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Potential of *Morus nigra* in Central Europe focused on micropropagation: A short review

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Abstract: This paper is focused on the description of the black mulberry (*Morus nigra*), its ecology and the possibilities of its *in vitro* propagation for quick and efficient obtaining of a large quantity of clones in a relatively short time for subsequent planting or sale. Due to ongoing climate change, it is considerable to use mulberry trees in horticulture, agroforestry and forestry under the conditions of Central Europe. The use of the mulberry is conditioned by the availability of planting stock. A proven and successful method of mulberry propagation is *in vitro* cultivation. Based on literature review, the recommended composition of planting media and other procedures for *in vitro* cultivation of mulberries are presented. The aim of the article is to inform foresters about the possibilities of using the black mulberry tree in our conditions and, using its example, to point out the possibility of using non-standard species of trees both as part of adaptation measures to the expected climate change and as one of the options for increasing the biodiversity of the landscape.

Keywords: black mulberry; climate change; *in vitro*; *Rosales*; silviculture

The order Rosales (*Rosales*) includes approximately 110 genera with more than 2 000 species. Within the mulberry family (*Moraceae*), well-known genera such as fig tree (*Ficus*) or mulberry tree (*Morus*) can be found. Plants of this family are characterised by their often simple, alternate leaves, which can be entire or palmately divided. The flowers are usually unisexual, arranged in characteristic spikes, heads or racemes. The fruits are different, for example berries or drupes (Nepal et al. 2012). Plants of the *Moraceae* family are distributed almost all over the world, mostly in tropical and subtropical regions. Some species are important economic crops, for example the fig tree (*Ficus carica*) or the white mulberry tree (*Morus alba*), whose leaves are an important and almost

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exclusive source of food for silkworm caterpillars (*Bombyx mori*), from whose cocoons silk fibre is traditionally obtained (Mikula 1989). Therefore, in various parts of the world, mulberry trees are often grown in gardens and plantations, focused on the production of silk. Of course, plants of this group also play an important role in the food chain of various species of animals, birds, and insects (Hejný, Slavík 1992, 1997).

A synthesis of the taxonomic literature on the genus *Morus* can distinguish thirteen distinctive species of mulberry trees, among which native to Asia are *M. alba*, *M. australis*, *M. cathayana*, *M. macroura*, *M. mongolica*, *M. nigra*, *M. notabilis*, and *M. serrata*, while *M. celtidifolia*, *M. insignis*, *M. microphylla*, and *M. rubra* come from America, and the African species is *M. mesozygia* (Nepal et al. 2012; Švagr 2021).

Distribution and description. The black mulberry tree (*Morus nigra*) is native to the region of Southwest Asia and is now widespread as a fruit tree in many parts of the world, including Europe, North and South America, North Africa, and South Asia. It is a medium-sized fruit-bearing deciduous tree that grows to a height of 10 m to 15 m with a crown diameter of up to 10 m. Its branches are often long and slanting, and in full foliage they create a dense to compact shade under the tree (Mareček 1994, 1997). The occurrence of mulberry in the Czech Republic is quite sporadic and is limited to the warmest areas (Figure 1).

The leaves of the black mulberry tree are simple, alternate, petiolate, deep green, stiff with a short and thick petiole, the leaf blade is broadly ovate, entire or deeply, irregularly incised and hairy on the face, dull, the base of the leaves is heart-shaped with a serrated, toothed edge. The size of the leaves can vary according to the age of the plant and different environmental conditions. In general diameter, black mulberry leaves are about 5 cm to 20 cm long and 4 cm to 15 cm wide (Mikula 1989; Hejný, Slavík 1997). On younger branches, the leaves can be smaller, on the contrary, on older branches and in the crown, they are larger and wider. Black mulberry leaves are used to treat headaches, migraines, muscle pain and to relieve inflammation (Yadav et al. 1990).

Black mulberry flowers in May to June and the flowers are relatively inconspicuous and arranged in small spherical greenish to reddish inflorescences with a simple structure consisting of a calyx, crown and stamens with anthers. Flowers are pollinated by wind or insects.

