

Zygotic embryogenesis and empty seed formation in European larch (*Larix decidua* Mill.)

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Abstract – Zygotic embryo development and its influence on empty seed formation of the European larch (*Larix decidua* Mill.) was studied within a seed orchard in the Slovak Republic. Histological study revealed a fertilization date early June. Although embryogenesis was most intensive in June, fully morphologically differentiated embryos were observed no earlier than in the second half of July. Multiple fertilization and simple polyembryony were relatively rare but their frequencies correlated with the efficiency of pollination. Only a small percentage of developing embryos reached full maturity. Degeneration included suppressed embryos from polyembryony as well as normal embryos occupying the optimal position within the corrosion cavity of the female gametophyte. The mean proportion of fertilized ovules without a viable embryo was 61% after open pollination and as high as 88% after controlled selfing. In total, embryo degeneration reduced the potential full seed percentage by almost 40% and is therefore considered the most important cause of unsound seed development in European larch.

larch / embryo / embryogenesis / empty seed

Résumé – Embryogenèse zygotique et formation des graines vides chez le mélèze d'Europe (*Larix decidua* Mill.). Le développement des embryons zygotiques et son influence sur la formation des graines vides du mélèze d'Europe (*Larix decidua* Mill.) ont été observés en condition de verger à graines en République Slovaque. L'étude histologique révèle que la période de fécondation la plus fréquente est le début du mois de juin. Bien que l'embryogenèse soit la plus intensive en juin, les embryons morphologiquement les plus différenciés sont observés dans la deuxième moitié du mois de juillet. Même si la fécondation multiple et la polyembryonie simple sont relativement rares, elles sont corrélées avec une pollinisation abondante. Le pourcentage d'embryons développés est très faible. La dégénérescence a affecté les embryons opprimés de polyembryonie ainsi que les embryons ayant la position optimale dans le gamétophyte femelle. La moyenne des ovules fécondés ne présentant pas d'embryon vivant a atteint 61 % après pollinisation libre et près de 88 % d'ovules après autopollinisation contrôlée. En moyenne, la dégénérescence des embryons a réduit le pourcentage potentiel de graines pleines de presque 40 % et c'est pourquoi elle est considérée comme la cause principale de la formation des graines vides chez le mélèze d'Europe.

mélèze / embryon / embryogenèse / graine vide

1. INTRODUCTION

European larch (*Larix decidua* Mill.) is a tree species with fast growth, valuable timber and a relatively high resistance to unfavourable environmental conditions and pests [35]. Despite the limited natural distribution, it is a frequent subject of introduction and breeding programmes over Europe and overseas [8, 10, 16, 21, 25]. Unfortunately, its natural and artificial regeneration is hampered by a high percentage of normal-appearing but empty seeds.

Since pre-fertilization ovule development of *Larix* Mill. occurs regardless of the presence of pollen grains on the stigmatic micropyle [22, 24], insufficient pollination has been considered the general cause of this problem. According to our previous

data [31], however, correlation between the efficiency of pollination and full seed percentage is relatively poor and it is therefore supposed that there exist additional factors affecting empty seed production.

In addition to the lack of pollination, other pre-zygotic events are known to influence mating success. These include pollination with non-viable pollen grains [5, 7, 32], abnormal pre-fertilization development of the female gametophyte [18, 19, 33] and disturbance of the pollination mechanism [19, 34].

Among post-zygotic events, the zygotic embryo development of larch is considered to be complex and vulnerable to failure [13, 14, 18, 19, 30]. Nevertheless, a closer look at post-zygotic processes is warranted, one that focuses on the types of losses during embryo formation and maturation. On this

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account, further detailed studies of zygotic embryogenesis could contribute to understanding some mechanisms of unsound seed formation in European larch.

