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# Preliminary Work on Generative Seedling Production and Clone Selection of European Black Poplar (*Populus nigra* L.)

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## ABSTRACT

European black poplar (*Populus nigra* L.) is a pioneer species that belongs to the *Salicaceae* family and occurs in riparian ecosystems. It is one of the most endangered forest species in its entire distribution area. In Croatia, black poplars are considered an economically important forest species, but mostly clones originate from crossing combinations with American and European black poplar (*Populus* × *canadensis* Moench), while a small number of clones are native black poplar. Studies on native black poplar are quite rare and the generative propagation has not been used. The aim of this study was to gain knowledge on the production of high-quality black poplar seedlings and to carry out the selection of genotypes with the aim of establishing a base for future breeding. The female black poplar tree was selected on phenotypic characteristics in the area of Forest Administration Osijek, Forest Office Valpovo. The tree was cut down in April 2019, and branches with half-open seed capsules on catkins were collected. The branches were transferred to the Croatian Forest Research Institute's greenhouse, where the catkins opened under the influence of the high temperature. Sowing was done in different substrates to test their effectiveness. Black poplar seedlings were selected and transplanted with regard to development and height growth. The results showed differences in height growth between plants sown in two different substrates and the occurrence of fungal diseases only on plants sown in pure sand. With subsequent multiple propagation using cuttings and selection by genotype, it is expected that it will be possible to identify several clones of native black poplar that will be introduced for use in forestry in Croatia. The use of quality plants grown from seeds will increase the genetic diversity and preserve the native black poplar gene pool.

**Keywords:** black poplar; seeds; gene pool; substrates; selection

## INTRODUCTION

European black poplar (*Populus nigra* L.) is a species belonging to the *Salicaceae* family. It belongs to extremely halophilic species, which as pioneer species inhabit exclusively areas without trees (Kajba and Romanic 2002). It has a wide distribution area (Zsuffa 1974) and is a dioecious species that flowers before leafing and is pollinated by wind. Individual trees can grow up to 400 years (Popivshchy et al. 1997). Black poplar is characterized by a great diversity of populations, from isolated tree as well as pure stands to mixed stands (Lefèvre et al. 1998). It mainly grows in wet floodplains along rivers. In nature, it is mainly propagated by seeds, while the vegetative method is most often used for economic purposes. A single female black poplar tree can annually produce thousands or even millions of non-dormant small seeds (about 1 mm long), with high germination (>90%) (Van Splunder et al. 1995, Karrenberg and Suter 2003), but short-term viability (Karrenberg and Suter 2003), which are dispersed by wind and water over a large distance. Sexual regeneration with seeds increases genetic diversity, population resistance to environmental changes and various diseases (Lefèvre et al. 2001, Barsoum et al. 2004). Unfortunately, European black poplar is one of the most endangered species in Europe (Wójkiewicz et al. 2021). The main reasons are the loss of natural alluvial habitat, especially the sandy and gravelly river coast that are necessary for the success of the seeds, and competition and introgression with non-native species from the genus *Populus* sp. (Fossati et al. 2004, Vanden Broeck et al. 2005, Ziegenhagen et al. 2008, Meyer et al. 2018). In riparian areas of Croatia, mainly planted clones of poplars are *Populus* × *canadensis* Moench and *Populus deltoides* Bartr. ex Marsh., which endangers *Populus nigra* L. gene pool. Restoration and protection of floodplain forests is one of the main priorities in biodiversity conservation and adaptation strategies related to climate change (e.g. EU Biodiversity Strategy 2030, EU Floods Directive 2007/60/EC). Floodplain forests are a hotbed of biodiversity and provide a number of ecosystem benefits including flood protection, erosion, soil thermal regulation with tree canopy cover, and water quality protection (Van Looy et al. 2013).

