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Variability of in vitro germination of *Picea omorika* pollen

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Abstract: Serbian spruce (*Picea omorika* /Panč./Purkyne) is a Balkan endemic and Tertiary relict, therefore it has an exceptional significance from the historical-geographical aspect as well as from the aspect of tree improvement and biodiversity conservation. Pollen was collected from 24 trees in two consecutive years and analyzed once a month. Germination of fresh pollen and pollen stored under different temperatures (room temperature: 23 ±1°C, +4°C, –15°C and –20°C) by in vitro method on a medium with 10% sucrose was analyzed. Fresh pollen germination was 53.63% and 68.06% for pollen collected in first and second year, respectively. After one year of storage at –20°C pollen germination was 22.36% and 60.72%, respectively. The differences between pollen collection years, temperature treatments, storage periods and individuals were statistically significant. Since pollen germination rates of individual trees between the first and the second year showed weak positive correlation, one could conclude that germination of fresh pollen grains is under the certain influence of environmental conditions. It is determined strong positive correlations between fresh and stored pollen at –20°C from both years of pollen collection.

Additional key words: Serbian spruce pollen, pollen conservation, treatments, germination

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Introduction

Serbian spruce, *Picea omorika* (Panč.) Purkyně, is an endemic-relic conifer of the family *Pinaceae*. It grows predominantly on calcareous soils, at the elevation of 300–1700 m, north exposition (Vidaković 1991). This species was once-widely distributed across Europe. It passed the glaciations period in the refuge, and today its natural range is restricted to west and southwest Serbia and east Bosnia and Herzegovina (Nasri et al. 2008), most often as small, geographically isolated populations which are threatened by extinction (Ballian et al. 2006).

Because of its theoretical and practical importance, natural populations of Serbian spruce were investigated from different aspects (Budimir 2003; Ballian et al. 2006; Milovanović et al. 2007; Aleksić et al. 2009 etc.)

The normal pollination process and seed yield depend on pollen quality and quantity (Tucović and Isajev 1982; Runions et al. 1999; Šijačić-Nikolić and Isajev 2001). The characteristics of male gametophytes are directly dependent on parental characteristics, but they are also affected by external factors deciding the flowering time and abundance, and pollen distribution and germination (Mergen et al. 1965; Owens et al. 1987; Caron and Leblanc 1992; Gómez-Casero et al. 2004; Schueler et al. 2005). *Picea orientalis* and *P. sitchensis* (Ho and Sziklai 1972; Runions et al. 1999) release pollen in the second half of April and the first half of May. *Picea omorika* pollinates in the second week of May (Grbović and Isajev 1997). The periodic-

ity and the variability in the abundance of flowering and pollen production have already been reported for *P. omorika* (Isajev 1987), as well as for *P. mariana* (Caron and Leblanc 1992). However, it had already been concluded that abundant flowering does not guarantee high germination of pollen (Grbović 1998). Nikkanen et al. (2000) also reported that in *Picea abies* there was no correlation between pollen viability and phenology, or between pollen viability and growth characteristics of trees.

Morphological, chemical and/or physiological properties of pollen grains are very often used in botanical (Ho and Sziklai 1972; van der Knaap et al. 2005), aerobiological and allergenic (Diaz de la Guardia et al. 2006), paleobotanical and palinological (Čolić 1965), as well as in plant breeding studies (Williams and Savolainen 1996). For the successful preservation of the viability of pollen, several methods were improved: storage at low temperatures (Kirby and Stanley 1976; Cram and Lindquist 1984; Braggio Morucchio et al. 1990; Lanteri et al. 1993), storage in liquid nitrogen (Withers 1991) and vacuum drying (Shoenike and Stewart 1963).

Most studies of variability morpho-physiological traits related to pollen of species level, but only a few authors dealt with individual variability (Kormutak et al. 1994, 2007; Nikolić 1996; Slobodnik and Krizo 1997; Nikolić and Tošić 2006). Reports about individual variability of Serbian spruce flowering and pollen germination are still rare and insufficient (Grbović 1998; Grbović and Isajev 1997; Šijačić-Nikolić and Isajev 2001; Batos and Nikolić 2004).

The research aims of this paper are: 1) analysis of individual and population variability of Serbian spruce fresh pollen germination rate and 2) determination of the possibility of its pollen germination preserved under different temperatures.

