THE EARLY-SPRING DEVELOPMENT OF MALE GENERATIVE ORGANS AND ABNORMALITIES IN POLLEN ONTOGENESIS OF EUROPEAN LARCH (*LARIX DECIDUA* MILL.)

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ABSTRACT

In central Slovakia, the early-spring phases of the European larch (*Larix decidua* Mill.) microsporogenesis start at the late diplotene from mid-February to early March, depending on the year and the observed individual. While the course of late diplotene–telophase I and prophase II–telophase II is rapid, the duration of interphase before meiosis II is longer. In general, tetrads of microspores appear in microsporangia approximately two weeks after the cessation of the winter rest and the shedding starts in early April. The five-celled mature pollen is smooth, cup-shaped, with ridge-like sculpturing of exine and a barely visible leptoma. The tapetum of European larch remains in a continuous layer until microsporogenesis is completed, suggesting the long-lasting secretory activity. The three basic categories of the abnormal ontogenesis of pollen (chromosomal irregularities in nuclei of meiocytes, irregularities in the mechanism of cell division during microsporogenesis, improper development of the male gametophyte) were relatively rare and therefore, their major impact on viability of pollen is not supposed.

Key words: *Larix decidua* Mill., microsporangium, microsporogenesis, pollen development

INTRODUCTION

In the Slovak Republic, European larch (*Larix decidua* Mill.) is interesting for commercial, environmental and aesthetic reasons. Despite its limited natural range in the Carpathian Mountains of Central Europe, it is frequently planted in seed orchards and its seed is used for reforestation. However, at most, only 30–40 % of seed yield contains filled seeds. Based on the older literature data from Sweden (EKBERG & ERIKSSON 1967; EKBERG *et al.* 1968; ERIKSSON 1968, 1970; ERIKSSON *et al.* 1970), abnormalities in pollen ontogenesis should be considered one of the serious causes of the lack of fertilization and development of unsound seeds, because the seed coat formation of larch is independent of the fertilization success (OWENS & MOLDER 1979; OWENS & BLAKE 1985; TRENIN 1986; OWENS *et al.* 1994).

Development of the male generative organs in relation to the dormant period in *Larix* Mill., as well as *Pseudotsuga* Carrière, *Tsuga* Carrière and *Thuja* L., is characterized by the relatively rapid differentiation of microsporangia and by the start of meiosis in autumn (ERIKSSON 1968; OWENS & BLAKE 1985). After leptotene and zygotene, meiosis ceases at the prolonged pachytene or diffuse diplotene stages at the onset of dormancy (OWENS & MOLDER 1971). Development of microspores is not completed until early spring, during which time temperatures are frequently below freezing. Therefore, the long-lasting microsporogenesis of these genera, and *Larix* in particular, might be very vulnerable to spring-frost-induced abnormalities that might result in non-viable pollen. However, data on the development and frequency of abnormalities vary with geographical regions and species (CHRISTIANSEN 1960; EKBERG & ERIKSSON 1967; EKBERG *et al.* 1968; ERIKSSON 1968, 1970; ERIKSSON *et al.* 1970; KOZUBOV 1974; HALL & BROWN 1976; HALL 1982; ROZHDESTVENSKII & SEMERIKOV 1995) so that generalizations are almost impossible. Because of this, the early-spring pollen development of European larch was investigated under the natural conditions of Central Europe.

MATERIAL AND METHODS

The early-spring development of the male generative organs was studied in „Kmeťová" seed orchard (Forest Enterprise in Slovenská Ľupča, region of Banská

Bystrica, central Slovakia) during the spring periods of 1997, 1998 and 1999. The studied seed orchard comprises of clones from two different localities of European larch in Slovakia: one from its natural range in the Low Tatra Mountains (clones marked with „Š" - Šumiac village) and the other from an allochthonous locality of uncertain origin (clones marked with „M" - Motyčky village). Branches with developing male strobiles were collected twice per week from five clones scored as the most fertile.

Cytological aspects of meiosis and development of the male gametophyte were observed two times per week from squash preparations after fixation and staining by acetocarmine. The microsporocytes, microspores or developing pollen grains from 2-3 male strobiles of each observed clone were investigated for each collection date. The microphotographs were taken using an OLYMPUS PM-20 photomicrographic system adapted to the OLYMPUS BX-50 optical microscope. Due to the destructive sampling, the time course of the development of the male generative sphere was estimated from the observed frequencies of individual stages on investigated slides.