European black poplar is one of the main species of the floodplain ecosystem that belongs to the economically important forest species in Croatia. In recent years, there has been a serious problem of survival, primarily due to the loss of habitat and the impossibility of natural regeneration. Therefore, this is the reason why it was decided to start with works that include the production of seedlings from seeds (by generative reproduction). According to our knowledge, there has been no other such method of producing European black poplar seedlings in Croatia. Also, there is still no protocol for the production of quality seedlings that would include good practices from the collection of mature branches from the mother trees, through the manipulation of seeds to the growing quality seedlings in the nursery. Due to the importance of this species in the context of the preservation of the biodiversity of fluvial ecosystems, as well as its economic value, it is necessary to establish the protocol in the future.

The main goal of this work is to gain practical experience in the production of quality black poplar seedlings as the first step in this process. Other objectives are: i) to compare the height development of seedlings in two sowing substrates and ii) to perform an initial selection of genotypes with the aim of establishing a base for breeding.

## MATERIALS AND METHODS

### Sampling Area of Reproductive Material

The largest areas of European black poplar in the Republic of Croatia are located in its eastern part, Slavonia and Baranja, along the Drava and Danube rivers. Although its total area has been decreasing and dispersing over the years, larger natural stands can still be found in this area. This is supported by the existence of the only black poplar forest seed object in Croatia in the category 'selected', register number HR-POP-SS-111/117, which is located in the area of Forest Office Osijek, right next to the estuary of the Drava and the Danube.

This research was carried out in the area of Forest Administration Osijek, in the area of the Forest Office Valpovo, on the banks of the Drava river in a natural stand of European black poplar was found, which is located in the management unit Valpovačke podravske šume, department/section 4b, on area of 9.35 ha. There are no cultures and plantations of other species from the genus *Populus* sp. nearby, so unwanted pollination could not occur, which bring us to conclusion that this is pure stand.

### Sampling Method

In the selected natural stand, based on good phenotypic appearance and health status, a female tree was selected in April 2019. The selected tree was fallen because it had already been marked previously, i.e., scheduled for felling in the same year. Ten 1 m long top branches with catkins (early ripening seed capsules) were cut from the felled tree to obtain seed material (Figure 1).

**Figure 1.** Collected branches with catkins containing black poplar seeds.

## Method of Obtaining Seed Material

The collected branches with catkins were transported to the Croatian Forest Research Institute on the same day, shortened to a length of half a meter and stored in a greenhouse. There were 4-7 catkins with seeds on each sampled branch. The branches were put in plastic containers with water and covered with breathable cotton material so that the seeds would not disperse after opening of the catkins. In a period of 24 hours under the influence of high temperature in the greenhouse (15-34°C) and sufficient amount of air moisture, the catkins with the seeds opened and the seeds came out (Figure 2). There was an average of 15 seeds per capsule, and about 15,000 seeds were obtained from this quantity of sampled catkins. Due to the large amount of seeds obtained, 1/3 of the total amount of seeds were used (approx. 5,000 seeds). The seeds of the black poplar are covered with a white silky hair giving a fluffy a cotton like layer, which the wind blows over long distances (Barsoum 2001). For better sowing efficiency the cotton like layer had to be separated from the seeds. This was done mechanically, i.e. by rubbing on a metal sieve with 2.38x2.38 mm holes.

## Sowing Method

Black poplar seeds are characterized by good germination and high vitality. However, the minimal energy reserves of the small seeds limit their longevity to a period of only a few days or weeks (Moss 1938, Muller et al. 1982, Johnson 1994, Van Splunder et al. 1995, Braatne et al. 1996, Karrenberg and Suter 2003), preventing long-term seed storage stock in the seed bank.

For these reasons, sowing was started within 24 hours after obtaining clean seeds. Two types of substrates were used for sowing. The first type was sterilized fine-grained sand, which is used for seed germination, and the second type was a mixture of Drava sand (granulation 0.2 mm) and peat (DURPETA PROFI MIX 1a, pH 5.5-6.5). The seeds were sown in 59x39x15 cm containers. When sowing, the seeds were evenly distributed and covered with a thin layer of the substrate. Since it is very important that the seeds have enough heat and moisture after sowing, this was achieved by sprinkling them twice a day. Sowing in different substrates was used for comparison, i.e., testing their effectiveness.

