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# Micropropagation and Reintroduction of *Nepeta rtanjensis*, an Endemic and Critically Endangered Perennial of Serbia

By

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With 6 Figures

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Summary

MIŠIĆ D. M., GHALAWENJI N.A., GRUBIŠIĆ D. V. & KONJEVIĆ R.M. 2005. Micropropagation and reintroduction of *Nepeta rtanjensis*, an endemic and critically endangered perennial of Serbia. – Phyton (Horn, Austria) 45 (1): 9–20, 6 figures. – English with German summary.

A micropropagation protocol was developed for the conservation of critically endangered Serbian perennial *Nepeta rtanjensis (Lamiaceae)*. Rooted shoots were obtained from one-node stem segments and shoot tips on a half-strength Murashige and Skoog (MS) medium without growth regulators. The best pH of the medium for axillary buds induction and for rooting of shoots was found to be at 7 and/or 7.2 respectively. The addition of cytokinins to the culture medium did not significantly stimulated auxillary bud production as compared to the control. On the contrary, on media supplemented with high cytokinin concentrations, only dwarf shoots with rudimentary roots were obtained. All tested concentrations of 6-benzylaminopyrine (BAP) and kinetine (Kn) in combination with 0.1 mg $l^{-1}$ indole-3-acetic acid (IAA)

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negatively affected the elongation and rooting of shoots. Plants micropropagated on hormone free medium and rooted in vitro were successfully acclimatized in greenhouse and in open field conditions. The result of successful acclimatization was the production of more than 7000 plantlets with normal sexually reproduction. They flowered, fruited and produced seeds which exhibited 47% germination. The survival rate of plants that were transferred to the open field for the acclimatization and exposed to the winter chill was 99%. The reintroduction of *N. rtanjensis* occurred in May 2004. One thousand plantlets were planted within the historic range of this plant species. The survival rate was also 99%.

## Zusammenfassung

Mišić D. M., Ghalawenji N.A., Grubišić D. V. & Konjević R.M. 2005. Die Micropropagation und Wiedereinbringung von *Nepeta rtanjensis,* einer endemischen und kritisch gefährdeten perennierenden Pflanze in Serbien. – Phyton (Horn, Austria) 45 (1): 9–20, 6 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Eine Vorschrift zur Micropropagation wurde entwickelt um die ernstlich gefährdete serbische perennierende Pflanze *Nepeta rtanjensis (Lamiaceae)* zu erhalten. Bewurzelte Sprosse wurden von Stammabschnitten mit einem Knoten und Sprossspitzen auf einem halb konzentrierten Murashige and Skoog (MS) Medium gewonnen. Der beste pH-Wert für ein Medium um Axillarknospen zu induzieren und um die Sprosse zu bewurzeln, lag bei 7 bzw. und/oder bei 7,2. Die Zugabe von Cytokininen zum Kulturmedium förderte nicht signifikant die Axillarknospenbildung gegenüber der Kontrolle. Im Gegenteil: Auf einem Medium mit hohen Cytokininkonzentrationen konnten nur zwergenhafte Sprosse mit rudimentären Wurzeln gewonnen werden. Alle geprüften Konzentrationen von 6-benzylaminopyrine (BAP) und Kinetin (Kn) in Kombination mit 0,1 $mg/l^{-1}$ indole-3-acetic acid (IAA) beeinflussten negativ das Längenwachstum und die Bewurzelung der Sprosse. Pflanzen, welche auf hormonfreiem Medium vermehrt wurden und in vitro anwurzelten, wurden erfolgreich im Gewächshaus und unter Freilandbedingungen akklimatisiert. Das Ergebnis der erfolgreichen Akklimatisierung war die Produktion von mehr als 7000 Jungpflanzen, welche sich normal geschlechtlich vermehrten. Sie blühten, fruchteten und bildeten Samen, welche 47% Keimrate aufwiesen. Die Überlebensrate von Pflanzen, welche ins Freiland zur Akklimatisierung verbracht wurden, und dem Winterfrost ausgesetzt waren, betrug 99%. Die Wiederausbringung von *N. rtanjensis* erfolgte im Mai 2004 wobei 1000 Jungpflanzen innerhalb des historischen Areals dieser Art ausgesetzt wurden. Die Überlebensrate betrug hier ebenfalls 99%.

## Introduction

*Nepeta rtanjensis* Diklić & Milojević, belonging to the *Lamiaceae* family, is an endemic and critically endangered perennial of Serbia. The small natural population of this species is estimated to contain only several hundred specimens (Diklić 1999), which are confined to four small natural populations scattered across the southern slopes of Mt Rtanj in SE Serbia. The natural habitats of this species are open calcareous stony grounds in the zone of oak forests *Quercetum freinetto-cerris*, at 650–850 m (Diklić 1999). Anthropogenic intrusion, as well as intensive cattle grazing,

severely hampered the species wild populations. Low viability and low germination rate of seeds, which are probably the consequence of fungal infection, further curbed the propagation rate and dispersion of species (RANČIĆ & al. 2002). The population size rapidly decreases making the plant acutely threatened. Considering the strictly endemic nature and decreased population, there is an urgent need to prevent this species from becoming extinct.