Once a week, this method was combined with the observations of the semi-thin sections. For this purpose, segments of the male strobiles were fixed in the buffered solutions of glutaraldehyde and osmium-tetroxide, dehydrated by a graded series of acetone, embedded in Durcupan and, after the 48-hour polymerization at 60 °C, were cut on a TESLA BS-490A ultramicrotome. Individual sections (1-1.5 μm thick) were stained in water solutions of basic fuchsine and toluidine blue (LUX 1981). After the preliminary observations, the selected preparations were processed by the OPTIMAS 6.5 software adapted for the ZEISS Axiophot II optical microscope with the SONY DXC-950P video camera and ITI PCI framegrabber (GUTTENBERGER *et al.* 2000).

The surface and the outward appearance of the mature pollen grains were observed by the PHILIPS ESEM scanning electron microscope. Before the beginning of the investigations, small amounts of pollen were applied to the aluminium stubs with double-side adhesive tape. Using an AGAR Sputter-Coater, they were coated with a thin layer of gold.

RESULTS

According to the previous findings of SLOBODNÍK (2000), the sporogenous cells in the male generative organs (microsporangia) European larch from central Slovakia enter the dormant period in early November. At that time, the nuclei of the meiocytes stain very weakly and the tapetum is characterized by an occasional occurrence of binucleate cells.

Immediately after the end of winter dormancy (mid-February to early March), late diplotene, diakinesis and metaphase I through telophase I were observed in the meiocytes in similar frequencies, suggesting rapid succession. More than two weeks differences were observed among individual years, but the differences among the observed clones were considerably smaller.

Based on the higher frequency of the interphase before meiosis II on the investigated slides from the second and especially the third sampling dates, duration of this stage appeared a little longer in comparison with the postdormant stages of meiosis I. During this stage, development of the tapetum and the microsporangium wall was almost identical to that during the diffuse chromosome stage at the beginning of the dormant period (SLOBODNÍK 2000). The epidermis was about 10 μm thick and the cells of the middle layers were flattened (Figure 1a). At that time, the size of the binucleate cells of the tapetum ranged from 15 × 20 to 20 × 25 μm.

Unlike the relatively long duration of interphase, prophase II through telophase II were quick. Within a few days meiosis of the microspore mother cells was completed and the tetragonal or tetrahedral tetrads of haploid microspores were observed as early as two weeks (i.e. at fourth sampling date) after the end of winter dormancy. The cells of the tapetum were still arranged in a continuous layer but in many cases, they were characterized by the vacuolated cytoplasm (Figure 1b).

After the separation of the microspores in the tetrads, the microspores enlarged and the starch grains began to accumulate in their cytoplasm. The tapetum was completely destroyed by this time and small clusters of tapetal protoplasts were observable among the microspores (Figure 1c). During the first two mitoses, formation of the two-layer pollen grain wall was completed and differences in timing of pollen development among individual trees were largely eliminated. The last mitosis before maturation of pollen was observed in late March (image not shown). For all investigation years, shedding of the mature, five-cell pollen grains with a diameter of about 65-70 μm began in early April (Figure 1d). The SEMs demonstrate the conspicuous ridge-like sculpturing of the exine (Figure 1e-f) and the leptoma at the distal end of the cup-shaped pollen grain (Figure 1f).

Disturbances and irregularities in the development of male generative organs

Some chromosomal irregularities were observed during the early-spring stages of microsporogenesis, including

not fixed at any constant date and is markedly influenced by the prevailing meteorological conditions. In tamarack (*L. laricina* (Du Roi) K. Koch), the differences in the dates of the early-spring meiosis might vary by more than two weeks among seasons (HALL 1982). Within individual microstrobiles, the microsporocytes in basal microsporangia start to divide first (OWENS & MOLDER 1979; HALL 1982). Results from the present study demonstrated that the time of the first post-dormant stages of microsporogenesis varied in European larch grown in seed orchards. The development in clones from one locality was always one week ahead of the development in clones from the other origin. This indicates that the pollen development is under genetic control.

In the early spring, cells of the tapetum remained in a single continuous layer until the microsporogenesis was completed. Their cytoplasm was granular and vacuolated but symptoms of degeneration were not observed before the unicellular microspore stage, when fragments of the tapetal protoplasts were observed frequently in the pollen loculus of microsporangium. Therefore, the tapetum in the male sporangia of European larch is, most likely, of the secretory type. However, during various stages of development there was complete premature destruction of the tapetal cell walls in some abnormal microsporangia. The abnormalities were probably similar to those observed and described by MIKULSKA *et al.* (1969) who characterized European larch as having a periplasmodial type of tapetum. My opinion is that such premature disintegration of the tapetal cells is an abnormal phenomenon, caused by some disturbances in the development of the male generative organs or by incorrect chemical treatment of the biological material or some other unknown factors.