Materials and Methods

Material collection

Pollen was collected from the trees in the Serbian spruce planted populations at the site Bele Zemlje (43°48'32" N; 19°44'25" E; Zlatibor Mountain, Serbia) at about 20 km air distance from the nearest Serbian spruce natural population. This forest culture was established at the site of *Quercetum frainetto – cerris*, at an altitude 680 m, from the seed from natural populations (Tara Mountain, Serbia). The culture was 60 years old and its area was 0.5 ha. Based on the general viability, 24 test trees were selected and the branchlets with microstrobiles were sampled during the period immediately prior to pollination (Pulkkinen and Rantio-Lehtimaki 1995), in the second week of May and transported to the laboratory in plastic sacks, taking care about the preservation of genotype (tree) identity. Pollen was collected from the same trees for two consecutive years. Mean air temperatures were 8.0°C and 9.7°C in the first and in the second year, respectively. Mean annual precipitations were 750.4 mm and 748.0 mm, respectively.

Collection and storage of pollen

The branches with microstrobiles were placed in the vases with water in the laboratory and within 24 h anther breaking and releasing of pollen grains took place. The collected pollen was dried during 48 h in the thermostat at 30°C, and purified by passing through a series of sieves, up to 0.2 mm. A part of the fresh pollen was immediately tested for germination, and the other part was stored in the sealed glass vials and in a desiccator over Silica Gel at 4 different storage temperatures: 1) room temperature: 23 ±1°C, 2) +4°C, 3) –15°C and 4) –20°C (Lanteri et al. 1993, Kormutak et al. 2007).

In vitro germination of pollen

Pollen germination test was performed on fresh pollen (immediately after collection) and then monthly over the next 11 months (stored pollen). The pollen stored in low temperatures were thawing at room temperatures for 2 h. According to Kirby and Stanley in vitro modified method (1976), pollen was sawn on a drop of germination medium on the slides which were placed on metal spatulas in closed Petri-dishes (Fig. 1). Pollen of each tree (10 mg of dry pollen in 0.1 ml medium) was sown in three repetitions, i.e. drops. The germination medium was a 10% sucrose solution in distilled water (Fig. 2). This concentration showed the best results in the earlier research (Grbović and Isajev 1997). Pollen grains of each tree were placed in separate Petri dish, whose bottom was covered with a thin layer of distilled water for the hydration of pollen granules. Pollen germination was evaluated 48 h after sowing by calculating

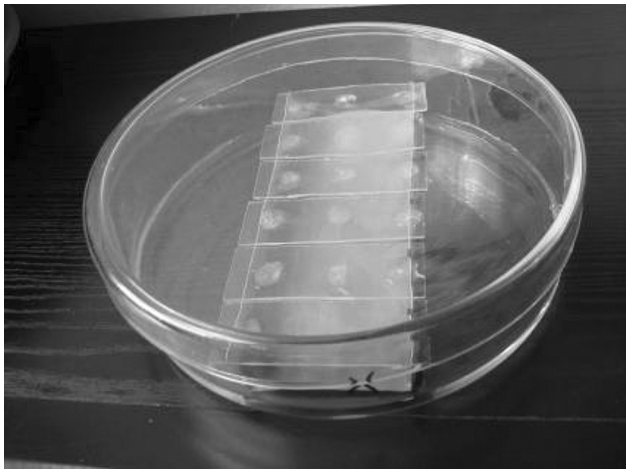

Fig. 1. Sowing of *Picea omorika* pollen in Petri-dish, in vitro method (Batos oryg.)

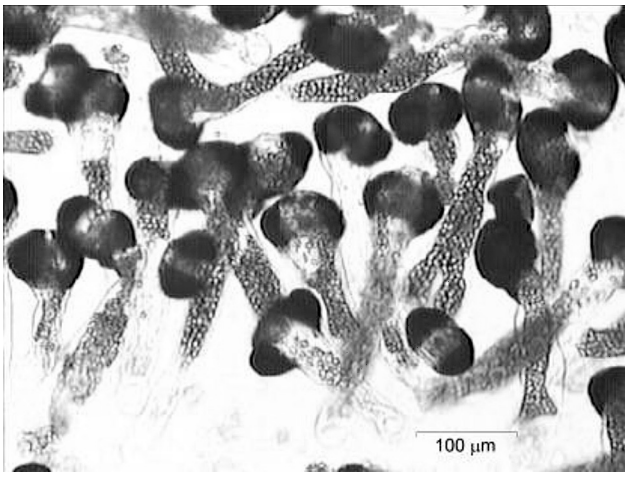

Fig. 2. The germination of *Picea omorika* pollen in medium of 10% sucrose. Bar refers to 100 μm (Batos oryg.)

