed age, as confirmed by the percentage of germinated seeds and protocorm development after 3 and 6 months (Tables 3, 4).

Table 3. The results of two-way ANOVA showing the F and P values for the effects of seed age and nutritious medium and their interaction on *Platanthera bifolia* seed germination after 3 months of seed culture

Source of variation	Dependent variable	d.f	Mean square	F Value	P Value
Seed age	Seed germination	2	6903.28	389.14	0.00
Medium	Seed germination	1	874.32	49.28	0.00
Seed age* Medium	Seed germination	2	972.27	54.80	0.00

Table 4. The results of two-way ANOVA showing the F and P values for the effects of seed age and nutritious medium and their interaction on *Platanthera bifolia* seed germination after 6 months of seed culture

Source of variation	Dependent variable	d.f	Mean square	F Value	P Value
Seed age	Seed germination	2	11747.93	492.790	0.000000
Medium	Seed germination	1	396.68	16.640	0.000124
Seed age* Medium	Seed germination	2	143.18	6.006	0.004014

Thus, the maximum germination outcome was obtained after 6 months from 30 DAA seeds inoculated onto mM medium, which also contributed to the highest yield of seedlings at advanced stages.

3.5. The relationship determination between three different assessments of P. bifolia seed viability

Minimal percentages were recorded in three viability assessments with 20 DAA P. bifolia seeds, suggesting that these immature seeds were insufficiently developed to germinate. The percentages of 30 DAA and 41 DAA full seeds were higher than their viability estimated by the degree of TTC staining in the tetrazolium test, as well as germination frequencies *in vitro* (Table 5).

Table 5. Three indicators of *Platanthera bifolia* immature seed viability: data from TTC test, assessments of seeds with embryo and *in vitro* germination test.

Experimental values are mean ± standard errors (SE)

Seed lots according to the age	Percentage of seeds with embryos, %	Viability (TTC test), %	Germination after 3 months*, %	Final germination after 6 months*, %
1. 20 days after anthesis	31.0 ± 2.4 b B	36.2 ± 0.9 c A	4.1±0.6 c C	0.0±0.0 c C
2. 30 days after anthesis	71.8 ± 3.6 a A	68.4 ± 4.2 a AB	37.7±2.8 a C	45.2±3.5 a B
3. 41 days after anthesis	75.5 ± 5.4 a A	51.6 ± 3.4 b B	25.0±1.7 b C	23.7±3.3 b C

* In order to estimate the total amount of seeds germinated *in vitro*, the average frequency between the two media was determined between 3 and 6 months of culture. According to Duncan's test, mean values followed by the same lowcase letters in the columns and by the capital letters in the rows do not differ statistically at $p < 0.05$.

For all ages of *P. bifolia* seeds tested, two methods of viability determination yielded much higher values than the germination test did. Significant differences were recorded: 1) between final *in vitro* germination values and the full seeds percentage: $F_{(2.33)} = 160.34$, $p = 0.000$; 2) between final *in vitro* germination values and the viability percentages, assessed by TTC test: $F_{(2.33)} = 74.8$, $p = 0.000$.

When establishing the relationship between the three indicators of seed viability, using Pearson's correlation, the only positive correlation was recorded between 30 DAA seed germination and embryo presence, $r = 0.662$ (Table 6), thus suggesting that the full seeds assessment could potentially predict the results of germination test *in vitro* for *P. bifolia* immature seeds the age of which was 30 DAA.

Table 6. Pearson's correlation coefficients and their statistical significance between seed quality measures in *Platanthera bifolia* for seeds with different degrees of maturity (20, 30, 41 days after anthesis)

Indicators/ Seed age (days)	Full (20)	TTC (20)	C G6 (20)	Full (30)	TTC (30)	C G6 (30)	Full (41)	TTC (41)	C G6 (41)
Full* (20)	1.000	0.345	0.090	0.170	0.061	-0.284	-0.146	0.101	-0.115
TTC* (20)	-0.345	1.000	0.081	0.212	-0.047	-0.343	0.368	0.083	0.274
C G6* (20)	0.090	0.081	1.000	-0.093	0.329	-0.455	-0.117	-0.147	-0.068
Full (30)	0.170	-0.212	-0.093	1.000	0.360	0.662	-0.385	-0.683	0.148
TTC (30)	0.061	-0.047	0.329	0.360	1.000	0.106	0.081	-0.441	-0.423
C G6 (30)	-0.284	-0.343	-0.454	0.662	0.106	1.000	-0.427	-0.423	0.125
Full (41)	0.146	0.368	-0.117	0.385	0.081	-0.427	1.000	0.310	-0.263
TTC (41)	0.368	0.083	-0.147	-0.683	-0.441	-0.418	0.311	1.000	-0.495
C G6 (41)	-0.116	0.274	-0.068	0.148	-0.423	0.125	-0.263	-0.495	1.000

* Full - embryo presence; TTC - embryo stainability; C G6 – mean seed germination measured after 6 months between 2 media treatments; seed age is indicated in brackets; quality measures marked in red are statistically significant between each other, $p < 0.05$)

Thus, 41 DAA seeds of *P. bifolia* are characterized by the beginning of maturation, at which the embryo slightly increased in size, while the volume of the seed, along with the proportion of empty air space, decreased. The seeds of this age were characterized by the additional formations, which may lead to their seed coat hardening (Fig. 2 C, E; Fig. 3 C).

