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In vitro reproduction of rare and endemic species of rowan tree

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ABSTRACT: The preservation and reproduction of gene resources of rare and endemic rowan species were the main aims of this study. Rowan species represent important woody trees from the aspect of forest biodiversity. There are only several endemic rowan species in the Czech Republic which are of hybridogenous origin from the whitebeam (*Sorbus aria*) range. Most of them have been described only at the end of the 20th century. For these species, the new procedures of vegetative reproduction were developed. *In vitro* cultures from dormant buds of 57 mature trees were established. The successful induction of organogenesis was achieved in a MS modified medium with 0.2 mg·l^{-1} of BAP, 0.1 mg·l^{-1} of IBA, 200 mg·l^{-1} of glutamine, 2 mg·l^{-1} of glycine, 200 mg·l^{-1} of casein hydrolysate, 30 mg·l^{-1} of sucrose, and 6 mg·l^{-1} of agar. The pH was adjusted to 5.8. NAA in the concentration of 13.5 mg·l^{-1} was efficient for the rooting of microcuttings. An efficient protocol for the reproduction of endemic rowan species by means of organogenesis induction in apical meristems of dormant buds is reported.

Keywords: *Sorbus*; micropropagation; organogenesis; preservation of gene sources

Various rowan species are scattered in one or a few populations up to several tens to hundreds of individuals. Their natural regeneration is usually very weak and little successful. Moreover, young seedlings and trees are permanently exposed to the risk of game grazing. They therefore belong to extremely rare and endangered woody species, and it is important to take steps for their reproduction and conservation. The rowans represent interesting subjects for a number of biological disciplines like taxonomy, ecology, karyology, and others. However, sufficient attention has not been paid to their micropropagation yet. An important source of rowan diversity is interspecific hybridization, in which phenotypically different but genetically related rowan species are crossed. Primary hybrids are not important from the evolutionary point of view, they are not usually fertile and if they rarely create a progeny, so they segregate. The fruitful hybridogenous species are much more significant. There is no segregation and their progeny has consistent characters (Kovanda 1961). Many endemic and hybridogenous species have recently been described in neighbouring countries such as Slovakia (Bernátová, Májovský 2003) and particularly in Germany (Bavaria) (Meyer et al. 2005).

Several new endemic apomictic species of hybridogenous origin, from the combination of *S. aria*, *S. torminalis*, *S. danubialis*, and *S. aucuparia*, have been described in the Czech Republic in the last decades (Kovanda 1961, 1984, 1996; Lepší et al. 2008). These are *S. quernea* Kovanda, *S. hardeggensis* Kovanda, *S. rhodanthera* Kovanda, *S. gemella* Kovanda, *S. alnifrons* Kovanda, *S. eximia* Kovanda, *S. bohemica* Kovanda, *S. milensis* M. Lepší, K. Boublík, P. Lepší *et* P. Vít, *S. sudetica* (Tausch) Bluff, Nees *et* Schauer, which have already been described in the 19th century. More detailed information about individual species, including morphological, karyological, and molecular assessment was presented by Vít (2006) and Vít and Suda (2006). Fig. 1 shows the map of Czech endemic rowan species distribution.

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For these species a new procedure of vegetative reproduction, micropropagation, was established. It consists of the induction of morphogenetic processes on primary explants. The establishment of appropriate culture conditions, particularly the chemical composition of nutrient media, concentrations of phytohormones, temperature, humidity and light regime are presumptions of successful organogenesis. Because these rowan species occur predominantly in the Czech Republic, in standardizing the cultivation methodology we utilized the experience of micropropagation of other rowan species, such as wild service tree (*S. torminalis*), service tree (*S. domestica*), and European mountain ash (*S. aucuparia*) (Malá et al. 1991, 2005, 2009).