the number of normally germinated pollen grains compared to the total number of grains in the microscope field of vision (magnification × 160). According to Arista and Talavera (1994), the pollen grains were considered germinated when the pollen tube was longer than half the pollen grain diameter. The temperature treatments in which the average pollen germination decreased to ≤ 5% were excluded from further testing. Results were presented for fresh and stored pollen (after 6 and 11 months of pollen preservation at –20°C) on the 10% of sucrose.

Statistical analysis

The statistical analysis included two- and three way analysis of variance, testing of individual differences (Fisher's LSD test, at $\alpha = 0.05$ significance level). The statistical analysis also included correlation analyses of fresh pollen harvested from two consecutive years as well as correlations between fresh and stored pollen (at –20°C), particularly for every year of pollen harvesting. For statistic analyses software package STATGRAPHICS Plus was used.

Results

Germination of fresh pollen

Germination of Serbian spruce fresh pollen from 24 trees accounted for 8.33% to 93.57% (53.63% on average) in the first year and for 48.50% to 90.21% (68.06% on average), in the second year, respectively (Fig. 3). The differences in fresh pollen germination between collection years and between individual trees were statistically confirmed (results were not presented).

Germination of stored pollen

The germination percentage of pollen from the first collection year, stored at a room temperature: 23 ± 1°C, decreased to less than 10% already in the first month (from 53.63% to 8.75%) (Fig. 4). In the fourth month of storage, the germination percentage of the pollen from the second collection year decreased from 68.08% to 33.52% and it decreased to below 10% (9.96%) in the sixth month (Fig. 5).

Pollen from the first collection year stored at +4°C lost gradually its otherwise poor initial germination percentage, by the eighth month (from 31.41% to 2.81%) (Fig. 4). The germination of pollen from the

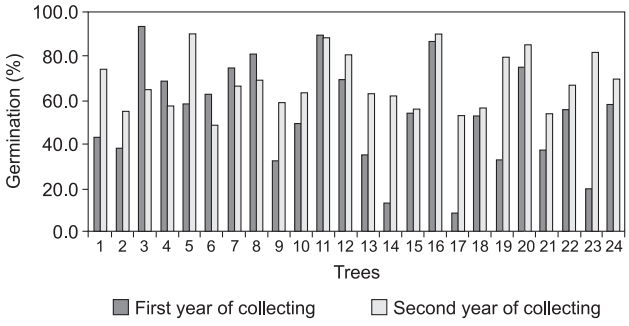

Fig. 3. Individual variation of fresh pollen germination from the first and second collection years

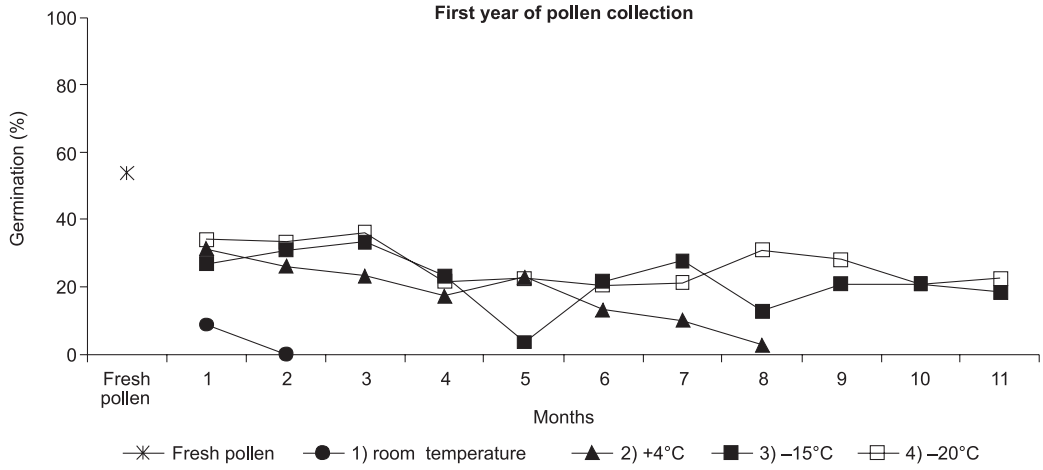

Fig. 4. Variability of pollen germination in the first year of collection, during a year of preservation at different temperature treatments