Formation of the cell walls generally was simultaneous during the development of tetrads. However, the successive type of wall initiation was described as the one way of the cell wall formation occurred in tetrad development of European larch (RODKIEWICZ *et al.* 1984). Although both simultaneous and successive types may occur in the microsporocytes of *Larix* (CHRISTIANSEN 1960; TRENIN 1986), the successive cell wall formation is considered atypical. According to the data of TRENIN (1986), this might be caused by a deceleration of microsporogenesis resulting from adverse meteorological conditions. In some squash preparations of the dividing microsporocytes, the dyad formation was observed as a possible first phase of the successive cell wall formation after the completion of the meiosis I. It was not determined whether such dyads are able to undergo the normal meiosis II and finish the successive cell wall formation or if further development stopped.

In Central Europe, pollen release of *L. decidua* occurs at the five-cell pollen stage. However, the division of the antheridial („generative") cell occurred only a short time before pollen release suggesting possible release of four-celled pollen grains as well. This phenomenon was described for *Larix* species (CHANDLER & MAVRODINEANU 1965; HO & ROUSE 1970; KOZUBOV 1974; SHIN & KARNOSKY 1995).

The occurrence of so-called twin pollen grains of larch (i.e. the pollen grains which contain two male gametophytes) has been reported by HO & ROUSE (1970) and TRENIN (1986). This kind of abnormality might be caused by incomplete cell wall formation during microsporogenesis due to low temperatures. TRENIN (1986) supposed that the twin pollen grains might be formed due to the vigorous development of a large first prothallial cell which could divide as an initial cell of a new male gametophyte. In accordance with these data, CHRISTIANSEN (1960) also observed the abnormal enlargement of the prothallial cell at the beginning of the male gametophyte formation. The occasional occurrence of this phenomenon has been also confirmed by present investigations.

Concerning the very poor pollen tube formation of *Larix* on traditional cultivation media (CHRISTIANSEN 1969, HO & ROUSE 1970, SAID *et al.* 1991, SHIN & KARNOSKY 1995), the exact *in vitro* test of the viability of pollen was not carried out in this study. However, the newer authors (FERNANDO *et al.* 1997, 1998; DUMONT-BÉBOUX & VON ADERKAS 1997; DUMONT-BÉBOUX *et al.* 1998, 1999, 2000) describe the successful *in vitro* pollen tube induction of *Larix* and closely related genus *Pseudotsuga*. In most cases, their methodology was based on the surface sterilization of male strobiles, slow pollen hydration at 100 % relative humidity and the so-called two-step method (pollen elongation and tube induction on two different media with the addition of minerals, sugars and/or polyethylene glycol). Nevertheless, pollen tubes developed only in a part of elongated pollen grains, scored as being alive (DUMONT-BÉBOUX & VON ADERKAS 1997; DUMONT-BÉBOUX *et al.* 1999, 2000). In present study, a gross estimation of pollen viability was carried out on the basis of pollen swelling and elongation after 5 days of growing on agar medium with 10 % sucrose. Proportion of the elongated pollen grains was very high in two consecutive years of 1997 (83.46 %) and 1998 (98.80 % on average). The reduced estimated viability of the pollen grains in 1997 may be ascribed to the low air temperatures in the early-spring period of that year (unfortunately, exact temperature data are not available). There were very small differences among the mean values of individual clones and therefore, the preference of genotypes as a consequence of different

viability of pollen (VENÄLÄINEN *et al.* 1999; NIKKANEN *et al.* 2000) is not probable in this case. Whereas female strobiles of European larch have been observed to be damaged by spring frost in Poland (LEWANDOWSKI & KOSIŃSKI 1987), the studies from Slovakia do not confirm such strong damage of male generative organs.

CONCLUSIONS

These results on early-spring development of male generative organs of European larch may have application e.g. in the optimal timing of pollen collection for the supplemental mass pollination, artificial hybridization etc., for various breeding programs and strategies in Central-European seed orchards. For the shedding of pollen grains in lab conditions, branches with maturing male strobiles should not be collected earlier than after the last mitosis in developing pollen (late March).

The abnormalities in development of pollen are not considered the significant cause of empty seed production of *L. decidua* in Central Europe. The frequency was not high for either abnormal chromosomal or cellular activities, such as chromosome bridges, lagging chromosomes, stickiness and fragmentation of chromosomes, spindle irregularities, nuclear divisions without replication of chromosomes, formation of univalents, giant pollen grains etc. Even in 1997, when the air temperatures in late March and early April were frequently below freezing, the estimated percentage of the pollen viability per individual was about 80 % at least.

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