2. MATERIALS AND METHODS

Female strobili of European larch were collected in 1998 from four grafted plants in the “Kmet’ová” seed orchard in Central Slovakia. This orchard was established in 1973 and is composed of the vegetative progenies of plus trees from the regions of Šumiac (South-Eastern part of Low Tatra Mts., autochthonous population of European larch – all the clones marked with “Š”) and Motyčky (Western part of Low Tatra Mts., allochthonous provenance of uncertain origin – the clones marked with “M”). For the purpose of embryological research, representative individuals from the most fertile clones were chosen after previously controlling for the production of female generative buds.

Semi-thin sections (~1.5 µm thick) were used for preparation of slides. Before the light microscopy studies, sampled ovules were deprived of integument and treated analogous to our previous papers [33, 34].

The zygotic embryogenesis process was compared by pollination method. In addition to exposing the female strobili to open pollination, self-pollination was tested: several branches containing both male and female generative structures were isolated together using waxed paper bags. Within the first three weeks of embryo development, 20–25 ovules from each clone (in dependence on the mode of pollination) were assessed to measure the occurrence of simple polyembryony and degenerating embryos. The relationship between the pollination success and the frequency of simple polyembryony was studied by non-linear correlation. Pollination efficiency was expressed by the mean number of pollen grains per pollinated ovule, quantified at the end of the pollination period and published in our earlier papers [31, 34].

3. RESULTS

3.1. Embryo and seed development in European larch

Sixteen-celled proembryos with four different layers were observed in early June, almost two months after the most intensive pollination and one week after pollen germination on the tip of nucellus. Initially (while the proembryo remained located at the chalazal end of fertilized archegonium), all four layers of the proembryo were of approximately equal size (Fig. 1a). By the consecutive suspensor’s expansion (Fig. 1b), the embryonal group was pushed from the fertilized archegonium into the central region of the female gametophyte, where the corrosion cavity was formed by dissolution of megagametophyte cells. Given the relatively long distance of this displacement (about 1.3–1.5 mm), a massive multicellular suspensor system was formed to make relocation of the embryonal tier possible and, concurrently, to keep its connection with the intracellular content of fertilized archegonium (Fig. 1c).

Formation and development of the non-differentiated multicellular (globular or ellipsoidal) embryonal mass were observed in the second and third week of June (Figs. 1c–1e). At the start of this phase of embryogenesis (a short time after displacement of the embryonal group to the central region of ovule), some of the four embryonal units of the former proembryo lagged behind and were suppressed (Fig. 1d).

When the non-differentiated embryo reached a length of about 350–400 µm, the first indications of histogenesis became

Table I. Results of testing the differences between the proportions of fertilized ovules with simple polyembryony depending on mode of pollination; p_1 ; p_2 = proportions of fertilized ovules with simple polyembryony, n_1 ; n_2 = sample sizes, φ_1 ; φ_2 = transformed variables, $\varphi_1 = \arcsin \sqrt{p_1}$; $\varphi_2 = \arcsin \sqrt{p_2}$, t_s = test statistic of the equality of two percentages ($t_s = [\varphi_1 - \varphi_2] / \sqrt{820.8 \cdot [1/n_1 + 1/n_2]}$), $z_{0.05}$; $z_{0.01}$ = critical values of normal distribution.

Pollination	p_1 ; p_2	n_1 ; n_2	φ_1 ; φ_2	t_s	$z_{0.05}$	$z_{0.01}$
Open	12.1%	44	20.37	1.16	1.96	2.58
Self	5.4%	49	13.44			

observable. The earlier stages of this process progressed relatively quickly and, consequently, all of the basic structural units of the embryo were formed around late June. The total embryo length was approximately 1.1 mm and its maximal width (in the region of cotyledons) ranged between 350 and 400 µm. However, the morphological development was still incomplete because of further enlargement and elongation of the embryo. Its definitive size and dimensions were not observed before the second half of July, when the full-sized embryo was approximately 2.1–2.3 mm long and about 0.7 mm wide (Fig. 1f).

3.2. Occurrence of simple polyembryony

The percentage of ovules with two or more fertilized archegonia (i.e. simple polyembryony) ranged from 0 to 25% depending on the mode of pollination and the clone. Although the differences between open pollination and self-pollination were not significantly different statistically (Tab. I), the frequency of ovules with simple polyembryony correlated with pollination efficiency (defined as the mean number of pollen grains per pollinated ovule).