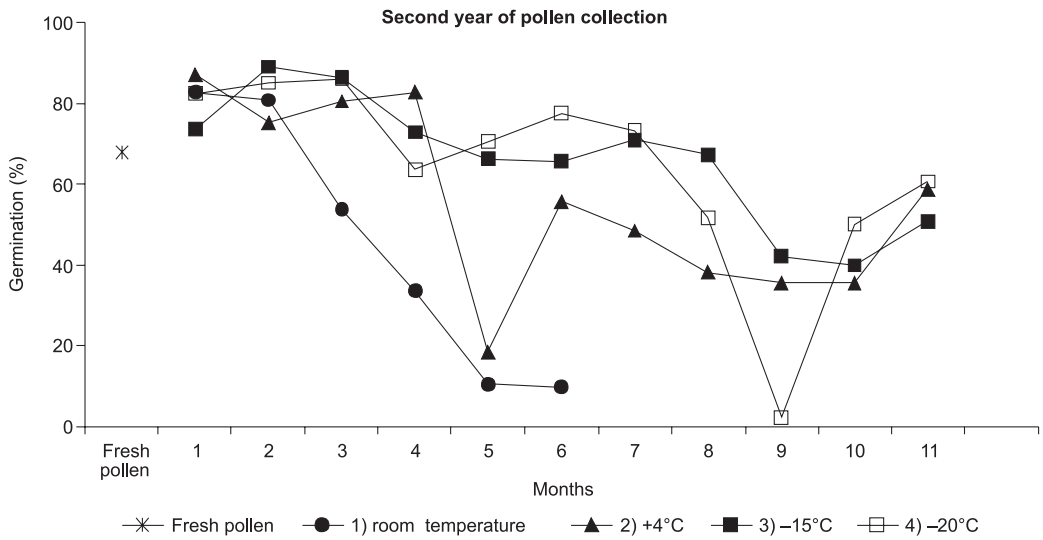

Fig. 5. Variability of pollen germination in the second year of collection, during a year of preservation at different temperature treatments

second collection year was high, above 80% (87.12%), till the fourth month (82.81%), and in the ninth month it dropped to 35.54%.

Germination of pollen stored at –15°C and –20°C alternately increased and decreased till the eleventh month. In the eleventh month, statistically significant differences in germination of pollen stored at –15°C and –20°C were obtained in the first collection year (18.28% and 22.36%, respectively) as well as in the second collection year (50.87% and 60.72%, respectively). Decrease of pollen germination after one year of storage was somewhat greater in the case of pollen stored at –15°C (65.91% and 25.26%, first and second year of collection, respectively) than at –20°C (58.31% and 10.78%, respectively) (Fig. 4, Fig. 5).

The germination of pollen stored in the first months of the second year, was even higher compared to fresh pollen germination (Fig. 4, Fig. 5). The differences in stored pollen germination between collection years, individuals and storage periods were statistically highly significant. The trees with high values of fresh pollen germination also had the best germination even after a year of pollen storage (results were not presented).

Correlations between years of pollen collection and between germination of fresh and stored pollen

The results of fitting a linear model to describe the relationship of fresh pollen germination between two collecting years were presented in Figure 6. The correlation coefficient was about 0.37 indicating a relatively weak relationship between years. It was approved statistically significant relationship at the 90% confidence level (ANOVA test, $p < 0.10$, results were not presented).

The relationship between pollen germination of fresh and pollen stored 11 months at –20°C (originated from the first year of collection) was presented in Figure 7. The correlation coefficient was about 0.61 indicating a moderately strong relationship between fresh and stored pollen. It was approved statistically significant relationship at the 99% confidence level (ANOVA test, $p < 0.01$, results were not presented).

The relationship between pollen germination of fresh and pollen stored 11 months at –20°C (originated from the second year of collection) was presented in Figure 8. The correlation coefficient was about 0.39 indicating a relatively weak relationship between fresh and stored pollen. It was approved statistically significant relationship at the 90% confidence level (ANOVA test, $p < 0.10$, results were not presented).