The relationship between the three different assessments of the seed viability was not consistent for 20 DAA and 41 DAA *P. bifolia* seeds, while for 30 DAA seeds the relation between the germination test results and the full seeds assessment was approved.

4. Discussion

It is a generally accepted fact that the germination of seeds of representatives of the tropical orchid flora, which have relatively large embryos, does not require a complex of interacting environmental factors for effective germination, while species of orchids of the temperate zone, which have a smaller embryo volume as a rule, exhibited low germination rates both *in situ* and *in vitro* (Prasongsom et al., 2022). The morphological changes identified during *P. bifolia* seed development are in good agreement with the assumption that the seed morphology is linked to dormancy, germination and seed structures' establishment (Alfaro Pinto et al., 2023).

Comparing the morphological characteristics of *P. bifolia* pods (size and color) and seeds (size and volume of seeds/embryos together with the air volume), it seems clear that we are dealing with seeds that have not entered the final stage of embryo development – full maturation stage. At this time, the embryo reaches its maximum size, the levels of auxin, cytokinin and gibberellin decrease, while the level of ABA reaches its maximum, which leads to a slowdown in mitotic activity and the beginning of storage products deposition (Yan et al., 2017).

The mean volume of free airspace detected in this study was from 70.59 to 79.64%, which appears to be the average between the values: 65.7%, obtained at the northern boundary of the species' distribution in Russia (Kirillova and Kirillov, 2017), and 88%, reported by Arditti and Gani (2000), when studying the European populations of this species.

Although the embryo occupied most of the 41 DAA seeds volumes, as compared to more immature seeds tested, the development of 41 DAA embryos is not yet complete, as evidenced by their continued elongation (Table 2). However, it can be assumed from the fact that the size of the seed is beginning to decrease that the first phase of maturity has already started. Decreased 41 DDA seed germination rate, along with the low stainability of these seeds in the TTC test, also confirm this assumption. Prior to vital staining, scarification with hypochlorite solution was reported to be necessary for mature seeds possessing hard coats to improve the effectiveness of the test (Van Waes and Debergh, 1986; Sawma and Moller, 2002). Intact embryos of *Cephalantera falcate* mature seeds, which were harvested 140 d after pollination, also were not stained by TTC solution, as reported by Yamazaki and Miyoshi (2006).

When testing the viability of immature seeds using the TTC test, it is possible to assess both the viability of the embryo and the viability of various seed tissues by their different stain ability during maturation. Thus, the higher intensity of TTC staining in embryos and testa cells of 20 and 30 DAA seeds was detected due to the pronounced dehydrogenase activity (Fig. 2 B, Table 3). Such an increased staining intensity of immature seeds was often noted in other orchid species of warm, e.g., *Dendrobium* species (Prasongsom et al., 2022), *Acianthera johannensis* (Duarte et al., 2019) and temperate climate, e.g., *Calypso bulbosa* (Yeung and Law, 1992).

Among *P. bifolia* 20 DAA seeds, 31% of seeds with embryos were recorded; however, the full seeds were characterized by an undifferentiated embryo showing strong autofluorescence and by the suspensor, which could be observed outside the micropillar end of the seed (Fig. 2 B). Autofluorescence indicates the non-completed development of the cuticular layer and the lack of phenolic compounds in the seed wall (Yeung, 2022).

The functions of the suspensor appear to be associated with obtaining nutrients from the cells of the inner layer of the outer integument, which is supposed to be an "endosperm substitute" (Donaldson, 2020; Yeung, 2022). The suspensor is eliminated at later stages of *P. bifolia* embryo development since it was not found in 30 and 41 DAA seeds. It seems that in 20 DAA seeds that are still so immature, the embryo is not developed adequately, and thus only minimal germination was observed after 3 months: 3.7% in mM and 4.5% in 1/4 mMS media, while no survivors were found after *in vitro* culturing for 6

months (Fig. 4B). Thereby, the development of the embryo aged 20 DAA was not autonomous in terms of the experiment.

The differences in seed characteristics involved rearrangement in the seed testa, which had completed the periclinal divisions. The physiological state of the embryo, which changes during seed maturation, can also affect the staining test. The influence of seed maturity on the ornamentation of the seed surface was revealed when the smooth periclinal walls of 30 DAA *P. bifolia* seeds changed to a reticulate structure in 41 DAA seeds. Thus, the reason why 41 DAA seeds exhibited viability values in the TTC test below the corresponding values of 30 DAA seeds may be related to the hardening of the seed coat structure during maturation, proved also by electron microscopy. These findings partially contradict both the results of Gamarra et al. (2008), who reported reticulate walls of *P. bifolia* seeds, and Efimov (2011), who characterized *P. bifolia* seeds as having smooth periclinal walls. It is very probable that the authors studied the seeds of different ages.