Reintroduction is a strategy recommended for conservation of rare, threatened and endangered plants and offers a good basis for the further work in preventing these species from becoming extinct. However, reintroduction is not to be considered as a substitute for the protection of existing plant populations. The source of plant material for reintroduction could be obtained by different propagation techniques. In cases where the use of conventional propagation methods is limited by factors such as strongly reduced populations, low production and viability of seeds and diseases, in vitro methods are recognized as a suitable alternative. In conservation terms, in vitro culture methods, based on the activation of pre-formed meristems (shoot tips and axillary buds), are the most favoured type of techniques. Considering that they are not associated with callus formation, they minimize the possibility of somaclonal variability and the risk of genetic alterations. Some rare and endangered plant species have previously been micropropagated, with the aim at producing plants for in vitro conservation, including *Centaurea paui* (CUENCA & al. 1999), *Dioscorea balcanica* and *D. caucasica* (ĆULAFIĆ & al. 1999), *Lilium speciosum* (CHANG & al. 2000), *Symonanthus bancroftii* (PANAIA & al. 2000), *Vanda coerulea* (SEENI & LATHA 2000), wild *Arachis* species (GAGLIARDI & al. 2000, 2002), *Cryptanthus sinosus* (ARRABAL & al. 2002), *Stackhousia tryonii* (BHATIA & al. 2002), *Ipsea malabarica* (MARTIN & PRADEEP 2003), *Rotula aquatica* (MARTIN 2003) and *Kniphofia leucocephala* (MCCARTAN & VAN STADEN 2003).

Due to very reduced populations of *N. rtanjensis* conventional conservation methods are ruled out. Therefore, in vitro preservation techniques are recognized as an efficient alternative. In this paper, as a part of conservation efforts, we describe a reliable method for large-scale in vitro multiplication of *N. rtanjensis*, aiming at production of plants for in vitro conservation, as well as for the population reinforcement of *N. rtanjensis*.

## Material and Methods

### Micropropagation

Plant material was collected in May 2001, at the locality Javor, on the southern slopes of Mt Rtanj, before the flowering period. One-node stem segments and shoot tips were used as primary explants for in vitro culture establishment.

In order to lower the pressure on wild populations, but also to eliminate the genotypic influence on phenotypic variability, all experiments for optimization of in vitro conditions were performed with single genotype. Afterwards, in vitro cultures of five other genotypes were successfully established.

Explants were washed under running tap water for approximately 2h, and then rinsed in 60% solution of ethanol. After surface sterilization in 20% solution of commercial bleach with two drops of liquid detergent for 10 min, explants were rinsed 5 times with sterile distilled water. The sterilized explants were aseptically transferred on basal medium containing half-strength MS (MURASHIGE & SKOOG 1962) salts and vitamins. The medium was supplemented with 100 mg $l^{-1}$ myo-inositol, 30 g $l^{-1}$ sucrose and 7 g $l^{-1}$ agar ("Torlak", Belgrade, Serbia and Montenegro). The pH of the medium was adjusted to 7, except for the study of the effect of pH, before sterilizing by autoclaving at 114°C for 25 min. The explants were transferred to the fresh medium once a month.

The effect of pH of the medium was studied by culturing one-node stem segments on media with pH adjusted within the range of 5.8 to 7.2. All treatments were repeated three times, with 25 explants per treatment.

The one-node stem segments obtained from in vitro-derived shoots were transferred to half-strength MS medium supplemented with 6-benzylaminopurine (BAP) or kinetine (Kn) within the range of 0.05 to 4.0 mg $l^{-1}$. In all treatments the media were supplemented with 0.1 mg l-1 indole-3-acetic acid (IAA). Treatments were carried out with 25 explants and each treatment was repeated twice.

All experiments lasted for one month. For all treatments, cultures were grown in 350 ml glass jars closed with transparent polycarbonate caps, with 60 ml culture medium each. All cultures were grown in a growth chamber under long day conditions (16/8 h light/dark cycle), at a temperature of $25 \pm 2$°C, and a relative humidity of 60–70%. Light was provided by white fluorescent "Tesla" Pančevo tubes (60 W, photon flux density 50 μmol $m^{-2}s^{-1}$).

## Statistical Analysis

Statistical analyses were performed using STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, USA). Data were subjected to Analysis of Variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $p < 0.05$. Mean values and standard errors are shown on figures. Within each treatment, values with the same letter are not significantly different at the $p < 0.05$ level according to the LSD test.