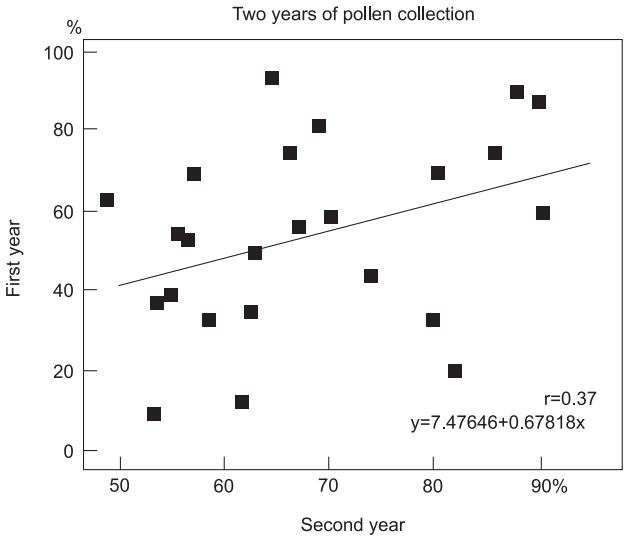

Fig. 6. Correlation between years of fresh pollen collection.

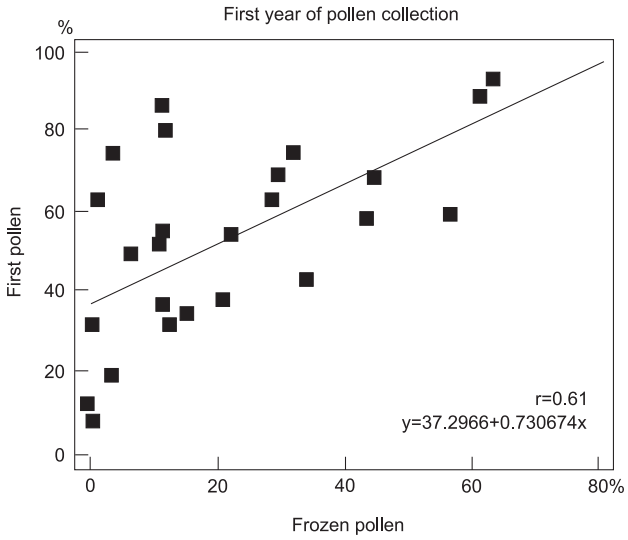

Fig. 7. Correlation between germination of fresh and stored pollen at –20°C (originated from the first year of collection)

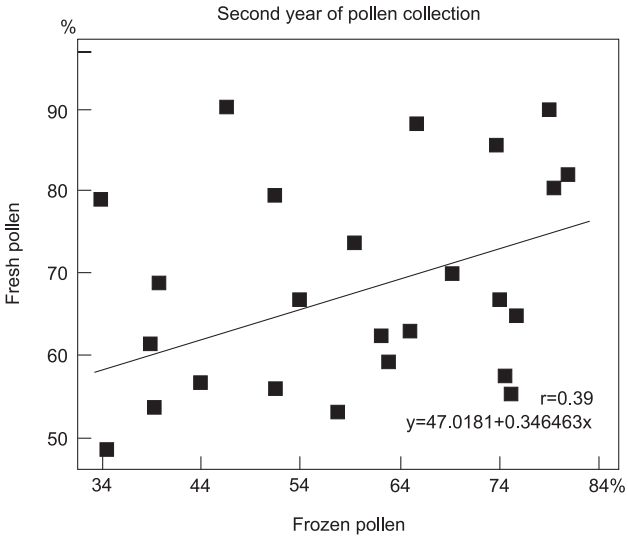

Fig. 8. Correlation between germination of fresh and stored pollen at –20°C (originated from the second year of collection)

Discussion

In our results *P. omorika* pollen germination (53.63–68.06%) is similar to the germability of *Picea mayerii* (61%, Wang et al. 2003). Šijačić-Nikolić and Isajev (2001) obtained far higher pollen germination of *P. omorika*, 72.97–82.60%, but on a small sample (ca 8 trees). Much higher values were also obtained in pollen of *P. pungens*, 97% (Cram and Lindquist 1984), *P. abies*, 80.7% (Lanteri et al. 1993), *P. abies*, 45.75–88.75% (Slobodnik and Krizo 1997).