MATERIALS AND METHODS

Field survey

A field survey was aimed to obtain information about the occurrence and phenotypic variability of rare and endemic rowan species. Selection of individuals suitable for reproduction was performed. The map and photodocumentation, including GPS coordinates, were acquired for all selected trees. The basic biometric measurement and evaluation of phenotypic characters and herbarium specimens were carried out. Plant material for micropropagation was collected from the following rowan species growing in localities (Fig. 1):

– *S. alnifrons* Kovanda – Templštýn near Moravský Krumlov (Dolnomoravský úval),
– *S. bohemica* Kovanda – Kletečná (České středohoří),
– *S. gemella* Kovanda – Konětopy, Louny District (Džbán),
– *S. hardeggensis* Kovanda – Hardegg hillside, Ice cave (Podyjí National Park),
– *S. quernea* Kovanda – Natural monuments Bílá skála, Jabloňka (Prague),
– *S. rhodanthera* Kovanda – Chlumská hora near Manětín (Žlutická vrchovina),
– *S. sudetica* (Tausch) Bluff, Nees *et* Schauer – Velká kotelní jáma (Krkonoše Mts. National Park).

Micropropagation

Plant material

Dormant buds of 57 mature trees of the abovementioned rowan species were collected during the winter and early spring months from (February to April) in 2009 and 2010. After 15 min sterilization in 1% NaClO (Savo, Bochemie CR) and washing three

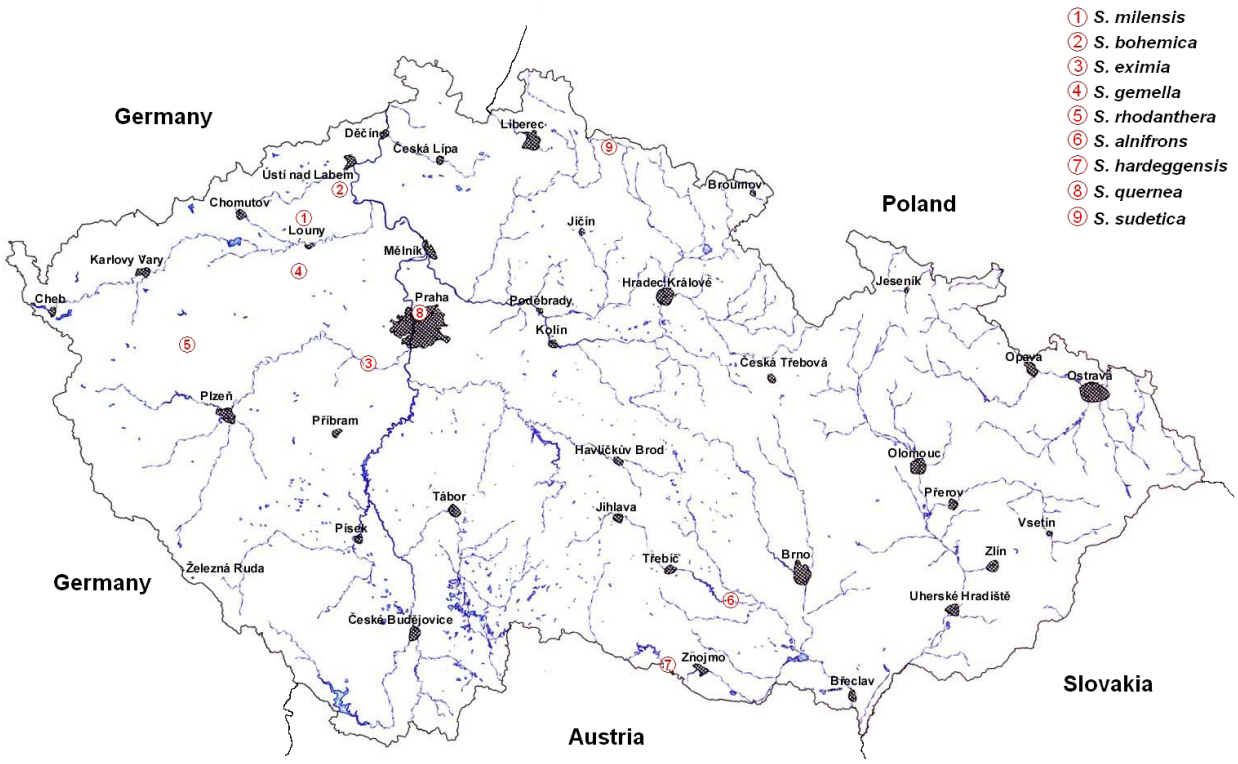

Fig. 1. Map of distribution

times in distilled water, the apical meristems of dormant buds (explants) were excised and used for the establishment of explant cultures.