## Acclimatization

One hundred plants that spontaneously rooted in vitro on a half-strength MS medium without growth regulators were transferred to non-sterile greenhouse conditions for acclimatization. Experiment was repeated three times. Before they were potted in soil, containing a mixture of 80% forest peat and 20% earthworm compost, plantlets were treated with 0.15% solution of "Previcur" (Aventis, Berlin, Germany) to prevent fungal contamination. Pots were shielded with plastic covers to initially maintain the plants at high humidity, and the plantlets were acclimatized by gradually opening the covers. After two weeks they were completely uncovered and har-

dened to the greenhouse conditions (temperature of 25 ± 2°C and relative humidity of 60–90%). Observations on acclimatization percentage were recorded 6 weeks after the transfer of rooted plants to the soil. For vegetative propagation in greenhouse conditions, one-node stem segments (2–3 cm long) and shoot tips (2 cm long) were used as cuttings. Basal part of explants were treated with 0.1% indole-3-butyric acid (IBA) in talc before transfer to the pots containing the mixture of "Floragard" peat (Floragard Vertriebes, Germany), forest peat and earthworm compost (2:1:1). One thousand cuttings were used for vegetative propagation. The survival rate was recorded 4 weeks after the onset of experiment. In September 2003, five hundred plants were transferred to the open field for the acclimatization and exposed to the winter chill. Plantlets were planted in an experimental field within the Institute for Biological Research "Siniša Stanković" in Belgrade. The survival rate was recorded in april 2004, i.e. eight months after the transfer to the open field.

### Seed Germination

Seven months after transfer to the greenhouse, *N. rtanjensis* plants flowered and subsequently fruited. Mature seeds were collected and stored in paper-bags at a room temperature for six months. After that period, seed germination was tested. Lots of fifty seeds were placed in 6 cm Petri dishes, each containing 2 ml of the solution of distilled water with 500 mg $l^{-1}$ nystatin. Seeds were kept under long day conditions (16/8 h light/dark cycle), at a temperature of 25 ± 2°C. The seeds were scored for germination 14 days after the onset of the experiment. Experiment was repeated three times with four replicates.

### Reintroduction

In April 2004, one thousand individuals of *N. rtanjensis* were planted within the area of species historic range, to a calcareous stony habitat at locality Javor. Plantlets present the progeny of six plants. The current location was selected because of its similarities to this species natural habitat, and is separated by less than 1 km from the natural population. Because of the terrain configuration plantlets were disposed randomly. To facilitate monitoring, we marked each plant with a numbered metal flake. Plantlets were healthy, free of pests and diseases. One month after the planting, the survival rate was recorded.

## Results and Discussion

### Micropropagation

Whole plants of *Nepeta rtanjensis* were obtained in a single-step procedure, on a half-strength MS medium without growth regulators. Shoots derived from axillary buds (Fig. 1a) and from shoot tips (Fig. 1b) elongated and spontaneously rooted in vitro.

Growth and development of *N. rtanjensis* in vitro was significantly influenced by the pH of the medium. Better axillary bud induction and rooting of shoots were achieved on media with pH adjusted to 7.2 and 7.0 respectively, when compared to the generally used pH of 5.8 (Fig. 2). Considering *N. rtanjensis* natural habitats, which are open calcareous stony grounds, these results confirm that this species is adapted to the alkaline soil.

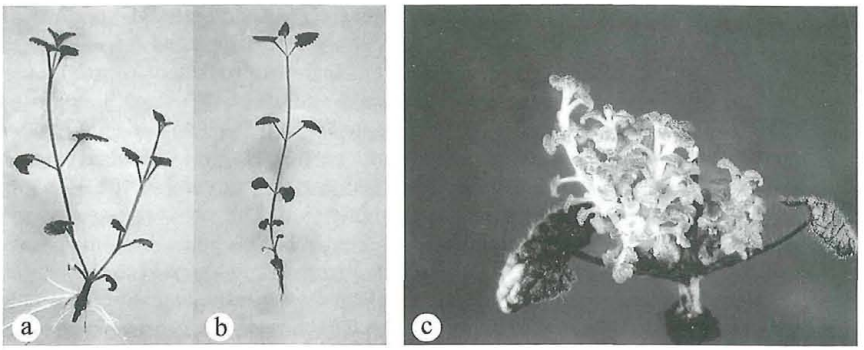


Fig. 1. Shoots of *N. rtanjensis* obtained from one-node stem segments (a) and shoot tips (b) on hormone-free ½ MS medium; (c) Axillary bud proliferation on medium supplemented with 4 mg $l^{-1}$ BAP and 0.1 mg $l^{-1}$ IAA.