## Further Seedlings Procedures

Germination of black poplar seeds occurs rapidly (usually within 24 hours), but the seeds must have sufficient moisture (Siegel and Brock 1990, Van Splunder et al. 1995). However, the seedlings do not tolerate shade, i.e. they are less competitive compared to natural vegetation (Gage and Cooper 2005). Therefore, the colonization of alluvial deposits and forming of young stoolbeds occurs during the retreat of floodwater. In addition to moisture, light is also important for successful germination.

With suitable conditions, i.e. enough moisture and light (heat in greenhouse conditions), the second day after sowing the seeds began to germinate. The radicle appeared first, then the cotyledons, which still had the seed coats on them (Figure 3).

Due to high humidity and high temperature in the greenhouse, fungal diseases (seedlings damping and common pinmould) appeared on about 1,500 seedlings only in pure sand substrate (Figure 4). After the appearance of fungal diseases, chemical treatment with Previcur Energy and Folicur EW 250 fungicides were applied in appropriate doses in pure sand.

With the appearance of the first leaves and the strengthening of the plants, the conditions were created for the first transplant into individual holes in 18 Bosnaplast plastic containers (32x21.5x18 cm, 33 holes in the block

**Figure 2.** Opened seed capsules on catkin from which black poplar seeds have emerged.

**Figure 3.** Seedlings of European black poplar.

**Figure 4.** The appearance of fungal diseases on black poplar seedlings.

(the volume of each hole is 220 cm$^3$)). When transplanting, plants that differed from the others were selected, i.e. all that had developed two leaves and reached an average height of 2 cm. A total of 499 plants were selected from the initial number, which was a rough estimate of 3,000 seedlings. From 5,000 sown seeds, about 3,000 seedlings were obtained, indicating a germination rate of 60%. Transplanting was done towards the end of June, before the summer heats. The containers were previously filled with a mixture of neutral peat (DURPETA PROFI MIX 1a) and Drava sand (granulation 0.2 mm) in a ratio of 250 l of peat and 50 l of Drava sand. The transplanted plants were moved from the controlled greenhouse conditions to outdoor conditions in the nursery where they were regularly watered, and their health condition was monitored (Figure 5).

At the end of the vegetation period, in November, the second transplanting of plants was started. The plants were transplanted into individual, larger containers (21.5x10 cm (volume 2 l)). The containers were filled with a substrate that is a mixture of neutral peat and Drava sand (the same substrate as for the first transplant). During the second transplant, an additional selection of cultivated plants was performed, during which 323 plants were selected out of a total of 499 plants after the first selection. Plants that had

**Figure 5.** Black poplar plants in individual containers.

a height increase of at least 1 cm since the first transplant and plants that had developed more than two leaves were selected. The plants in the containers were arranged in the open germinator in the nursery of the Croatian Forest Research Institute, where they were watered and health condition was monitored on a regular basis.

### Seedling Measurements and Data Processing

In November 2019 and 2020, during plant dormancy, plant heights were measured using a wooden meter with a precision of 0.5 cm. The heights were measured to determine the height difference between the plants depending on the substrate used when sowing the seeds, i.e. to determine the efficiency of the applied substrates, but also for the additional selection of individuals that showed the greatest height increase.

Student's t-test in MS Excel was used to compare the average heights of seedlings according to substrate type. For this statistical analysis, a significance level of 5% ($p<0.05$) was considered statistically significant.

### Selection of Genotypes

One of the basic methods in plant breeding is the selection of genotypes with regard to desired properties. In this research, using seed material taken from one mother tree, a large number of plants with relatively high genetic diversity were obtained (compared to seedlings obtained by vegetative propagation). After germination and until the first transplant, there were visible differences in the progress of plants that had the same conditions for growth. Selections were gradually made from the initial large number of plants, i.e. the most productive individuals were selected (as previously explained). The selection criteria were height growth and the development of the plant, i.e. the number of leaves. Therefore, in order to select the most productive plants at the end of the growing season, the plants that differed in height growth were gradually transplanted into pots with more soil and their progress was further monitored.