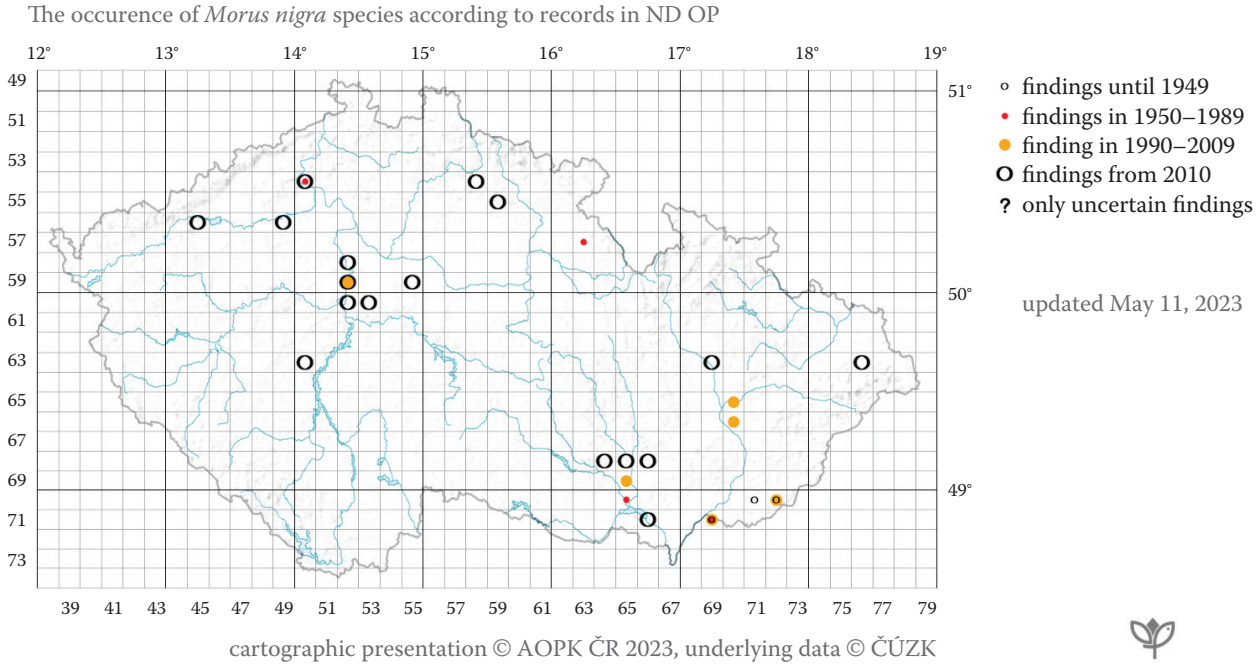

Figure 1. Distribution of black mulberry in the Czech Republic

AOPK ČR – Nature Conservation Agency of the Czech Republic; ND OP – Discovery Database of Nature Protection; ČÚZK – State Administration of Land Surveying and Cadastre

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The fruits of the mulberry tree are 25 to 30 mm elongated clusters of dark red to black mulberries (the achenes enclosed in a fleshy perianth) and resemble dark blackberry fruits with a pleasantly sweet taste. The fruits, which gradually ripen from the end of July to the beginning of September, are harvested either by hand or with stretched sails. The fruit is suitable for immediate consumption or can be processed into jams, marmalades, syrups, compotes, wine, sweet flour or simply frozen (Mareček 1997). The fruit contains a large amount of anthocyanin dye, free organic acids, invert sugar, pectins and other minerals. Black mulberry fruit is rich in antioxidants and vitamins and can help lower blood pressure, improve digestion and support the immune system (Yadav 1990).

The root system of the black mulberry tree is relatively shallow, but at the same time very extensive and wide. Its roots reach a depth of approximately 1 m to 1.5 m, where it creates a dense root network that allows the tree to be well anchored in the soil. However, the roots are very sensitive to unsparing interventions. Mulberries prefer well-drained, loamy-sandy soils with a neutral to slightly acidic reaction. The soil should be sufficiently moist, but at the same time well-drained to avoid stagnation or salinity. The tree is relatively tolerant of drier conditions, but the soil should be sufficiently moist for its optimal growth and development (Tewari 1999). Black mulberry root extract is used to treat joint pain, inflammation and bleeding gums (Yadav 1990).

At a young age, the mulberry tree is relatively sensitive to waterlogging of the soil and does not like soil with a high content of organic matter. It prefers sunny to semi-shady habitats where there is enough light for photosynthesis, i.e. for its own growth and fruit ripening. A site that is too shady can lead to smaller mulberry yields and overall slower tree growth.