The frequency of simple polyembryony increased very markedly when the pollination efficiency reached 3–4 pollen grains per pollinated ovule (Fig. 2). However, the percentages of multiple fertilization and simple polyembryony became irregular when 4 to 8 pollen grains were observed on the stigmatic hairs. Therefore, the highest correlation coefficient ($r = 0.94$) was computed when the threshold value of 4 pollen grains per pollinated ovule was taken into consideration. Although the correlated variables consisted of a restricted number of cases, the r -value is significant at $\alpha = 0.05$.

Nevertheless, the low rate of multiple fertilization does not correspond to the relatively high ascertained number of archegonia per ovule, which ranged from 2 to 5 with the mean values of individual clones ranging from 2.25 to 3.5 with a total weighted mean of 3.12.

3.3. Degenerating embryos

Despite the relatively high proportion of fertilized ovules (on average, more than 50%), only a few embryos achieved the stage of the full maturity. A great deal of embryos abnormal in appearance and showing consecutive degeneration were observed, especially in the phase of the non-differentiated multicellular embryonal mass (at about the mid-June), when the

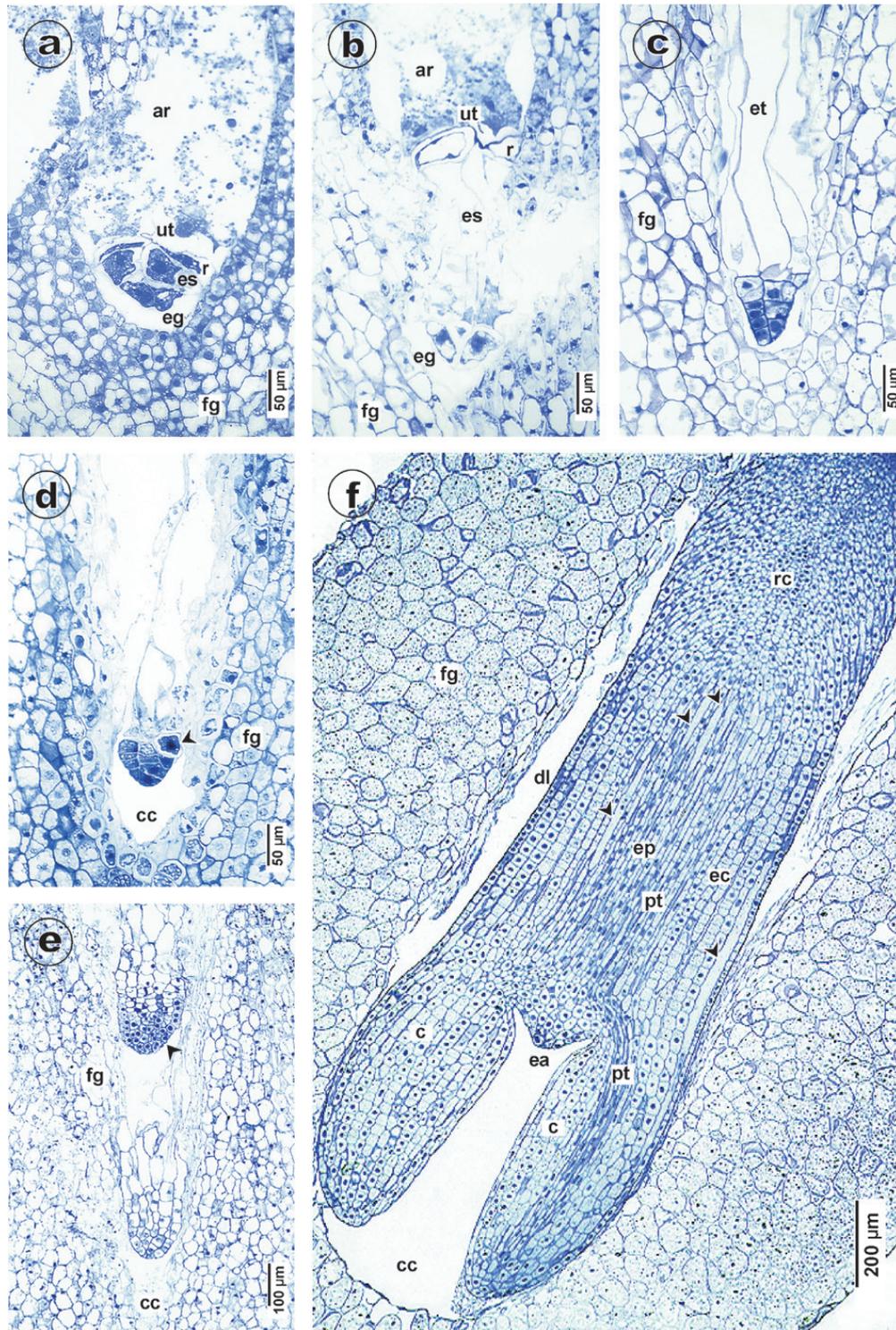


Figure 1. Development of embryo (ar: archegonium, fg: female gametophyte, ut: upper tier, r: rosette, es: embryonal suspensor, eg: embryonal group, et: embryonal tube, rc: root cap, ep: embryonic pith, ec: embryonic cortex, dl: dermal layer, ea: embryonic apex, c: cotyledons, pt: provascular tissue, cc: corrosion cavity). (a) Four-layer proembryo in the chalazal region of fertilized egg cell. June 8. (b) Elongation