## RESULTS

By measuring the height of seedlings grown in two different sowing substrates, and comparing them, a statistically significant difference was obtained. In 2019, the p-value was 3.0007e-18, while in 2020 it was 1.3309e-06.

The results shown on the Box-Whisker diagram (Figure 6) show the differences in the height of black poplar seedlings in different substrates in 2019 and 2020. The average height of seedlings in 2019 was 9.42 cm for the substrate "sand and peat" and 7.05 cm for the substrate "sand" (Table 1). In 2020, the average height of seedlings was 14.47 cm for the substrate "sand and peat" and 11.22 cm for the substrate "sand". Interquartile values show the range of data in 2019 from 7.5 to 11.5 cm in height for the sand + peat substrate, and from 5.5 to 8.0 cm for the sand substrate. In 2020, the interquartile values show a range of data from 9.87 to 17.0 cm in height for the sand + peat substrate, and from 7.0 to 14.62 cm for the sand substrate.

In addition to the results presented, where differences in height growth are visible, it is important to note that fungal diseases appeared only on plants that were sown in pure sand. Obtaining seeds using the applied method of branch collection and catkins manipulation proved a successful method, as sufficient quantities of branches with half-open catkins were collected, from which, after opening in the greenhouse, a quantity of about 15,000 black poplar seeds were separated by mechanical method of the “cotton layer” using a metal sieve. The success of seed germination was equal in both substrates, that is, there was no significant difference in germination between different substrates.

**Table 1.** Average height of black poplar seedlings in different substrate.

| Substrate | N | Height in 2019 (cm) | Height in 2020 (cm) |
|---|---|---|---|
| Sand + peat | 160 | 9.42±3.03 | 14.47±6.87 |
| Sand | 224 | 7.05±2.03 | 11.22±6.03 |

Note: Average ± standard deviation; N – the number

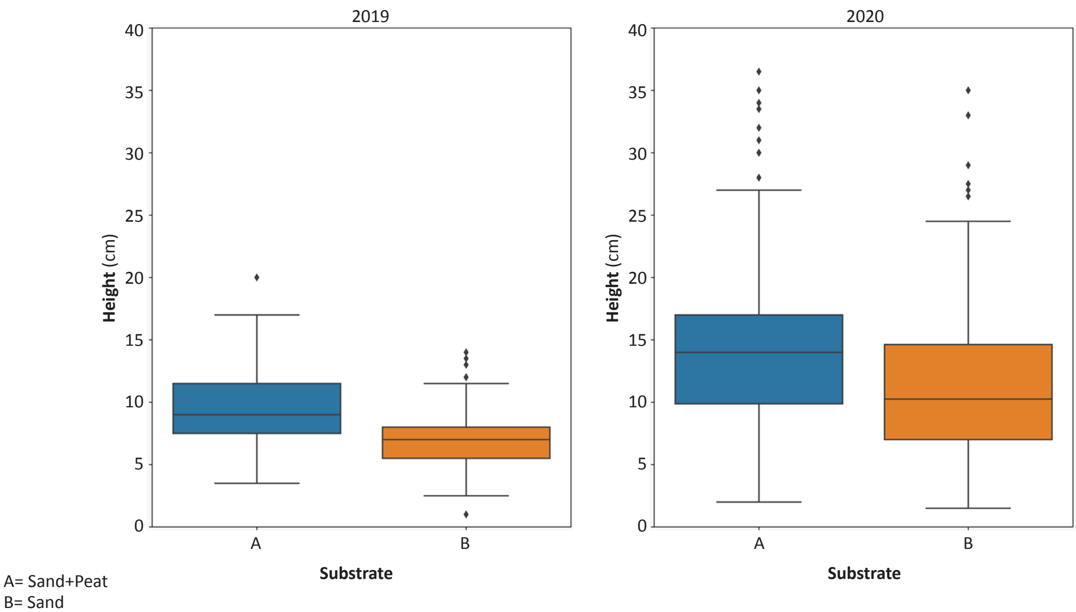


**Figure 6.** Height differences of black poplar seedlings in a different medium in the years 2019 and 2020.

Differences between seedlings and substrates were expressed later when plants needed more nutrients to thrive.