Organogenesis

For micropropagation of endemic rowan species, the organogenesis was induced in explants (Malá et al. 1999). From each selected clone, 30 to 50 explants were placed on the modified MS medium (Murashige, Skoog 1962) with 0.2 mg·l^{-1} of BAP (6-benzylaminopurine) and 0.1 mg·l^{-1} of IBA (β-butyric acid), 200 mg·l^{-1} of glutamine, 2 mg·l^{-1} of glycine, 200 mg·l^{-1} of casein hydrolysate, 30 mg·l^{-1} of sucrose, and 6 mg·l^{-1} of agar. The pH was adjusted to 5.8. The explants were cultured under defined conditions [white fluorescent light (36W/33 Philips tubes, Eindhoven, the Netherlands; irradiance of 30 μmol·m^{-2}·s^{-1} with 12 h photoperiods), and 24°C].

Induction of shoots on primary explants was reached after 4–5 weeks. Multiplication of explants was reached on the MS medium of the same composition. Explants were regularly transferred into the fresh medium (one passage took approximately 4–5 weeks). Multiplied shoots were used for further multiplication or for rooting. Shoots from the multi-apex cultures were used to complete the cultivation of plants, to induce rhizogenesis. The MS medium with one third concentrations of micro- and macroelements, with reduced sugar content (10 g·l^{-1}) and auxins IBA (6.5 or 13.5 mg·l^{-1}) or NAA (α-naphthylacetic acid) (13.5 mg·l^{-1}) was used for rooting. The shoots for rooting on the agar media with auxins were cultured first for 7 days in darkness for the acceleration of root growth and then were transferred onto a hormone-free medium (one-third strength MS) and exposed to light (36W/33 Philips tubes, Eindhoven, the Netherlands; irradiance of 30 μmol·m^{-2}·s^{-1} with 12 h photoperiods), and 24°C. The mixture of clones was used in each series of rooting. The smallest number of shoots used for rooting was 25 pieces. Then, the rooted plantlets were transferred into perlite and irrigated by the basal MS medium without phytohormones and saccharose diluted with distilled water 1:10 and cultured in the same constant conditions (see above). After 2 weeks, the plantlets were transferred into the non-sterile peat substrate and gradually acclimatized for outplanting. The experiment was repeated twice.

The data were explored by two-way analysis of variance (ANOVA), and their significance was evaluated by the Tukey-Kramer test using QC Expert and NCSS software.

RESULTS AND DISCUSSION

Explant cultures from all 57 collected clones of mature rare and endemic rowan trees (5 clones of *S. alnifrons,* 8 clones of *S. bohemica,* 7 clones of *S. gemella,* 15 clones of *S. quernera,* 7 clones of *S. hardeggensis,* 8 clones of *S. rhodanthera,* 7 clones of *S. sudetica*) were established during 2 years. The modified MS medium that was previously applied for effective micropropagation of other rowan trees (Malá et al. 1991; Lall at al. 2006; Ördögh et al. 2006; Yang at al. 2012) was used.

For induction of organogenetic activity, the application of a low concentration of cytokinin BAP appeared to be substantial. Similar results were described by Chalupa (2002) for micropropagation of European mountain ash (*S. aucuparia*). Low concentrations of BAP were introduced with regard to possible rooting inhibition that was observed *e. g.* in service tree (*S. torminalis*) (Malá et al. 2009). During 4–5 weeks, the growing cultures developed 3–4 new shoots about 4 cm in length per culture (Figs 2 and 3). No statistically significant differences among multiplied species were observed (Fig. 4). The influence of IBA and NAA