of embryonal suspensor and displacement of the embryonal group out of fertilized archegonium. June 8. (c) Formation of multicellular embryonal mass inside the corrosion cavity. June 8. (d) Globular embryonal mass: one of the embryonal units seems to be suppressed (marked with arrowhead). June 8. (e) Continuing development of non-differentiated embryo and appearance of simple polyembryony with the possible substitution of the more vigorous distal embryo (marked with arrowhead) for the less vigorous proximal one. June 17. (f) Differentiated embryo (arrowheads: elongated cells with an assumed secretory activity). July 30. Note: root meristem, column and pericolumn are not visible due to the oblique section.

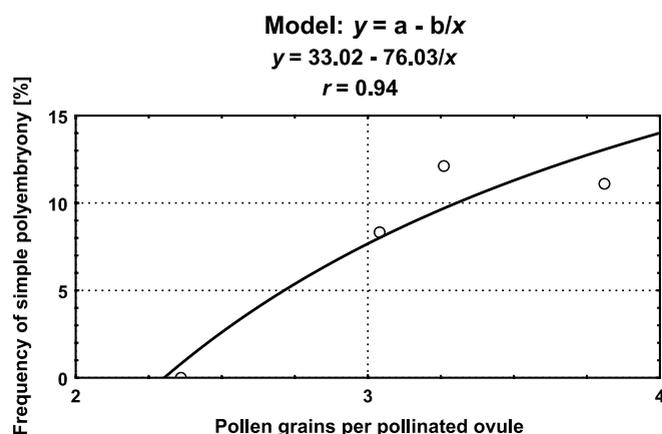


Figure 2. Correlation between the efficiency of pollination and percentage of fertilized ovules with simple polyembryony.

following three essential types of the abnormal-looking embryos were distinguished (Figs. 3a–3c):

- (i) Embryos with an abnormal location in the corrosion cavity (Fig. 3a).
- (ii) Embryos with apparent anatomical and cytological symptoms of degeneration (Fig. 3b).
- (iii) Dwarfed embryos, retarded apparently in their growth (Fig. 3c).

Abnormal appearance and symptoms of degeneration were also characteristic of all the suppressed embryos from polyembryony. However, in some cases of simple polyembryony, the embryo with the most favourable position in the corrosion cavity had the attributes of abortion while the second, more distally situated embryo was larger and more vigorous (Fig. 1e). This fact supports the assumption that simple polyembryony offers the possibility of embryo replacement after degeneration.