Use. Black mulberry will certainly not be grown primarily for the production of wood, but it could find use both as an ornamental tree in gardens and parks and also as part of green belts (borders, draws) between fields, as it usually has an extensive root system that effectively limits soil erosion while increasing the soil's capacity to retain water in the soil and landscape. The mulberry tree can also be used in continuous rows of trees along roads. Fruiting trees attract animals and birds to their sweet fruits and will certainly increase the diversity of our landscape (Stehlík 1966). The listed properties of the mulberry tree predetermine it for use in agroforestry (Karami et al. 2010). In recent decades, Moravian winemakers have been planting mulberry trees at the edges of vineyards, where they represent an alternative source of food for birds, thereby contributing to increasing the protection of the vines.

The hard heartwood of mulberry trees with a distinct pattern is valued for its strength, hardness and beautiful colouring. The wood has a fine texture and a dark colour with a strong contrast between the heartwood and sapwood. It is used in the sports industry in the production of hockey sticks, cricket bats, tennis and badminton rackets, as well as for the production of the bodies of traditional Turkish musical instruments, saz and darbuka, or Iranian musical instruments such as tar, setar, kamanche, as well as barrels for spirits and, last but not least, for the production of decorative objects such as wooden spoons, knives, book stands and the like (Karami et al. 2010). Fresh, long and flexible twigs can find use in the field of basketry or furniture (Se Golpazegani et al. 2017).

Threats and diseases. Mulberry trees, like any other trees, are exposed to various pests and diseases. Trees infested with mistletoe *Viscum cruciatum* tend to have reduced growth and fruit yield, and parts of the tree may show various growth deformities. Necrotrophic fungal pathogens *Ciboria carunculoides* or *Scleromitrula shiraiana* cause sclerotic and thus devastating diseases of mulberries (Lv et al. 2021). Mulberry leaves can be attacked by silkworm larvae (*Bombyx mori*) or hop weevil (*Tetranychus urticae*), and mulberries can also be an intermediate host for silkworm larvae (*Pseudaulacaspis pentagon*).

Cultivation and propagation *in vitro*. In order to produce quality trees for further use for planting or sale, it is necessary to propagate these trees. The simplest variant is the cutting method (Mikuška 2002; Švagr 2019). Ripe or semi-ripe cuttings separated from the mother plant with a length of about 15 cm to 20 cm root only in a suitable environment (Kalyoncu et al. 2009). This is, for example, their planting in a moist and permeable substrate with the addition of a certain amount of plant hormones, most often indolyl-3-butyric acid (IBA) in concentrations of 100 ppm to 3 000 ppm, which supports the seasoning of mulberry cuttings (Karabulut, Saraçoğlu 2022).

The method, which is more demanding on resources, uses a laboratory environment for plant

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propagation and is referred to as *in vitro*. It is a set of laboratory techniques where, after sterilisation of the collected material, donor plants are grown from the top or side buds of a new plant on a nutrient medium that is enriched with plant hormones, an energy component, or other organic substances. The composition of nutrient media can be different, as well as the concentration of phytohormones, and each plant reacts differently to these media during its growth. In plant biotechnologies, nutrient media are most often used Murashige and Skoog (1962; MS), Gamborg et al. (1968; B5), Schenk and Hildebrandt (1972; SH), Lloyd and McCown (1980; WPM), or Driver and Kuniyuki (1984; DKW). In terms of their effect on plant tissues, phytohormones are divided into two basic groups, cytokinins and auxins. Cytokinins stimulate cell division, slow down aging and degradation of chlorophyll, stimulate chloroplast differentiation, stimulate branching and increase the strength of nutrient attraction. Examples of cytokinins are benzylaminopurine (BAP), kinetin (KIN), zeatin (ZEA), and thidiazuron (TDZ). Auxins stimulate cell division, participate in cell elongation, determine apical dominance, stimulate root formation and are an important factor in gravitropism and phototropism. Examples of auxins are naphthylacetic acid (NAA), indolyl-3-butyric acid (IBA) and indolyl-3-acetic acid (IAA).

The result of laboratory work in *in vitro* conditions should be the acquisition of information on the most suitable medium and concentration of phytohormones for the successful and maximally rapid propagation of the donor plant under controlled conditions with the subsequent successful transfer of these plants to the *ex vitro*, i.e. planting in the soil environment.

Pattnaik et al. (1997) summarise in their work that they successfully multiplied shoots from both apical and lateral buds of *Morus cathayana*, *Morus lhou*, and *Morus serrata* explants on MS medium containing 0.5 mg·L^{-1} to 1 mg·L^{-1} BAP. The additional addition of gibberellic acid at a concentration of 0.4 mg·L^{-1} together with BAP induced faster growth and number of buds on individual explants. However, the initiation of shoot multiplication of the target culture was largely influenced by the type and age of the explant.