Fig. 2. Multi-apex explant culture of *S. quernea*

Fig. 3. Multi-apex explant culture of *S. bohemica*

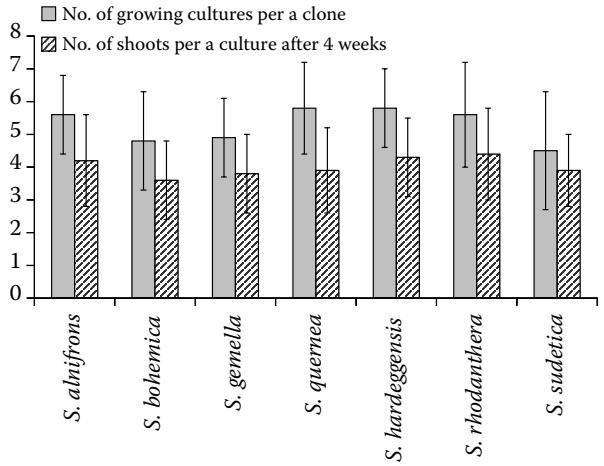

Fig. 4. Survey of *Sorbus* species growing *in vitro*

on rooting was examined. Both auxins were added in a concentration of 13.5 mg·l^{-1}, which proved efficient in other experiments (Malá et al. 1999, 2005). Contrary to Nikolaou et al. (2008), a very low number of plantlets was obtained with IBA after 4 weeks. Comparing the effectiveness of auxins IBA and NAA, it was found that IBA at both concentrations of 6.5 mg·l^{-1} and 13.5 mg·l^{-1} was not efficient in the rooting of rowan trees cultured in the same cultivation conditions. Using IBA, a low percentage of rooting microcuttings (20%) was obtained after 12 weeks. Contrarily, (on average) 66% rooting was reached within 4–6 weeks, when 13.5 mg·l^{-1} of NAA was used (Fig. 5). There are differences in the rooting ability of rowan species. The lower rooting efficiency could depend on the presence of cytokinin BAP used in the induction medium during organogenesis induction (Malá et al 2009). On the other hand, BAP is effectively used for organogenesis induction.

The micropropagation has proved very effective for the reproduction of the above-mentioned rowan species. The multi-apex cultures collected from all individuals growing on the multiplication medium were successfully established and stored in the Bank of

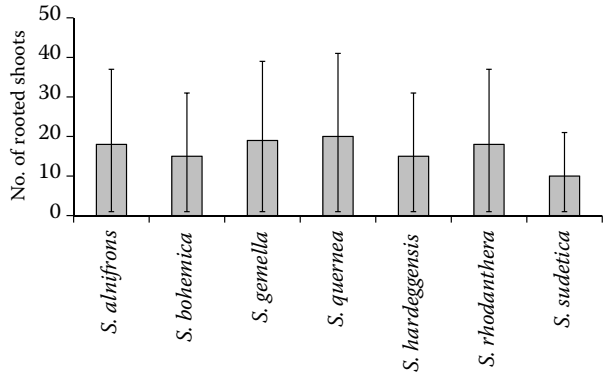

Fig. 5. The rooting of microcuttings, average number of rooted shoots (two replications)

Explants of the Forestry and Game Management Research Institute. The reproduced microcuttings were used for development of a rooting method. The rooting was more successful in shoots cultured with NAA, which was already successfully used in poorly rooting *S. domestica* (Arrillaga et al. 1991; Ďurkovič, Mišalová 2009), even if differences among some clones were observed (Fig. 5). After acclimatization, the rowan plantlets could be transferred to outdoor natural conditions.

Conclusively, the study was aimed to contribute to preservation and reproduction of gene resources of rare and endemic rowan species, important from the taxonomical point of view, even in terms of maintaining biodiversity. The data on their distribution and variability in different site conditions have been obtained and a possibility of vegetative reproduction has been examined and tested. The organogenesis has been proved very effective for the micropropagation of the above-mentioned rowan species. Original procedures of micropropagation were developed. The growing explant cultures are now stored in the Bank of Explants in the Forestry and Game Management Research Institute.

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