## DISCUSSION

The methodology of collecting and sowing black poplar seeds and growing seedlings in nurseries needs to be improved, and respectively the best protocol for growing seedlings from seeds needs to be developed. Cultivation of seedlings from seeds is not common in Croatia, nor has it been practiced so far, due to the fact that productive clones are produced mainly from cuttings that are used either for the production of seedlings or renewal of cultures. These are American black poplar clones or hybrids of American and European black poplar that are unsuitable for use in protected natural areas. Collection of seed material, further manipulation of seeds and growing of plants in nurseries is a complex process that needs to be developed and adapted to specific conditions. Guilloy-Froget et al. (2002) emphasizes the importance of recognizing the time period of seed maturation, which in the case of black poplar lasts from 2 to 9 weeks, depending on the climatic conditions in the current year. This is important because it is necessary to determine the right time to collect mature seeds for sowing success. Also, it is necessary to observe the catkins on the female trees often and collect branches with catkins or just catkins before they fully open. In this research, the beginning of the opening of catkins was noticed in a timely manner. A time gap of only two or three days could have affected the complete opening of catkins and, in that case, the seeds would have been dispersed all around. Other options for collecting seed material are the climbing of certified climbers on trees or the placement of seed catchers in the form of wooden cubes coated with glue under the crowns of trees, the so-called. 'seed traps', (Cooper et al. 1999, Gage and Cooper 2005). The method of felling the selected tree is suitable if it is a single tree or a forest stand at the end of the rotation. This method proved to be successful because it was possible simply to collect a sufficient amount of seed material. However, it should be emphasized that the seed material should be collected from a larger number of trees and in different habitat conditions. This would achieve a significantly higher level of genetic diversity of the offspring and thus a better and wider basis for selection. Collected quivers can be left in paper bags at room temperature (Gladwin and Roelle 1998) or in a greenhouse at a higher temperature which affects earlier opening and releasing of the seeds. Before sowing, the seeds must be separated from the cottony layer using a metal sieve.

Germination of seeds and initial growing of seedlings in a greenhouse allows controlled homogeneous growth conditions for all plants. This work showed the disadvantages of using pure sand as a substrate for sowing and the subsequent cultivation of seedlings, while the mixed substrate (neutral peat and sand) proved to be significantly better, probably due to the higher content of nutrients, which made it possible to achieve an average higher height growth of seedlings in that substrate. For better plant development, the substrate mix for sowing or soil texture for subsequent poplar transplanting should have a sand content between 55 and 70% (Pinno and Bélanger 2009). Sher et al. (2002) states that poplar seedlings grow best in a substrate with more clay in the first year, and better in a substrate with more sand in the second year. Other studies mention that fast-growing species accumulate more biomass in mixed, heterogeneous substrates than in homogeneous substrates such as pure sand (You et al. 2014, Luo et al. 2016). In certain works, it is stated that the use of a mixed substrate, which contains perlite, peat and sand, increases the height of seedlings and the number of leaves (Albaho et al. 2009). Although sand proved to be a good medium for germinating seeds, i.e. the seeds that were sown germinated very quickly, the plants later had little progress due to the lack of nutrients.

Another disadvantage of pure sand compared to mixed substrate is the occurrence of fungal diseases (seedlings' damping and common pinmould (*Mucor mucedo* L.)) because all seedlings that get symptoms of fungal infection were grown in pure sand. One of the possible explanations can be the property of sand to retain moisture and heat for a longer period, while in the case of a mixed substrate containing peat, there is a faster loss of moisture, and thus the substrate dries. In conditions of frequent irrigation, the substrate becomes saturated, and the high temperature creates ideal conditions for the appearance of fungal diseases. The occurrence of fungal diseases only on plants sown in pure sand leaves space for future research of this phenomenon.