In the work of Annis et al. (2003) with the culture of *Morus alba* on MS medium supplemented with BAP and KIN, a high frequency of formation of new shoots from the base of lateral buds (80%) and from apical buds (70%) was observed. Sticks *in vitro* were rapidly propagated by culturing both lateral and apical explants on MS with 2 mg·L^{-1} BAP and 0.2 mg·L^{-1} NAA. This combination proved to be the best when multiplying spikes. Excellent results were also achieved by culturing both types of explants on MS medium supplemented with 2 mg·L^{-1} BAP with 0.2 mg·L^{-1} NAA, 25 mg·L^{-1} asparagine, and 1 mg·L^{-1} glutamine. This specific medium resulted in shoot elongation and lateral bud emergence in *in vitro* grown explants.

Tewari et al. (1999) searched for suitable nutrient media with additives for the growth of *Morus multicaulis* and cultivars K2, RFS175, SI *Morus indica*. The presence of cytokinins was essential for bud development, and TDZ at 0.1 mg·L^{-1} was found to be more effective than BAP for bud and shoot multiplication in *Morus indica* for cultivars RFS175 and K2. In the case of cultivar S1, a concentration of 0.5 mg·L^{-1} TDZ was preferable as a substitute for BAP. Although TDZ increased shoot multiplication efficiency in *Morus indica*, the use of 2.5 mg·L^{-1} BAP was found to be more appropriate in the case of *Morus multicaulis*. TDZ significantly reduced the number of days required for bud development, but also increased the percentage of new buds and the number of shoots per explant in *Morus indica*.

Aroonpong et al. (2015) searched for a micropropagation system of *Morus alba* var. *Shidareguwa*, which is often used as a landscaping plant and has little rooting ability when propagated conventionally. The highest number of shoots was cultivated on MS medium with 20 g·L^{-1} sucrose and 1 mg·L^{-1} BAP for 20 days, the highest shoot multiplication was achieved using MS medium with 30 g·L^{-1} sucrose and 1 mg·L^{-1} BAP for a period of 30 days.

Attia et al. (2014) recommend a medium with added 2 mg·L^{-1} BAP for *Morus alba* MS shoot multiplication, Zaki et al. (2011) then a concentration of 5 mg·L^{-1} BAP for *Morus nigra*. Medková (2013) recommends a medium with 1 mg·L^{-1} metatopolin (mT) for *Morus nigra* WPM. Ivanička (1987) used for shoot proliferation of *Morus nigra* MS medium supplemented with 1 mg·L^{-1} BA, which was evaluated significantly better than the use of the phytohormone GA for *in vitro* propagation of *Morus nigra*. Ďurkovič et al. (2012) study deals with the best shoot proliferation performance by using MS medium supplemented with 0.5 mg·L^{-1} or 0.7 mg·L^{-1} BA in combination

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with 0.1 mg·L^{-1} IBA. The highest shoot elongation rates were found with 0.1–0.3 mg·L^{-1} BA treatment.

Explants of *Morus cathayana*, *Morus lhou* and *Morus serrata* according to Pattnaik et al. (1997) were successfully seasoned using 1/2 concentration of MS medium with 1 mg·L^{-1} IBA or IPA or IAA, and the plants propagated in this way were successfully acclimatised and transferred to the garden substrate. According to Annis et al. (2003), overall 80% of rooted *Morus alba* plants were obtained from explants cultured on 1/2 MS supplemented with 1 mg·L^{-1} NAA, then plants with well-developed roots were transferred to a garden substrate where 70% survived.

Tewari et al. (1999) state that rooting of *Morus multicaulis* and cultivars K2, RFS175, SI *Morus indica in vitro* was significantly improved by adding activated carbon to 1/2 concentration of the MS medium. A significant increase in rooting percentage and reduction in days required for rooting was observed using 0.05% activated carbon for *Morus multicaulis* and *Morus indica*, cultivar S1, and 0.1% activated carbon for *Morus indica*, cultivars K2 and RFS175. All plants grown *in vitro* were successfully acclimatised and transferred to the garden substrate (the proportion of successfully surviving individuals is not stated in the thesis).

Aroonpong et al. (2015) suggested in their work the cultivation of *Morus alba* var. *Shidareguwa* using 1/2 MS medium with 30 g·L^{-1} sucrose without auxins for 30 days. Acclimatisation of explants grown *in vitro* then statistically showed a 94% survival rate in greenhouse conditions.