It is well known that the reason for the lack of seed germination may be the non-optimal composition of the culture medium (Fast, 1982). In modified Malmgren and MS media with the decreased content of macro- and microelements, initiation of seed germination and protocorm formation enhanced in *P. bifolia*. Earlier, Tonecki and Dobrzynski (2008) studied the effect of 1/3 MS on *P. bifolia* seedlings' development, while Vejsadova (2009) used an improved culture medium for this species' seed germination. However, the authors did not emphasize that the response of the orchid to the medium composition depended on the age of seeds used for sowing.

Six months after sowing, protocorm development of both 30 DAA and 41 DAA seeds slowed down on 1/4 mMS medium containing only inorganic nitrogen sources. The lower germination percentages of *P. bifolia* seeds on 1/4 mMS medium could be attributed to high ammonium content in comparison with Malmgren medium, which is enriched in organic additives such as a mixture of amino acids and CW, providing additional sources of organic nitrogen and carbohydrate, which seem to be more efficient than inorganic nitrate and ammonium salts involved in MS (Dulic et al., 2019; Mose et al., 2020). Being added in Malmgren medium, CW supported the advanced *P. bifolia* seedling development but did not promote a high germination rate during the first 3 months, as compared with 1/4 mMS, which did not include the additive.

According to the previous reports, organic nitrogen sources, as opposed to inorganic forms, have a positive effect on seed germination in *Gymnadenia conopsea* (Ostojic et al., 2022), while CW as carbohydrate additive increased the germination of *Cypripedium macrantos* immature seeds (Huh et al., 2016).

The assumption about specific requirements during the formation of *P. bifolia* protocorms is in line with the results obtained by Ponert et al. (2013), who observed that even extremely low nitrate concentrations prevented the development of *Pseudorchis albida* seed outcomes.

Obviously, 41 DAA seeds had morphological or physiological dormancy since 30 DAA *P. bifolia* seeds yielded significantly higher germination percentages owing to the permeability of the seed testa. In fact, the highest germination rate was obtained with 30 DAA seeds, and after maintaining the seedlings on mM medium, they were characterized by a long lifespan *in vitro*, while 41 DAA seeds exhibited reduced seed germination after 6 months of culture, with the same percentages of seeds remaining at Stages 2, 3 and 4 as recorded by 3 months, regardless of which medium was used to prompt germination. Similar results were obtained for immature seeds of *Dendrobium nobile*, when an efficient nutrient uptake for the species germination was observed due to the incomplete cuticle formation (Vasudevan and van Staden, 2010).

The percentages of 30 DAA seeds with embryos and their embryos stainability approved by TTC test are consistent with their germination frequencies *in vitro*, while for the 20 DAA and 41 DAA seeds these data are not consistent.

This is partly in line with Hirano et al. (2005), who reported that the viability test based on TTC staining was not an accurate predictor of germination for immature seeds of terrestrial orchid *Bletilla striata*. The lack of correlation between seed viability and percentages of germinated seeds and formed protocorms is also in line with the results reported by Rasmussen (1995) for *Epipactis helleborine* or by Vujanovic et al. (2000) for *Cypripedium* species, who concluded that special requirements must be met to achieve germination rates close to those expected from seed viability tests. Lee and Yeung (2023) and Soch et al. (2023) showed that when seeds are sown close to their maturation age, their pretreatment by bleaching can improve the hydrophilicity of the seed coat and ensure the permeability of the staining agent. Their findings were confirmed in the present study. In assessing the viability of temperate orchid seeds using the TTC test, the age of the seeds correlating with the degree of seed testa permeability was to be taken into account for better interpretation of germination studies.

5. Conclusion

Morphometric analysis indicates that the color and morphology of both *P. bifolia* fruits and seeds, as well as their dimensions and the presence of ornamentation on the seed coat, are valuable characteristics for understanding the mechanisms of seed dormancy during maturation and predicting the success of seed germination *in vitro*.

The TTC test has not been successful with 20 DAA and 41 DAA *P. bifolia* seeds: 20 DAA seeds exhibited mostly extra stained ability due to the undeveloped embryo structures, while the penetration of tetrazolium into 41 DAA seeds was likely hampered by the thickened testa, resulting in lower results especially for *in vitro* germination test, compared to the same test for 30 DAA seeds.

The differences between stain-determined viability and the percentage of seeds with full embryos were non-significant for 30 DAA seeds, suggesting that both viability assessments could potentially predict the recruitment outcomes of *P. bifolia* immature seeds of this age.

Since the germination rate of *P. bifolia* seeds has been proven to depend on their age, it is important to record the maturity of orchid seeds by the time they are harvested after the anthesis and to study the embryo development

and seed coat ornamentation, which can be the key to improving the success of orchid seed germination *in vitro*.

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