In general, the proportion of the female gametophytes with complete degeneration of embryos was minimal in ovules with simple polyembryony, whereas the absence of viable embryos was much more frequent in cases of single fertilized archegonium per ovule.

As shown in Table II, complete degeneration of embryos occurred in 44.4–83.3% of the fertilized ovules exposed to the open pollination (the minimum and maximum values were related to the clones marked as Š13 and M10, respectively), whereas after the controlled self-pollination the percentage of the fertilized ovules with the completely degenerated embryos was considerably higher: it ranged from 81.3% in M10 to 100.0% in the clone marked as Š13. The differences in percent complete degeneration between open pollination and self-pollination (61.1% vs. 87.7% on average, respectively) were significant at $\alpha = 0.01$ (Tab. III). When calculated for the total number of ovules (fertilized as well as unfertilized ones), all the resultant proportions were lower and the total differences changed due to the different success of fertilization in individual samples. On average, embryo degeneration reduced the expected potential full seed set by almost 40%.

4. DISCUSSION

In various species of *Larix*, fusion of gametes and first divisions of zygote take place several weeks after pollination, a short time after relocation of engulfed pollen grains from the micropylar canal to the tip of nucellus. According to our observations, the estimated most frequent fertilization date occurs in early June.

The differences between the percentages of unfertilized ovules (especially between open pollination and self-pollination) might suggest the existence of some kind of a pre-zygotic incompatibility between pollen grains and the female gametophyte [9, 24, 36, 37]. However, our results are mostly influenced by an occasional lack of pollination [31], whereas the

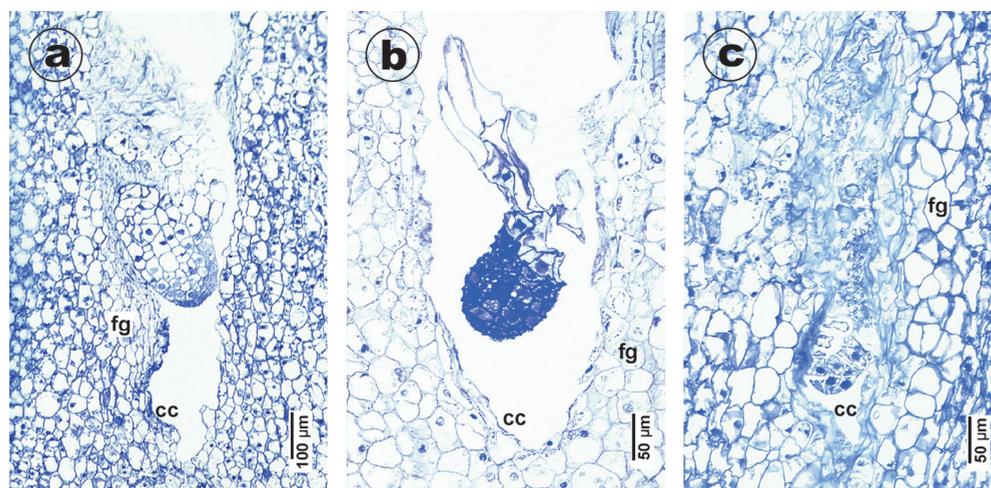


Figure 3. Appearance of degenerating embryos (fg: female gametophyte, cc: corrosion cavity). (a) Abnormally located and partially deformed embryo. June 17. (b) Degenerated embryo with darkly staining cells. June 22. (c) Abnormally small embryo retarded in its growth. June 17.

Table II. Percentage of ovules with complete degeneration of embryos depending on mode of pollination and clone.