For seasoning *Morus alba*, according to Attia et al. (2014), it is suitable to use 1/2 MS medium with 2 mg·L^{-1} IBA. According to Medková (2013), MS medium with 1 mg·L^{-1} CEP is recommended for *Morus nigra*, while Gogoi et al. (2017) recommend 1/2 MS medium with the addition of 5 mg·L^{-1} AC and 0.5 mg·L^{-1} NAA for *Morus indica*.

Anis et al. (2003), who investigated the use of auxins IBA and NAA at concentrations of 0.5, 1, and 2 mg·L^{-1}, recommend a concentration of 1 mg·L^{-1} NAA for *Morus alba*, when 80% of plants with an average root length of 5 cm were seasoned. Yadav et al. (1990) examined the seasoning of *Morus nigra* with individual auxins IBA, IAA, and NAA, for a duration of only 4 weeks at relatively low concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, and 1.5 mg·L^{-1}. Seasonings on 1/2 MS medium with the addition of 0.25 or 1 mg·L^{-1} IBA, then 1 mg·L^{-1} IAA or 1 mg·L^{-1} NAA are the best rated, unfortunately, the results do not indicate the percentage success rate of the plants. Similarly, Švagr (2019) states that for *Morus nigra*, it is enough to use 1/2 MS medium with lower (0.4 mg·L^{-1} and 0.8 mg·L^{-1}) concentrations of IBA or NAA for 8 weeks for optimal development of the root system. Ivanička (1987) utilised growth hormones IBA and NAA for the rooting of *Morus nigra*, and for the rooting of seedlings, he recommends either using half the concentration of MS media with 0.2 mg·L^{-1} IBA or a combination of 0.2 mg·L^{-1} IBA and 0.2 mg·L^{-1} NAA. With this combination, he achieved a rate of 88% for successfully formed roots. Ďurkovič et al. (2012) investigate that the highest frequencies of *in vitro* rooting for *Morus nigra* were found when using MS medium supplemented with 0.1 mg·L^{-1} or 0.2 mg·L^{-1} NAA with rooting frequency over 90%.

A very interesting article was written by Duarte et al. (2019), in which the plants of *Morus nigra* were acclimatised to the *ex vitro* environment either in closed mini-greenhouses or left open on growth racks in the growth room. A notable difference was observed in the shoot development after seven days of inoculation and 21–28 days of growth in a growth room and under greenhouse conditions, with about 100% of nodal explants producing new shoots in the growth room compared to only 80% under greenhouse conditions. However, the highest impact of environmental conditions was observed on the rooting, where only 40% of the nodal segments produced roots in the greenhouse compared to 90% in growth room conditions. Although up to 80% shoot regeneration from the nodal explants was recorded after 14 days of culture in greenhouse conditions, the rate later reduced to 70% and 40% after 21 and 28 days, respectively. This could be attributed to the death of plantlets after cultivation for 14 days in greenhouse conditions, possibly due to excessive ambient light intensity (above 68 000 lux), which caused maximum daytime temperatures reaching between 35.1 °C to 36.7 °C. This temperature was around 10 °C higher in comparison to the standard growth room conditions, i.e. 25 ± 2 °C temperature at a light intensity of 2 500 lux (Duarte et al. 2019).

The necessity of acclimatizing plants taken from *in vitro* is described in the work of Ďurkovič et al. (2009) and the study discusses poor control of water loss and clearly demonstrates the inability of non-acclimatised plantlets to avoid desiccation. Full acclimatisation *ex vitro* could be expected after ± 56 days (Mišalová et al. 2009).

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CONCLUSION

Black mulberry is a very interesting tree with many possibilities of use, as well as a high value for the development of biodiversity and other functions, especially in the area of soil protection against erosion. The cultivation of mulberry trees has been associated with humans throughout the history of the human race. The current and expected climate change, which will probably bring the Central European climate closer to Mediterranean conditions, offers the possibility of at least a partial use of the mulberry tree in Central Europe as well.

A proven and successful method of mulberry propagation is *in vitro* cultivation, which makes it possible to grow target plants with the donor's desired genotype. It follows from the literature that the suitable composition of nutrient media for mulberry cultivation varies slightly in individual growth phases. MS medium with 1–2 mg·L^{-1} BAP can be recommended for multiplication of mulberry shoots, and 1/2 MS medium with 0.4 mg·L^{-1} to 0.8 mg·L^{-1} IBA or NAA can be recommended for seasoning. For *ex vitro* transfer of plants, it is advisable to first place the plants for 1 month in containers with perlite and water with 1/10 concentration of MS medium, and then transplant them into containers with garden substrate and place them in a foil plant or greenhouse.

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