Relatively great range of the pollen germinability of *Picea omorika* could be the consequence of great individual variability in male flowering which was found by Isajev (1987). Differences in pollen germination between collection years found in our investigations were also reported in *Picea abies* (Nikkanen et al. 2000).

Serbian spruce pollen is not able to preserve germination at room temperature (Figs 3, 4). Pollen germination of some pines: *P. brutia, P. canariensis, P. halepensis* and *P. pinaster* is also lost very fast at the room temperature (Braggio Morucchio et al. 1990). In our research, Serbian spruce pollen retained high germination at the temperature of +4°C only for a few months. *Abies pinsapo* pollen could not be preserved at +4°C till the following flowering season, too (Arista and Talavera 1994). But, germination of pollen of some conifers could be successfully conserved at +4°C over one year storage period, like *Picea pungens,* 78% (Cram and Lindquist 1984); *Larix leptolepis,* 45% (Said et al. 1991); and *Pinus radiata,* 84% (Siregar and Sweet 2000). However, pollen of *Picea abies*, *P. glauca*, and *Pinus banksiana*, stored at +4°C, and aditinally under vacuum treatment, was viable even after 5 years' storage (Shoenike and Stewart 1963).

The highest germination at the lowest temperature (–20°C) was obtained in our investigation of *Picea omorika* pollen in about 11 months of storage (26.54% and 64.02%, first and second year of pollen collection, respectively). Pollen of other coniferous species was also successfully stored at –18°C or –20°C in the case of *Picea pungens* (Cram and Lindquist 1984), *Pinus canariensis, P. pinaster, P. brutia, P. halepensis* (Braggio Morucchio et al. 1990), *P. nigra* (Lanteri et al. 1993) and *P. radiata* (Siregar and Sweet 2000). Pollen of some other species, *Picea abies* and *Pinus nigra* was successfully stored at lower temperatures (–80°C or –196°C) without major differences according to temperature rates (Lanteri et al. 1993).

The increase in pollen germination during storage at low temperatures, in addition to Serbian spruce (our results), was also found in *Picea abies* and some pines (Lanteri et al. 1993; Caron and Powell 1995). The increase in germination percentage in the initial period of storage can be affected by pollen exposure for a short period to a predetermined temperature. In our study, there was a decrease in pollen germination percentage during the storage in winter months. This phenomenon is stated in literature as "winter dormancy of pollen" and it is explained by the decrease in activity and preparation for the following vegetation period (Popnikola 1973 and refs. cited therein). In his analysis of Macedonian pine pollen, he founds the drop in pollen germination percentage till November and December and then its increase in January, February, March and April, during pollen storage at +4°C. The same author reports that Scots pine (*Pinus sylvestris* L.) pollen loses its germinability completely during the winter, but the percentage increases gradually with the approaching of the flowering period.

Our investigations of in vitro germination of *Picea omorika* pollen could not completely explain pollen germination in natural conditions, because of higher variation of ecological conditions in open area. The most significant external effects are temperature, light and moisture (Mergen et al. 1965; Owens et al. 1987). Microsporogenesis which occurs within the anther of gymnosperms may be affected by both genetic and environmental stress (Gómez-Casero et al. 2004 and references cited therein). In the case of *Pinus sylvestris* in the pollination process chemical interaction between pollen occurs and this interaction may vary according to genotype and on the combination of genotypes interacting (Varis et al. 2010).

Beside theoretical, investigation of pollen biology, especially of pollen germination of forest trees, has practical importance in propagation and hybridization of plants with desired traits. Artificial pollination is often aggravated by the periodicity and lack of coincidence of flowering periods, physiological incompatibility or sterility, or geographical isolation of the populations. In some cases, this can be improved by using the stored pollen from the preceding year (Lanteri et al. 1993). Also, pollen germination can be important for obtaining interspecific and intraspecific hybrids, which can exhibit reduced pollen germinability (Kormutak et al. 2007).

Germination of Serbian spruce fresh pollen in the analyzed population is not very high. It was proven that trees with high fresh pollen germination were simultaneously the trees which could preserve high germination even after long time storage. So, this fact could be used for selection and artificial hybridization of individuals with some desired traits (early or last flowering, good height increment, good diameter increment, abundant seed crop). But, preservation of high level of variability in pollen germination of *Picea omorika* is necessary for conservation of its diversity.

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