Pollination	Clone	Proportion of ovules with the complete degeneration of embryos		Unfertilized ovules (%)
		From fertilized ovules (%)	From total number of ovules (%)	
Open	M5	50.0	40.0	20.0
	M10	83.3	55.6	33.3
	Š13	44.4	28.3	36.4
	Š14	66.7	35.6	46.7
	Total	61.1	39.9	34.1
Self	M5	91.7	28.2	69.2
	M10	81.3	51.7	36.4
	Š13	100.0	44.5	55.5
	Š14	77.8	32.4	58.3
	Total	87.7	39.2	54.9
Total		74.4	39.5	44.5

Table III. Results of testing the differences between the rates of fertilized ovules with complete degeneration of embryos depending on mode of pollination; p_1 ; p_2 = rates of fertilized ovules with complete degeneration of embryos, n_1 ; n_2 = sample sizes, φ_1 ; φ_2 = transformed variables, $\varphi_1 = \arcsin \sqrt{p_1}$; $\varphi_2 = \arcsin \sqrt{p_2}$, t_s = test statistic of the equality of two percentages ($t_s = [\varphi_1 - \varphi_2] / \sqrt{820.8 \cdot [1/n_1 + 1/n_2]}$), $z_{0.05}$; $z_{0.01}$ = critical values of normal distribution.

Pollination	p_1 ; p_2	n_1 ; n_2	φ_1 ; φ_2	t_s	$z_{0.05}$	$z_{0.01}$
Open	61.1%	45	51.42	3.05	1.96	2.58
Self	87.7%	49	69.45			

percentage of pollinated, but unfertilized, ovules is almost constant [34]. Therefore, the existence of a pre-zygotic incompatibility between ovules and self-pollinating pollen grains is not assumed and, most likely, the first fertilizing male gamete is derived from the first pollinating pollen grain, according to earlier data related to Douglas fir [39]. Conversely, recent data from Chinese authors [15] suppose the existence of post-pollination pollen selection in *Larix principis-rupprechtii* Mayr.

The phases of proembryogenesis, early embryogenesis and late embryogenesis are usually distinguished during the development of the zygotic embryo. However, analogous to the phases of mitotic cell division, older literature defines zygotic embryogenesis as prostage, metastage, anastage and telostage [29]. While the progression of proembryogenesis or prostage is usually quick, metastage or early embryogenesis has a markedly longer duration – nearly two weeks, or 36% of the total time of all the four phases, according to the last cited author. Our results support the investigations of Kosiński [18, 19], who found the most frequent disturbances and losses of developing embryos at the stage of the suspensor's expansion and the displacement of embryonal group into the corrosion cavity. On the other hand, the late embryogenesis (anastage and telostage) is characterized by the intensive quantitative growth and histogenesis [1, 12, 23, 29, 38].

In the context of our results, an extremely high percentage of self-pollinated ovules with complete degeneration of embryos may be interpreted as the negative selection of undesirable genotypes as early as in the embryonal phase. It seems to be probable (concerning the poor pre-zygotic incompatibility) that the genetically undesirable pollen grains of *Larix* may be transported to the tip of nucellus, produce normal pollen tubes and participate in fertilization. Nevertheless, the percentage of fully developed seeds decreases due to the frequent embryo degeneration at the post-zygotic stages.

Since a high percentage of selfed-embryo abortion (and consequently, the significant decrease of the full seed proportion after self-fertilization) was ascertained in various species of *Larix* [4, 17, 19, 26, 28], this mechanism may be considered a reproductive strategy within this genus. It seems to be controlled by lethal and sublethal genes [17, 26] and may also be considered the most significant reason for the high outcrossing rates, estimated after genetic analyses of full-developed seeds [2, 3, 6, 11, 20, 27]. On the other hand, embryo degeneration may also be relatively frequent after cross-pollination (e.g. in ovules which were exposed to the pollen grains from surrounding trees with a high degree of relatedness). Therefore, besides the maximal possible restraint of self-fertilization, the combinations of genotypes (clones) with a minimal degree of the mutual genetic relationship should be preferred in breeding populations and conversely, the combinations, in which the percentage of embryo degeneration is permanently high following consanguineous mating, should be eliminated. Such consistent selection might contribute to a significant increase in vital seed proportion and thus, to the successful breeding and enhanced economical effectiveness of the seed orchards of larch.

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