The phase of transplanting plants represents another possibility for progress in future research. It was carried out twice during the year, which could have been the cause of the weaker progress of the seedlings due to stress during the growing season. The first transplanting was done in June, when the seedlings were transferred from the greenhouse to outdoor conditions under the shade and the second in November of the same year. In view of the above, it is recommended to use pots or containers with a larger capacity immediately after sowing, so that transplanting is done less often. Also, it can be recommended to transplant once a year (if necessary) during the non-vegetation period. The use of small pots or containers can negatively affect root health and stability (Lindström and Rune 1999, Cedamon et al. 2005). In addition, some authors list a number of suggestions for initial germination and later transplanting, such as keeping the seeds in clean water, then later keeping the seedlings in water for 8-10 days to allow their roots to elongate quickly. After that, such seedlings were immediately transplanted into natural conditions on different types of soil without previous cultivation in soil substrates (Hao et al. 2012).

It is also important to point out the problem of lack of quality water for watering seedlings in greenhouse conditions, because water from the water supply was mainly used. Such water has less nutrients, and it is also chlorinated, which is why it is not of the same quality as the water from lakes or rivers, which is usually used to irrigate plants in large nurseries. In addition, with the aim of better seedling growth, one should try to use a more natural substrate, such as alluvium, because research has shown that mycorrhizal associations can be important for better growth of black poplar (Lodge 1989).

The generative method of reproduction promotes the preservation of genetic diversity, which is necessary for adaptability, i.e. the long-term survival of a species in changing habitat conditions. Collecting seeds from more (min. 30) female trees significantly increases the possibility of finding and selecting the most productive genotypes for further production.

There is high importance to make diagnostic molecular genetic markers for characterization of taxonomic status. It should be used in all cases to confirm the taxonomic identity and non-hybrid nature of the selected trees as pure *Populus nigra* L. individuals.

Considering the trends in the protection of natural ecosystems, climate change, but also the tradition of productive forestry production, it is recommended to intensify the cultivation of generative seedlings in outdoor conditions, in nurseries specialized in poplar production. Such nurseries are located on high-quality land, in natural poplar habitats, and plants are irrigated with water from rivers or artificially raised lakes. In such conditions, plants from seeds achieve maximum growth and later it is possible to select genotypes and then propagate vegetatively and test them in clonal plantations in various habitat conditions.

## CONCLUSIONS

Based on the conducted research, the following conclusions can be drawn:

- Average height growth was significantly higher in European black poplar plants grown in sand + peat substrate, compared to plants grown in pure sand. This can be explained by the fact that neutral peat mixed with pure sand enables better plant growth, as it contains nutrients that young plants need for growth. On the other hand, pure sand is not so rich in nutrients, and frequent irrigation leads to faster washing away of the already modest amount of nutrients. Although pure sand proved to be a good initial substrate for seed germination, as it allows for longer retention of moisture and breathability, it turned out that soon after emergence the plants must be transplanted into a soil substrate with more nutrients.
- Pure sand in conditions of frequent watering and high temperature favors the occurrence of fungal diseases, as they have all been recorded on plants sown in sand. A possible cause of such a phenomenon is longer retention of moisture and warmer micro-conditions of this type of substrate.
- The use of individual or larger containers enables more space for the development of the root system of seedlings. More frequent transplanting during the year also leads to plant stress, after which the plants progress more slowly, due to the adaptation to new conditions, and plant death is possible. Transplanting is best done only once a year and during the rest of the vegetation.
- Breeding of European black poplar seedlings from seeds is a rarely used method of reproduction and it needs to be improved, i.e. it is necessary to find the best protocol for breeding quality seedlings in our conditions.
- By using quality seedlings from seeds, the genetic diversity of planting forest reproductive material will be increased, the gene pool will be better preserved in long term, and with genetic testing and selection, we expect to identify one or more clones whose productivity will be competitive to the poplar's clones currently in use in forestry of the Republic of Croatia.

## Author Contributions

ZV, SB2 and MI conceived the research, NZ processed the data and performed the statistical analysis, ZV and ML carried out the field work and measurements, SB2, AGM, ML, NZ, SB1, MI helped to draft the manuscript, ZV and SB2 wrote the manuscript.

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## Conflicts of Interest

The authors declare no conflict of interest.

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