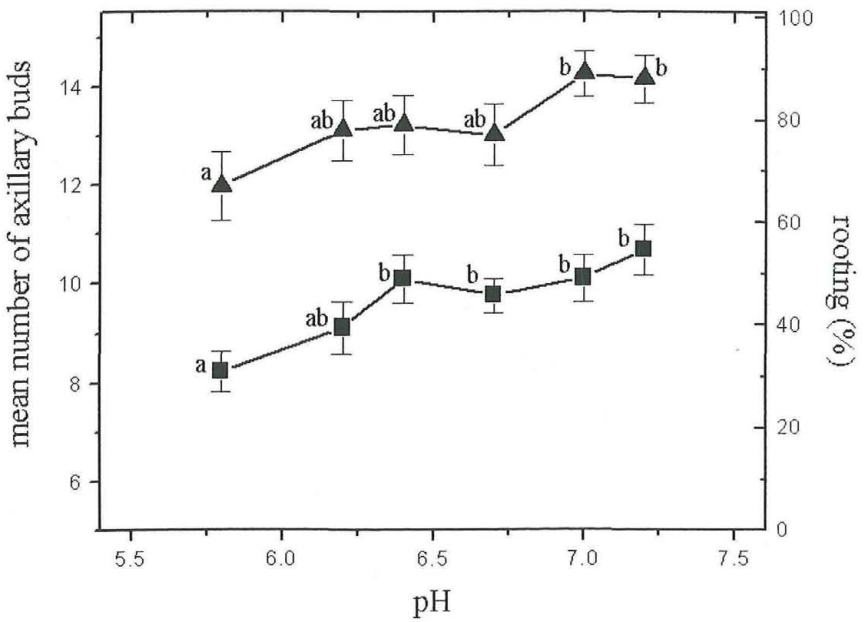


Fig. 2. Axillary bud formation (■) and rooting (▲) of *N. rtanjensis* shoots obtained from nodal explants, cultured for 30 days on ½ MS media with different pH regimes.

In order to increase the multiplication rate, the basal media was supplemented with combinations of different BAP and Kn concentrations (0–4 mg $l^{-1}$) and 0.1 mg $l^{-1}$ IAA. It was previously reported that low concentration of an auxin in combination with a cytokinin positively modifies

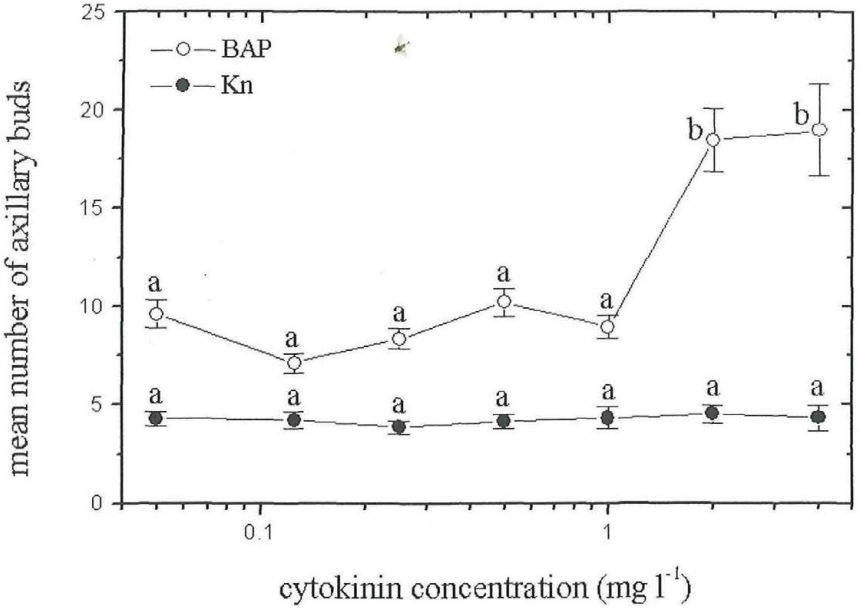


Fig. 3. The effect of BAP (○) and Kn (●) on axillary bud formation. Media was supplemented with 0.1 mg $l^{-1}$ IAA in all treatments. The control group of explants formed approximatlly $8.7 \pm 0.76$ axillary buds per explant.

the shoot induction frequency and their growth (YUAN & al. 1994, VANDEMOORTELE & al. 1996, SINGH & SEHGAL 1999, MARTIN 2003). As has been reported for many plant species (VANDEMOORTELE & al. 1996, KAUR & al. 1998, MESZAROS & al. 1999, CUENCA & al. 1999), media supplemented with different BAP concentrations were more efficient in initiation and subsequent proliferation of axillary buds than those supplemented with Kn. The increase in BAP levels stimulated *N. rtanjensis* axillary buds formation (Fig. 1c and Fig. 3), but also negatively affected shoot elongation (Fig. 4), which suggests an inverse relationship between the number of shoots and shoot elongation. Kn did not promote axillary bud proliferation. Similarly to the results reported for *Lavandula latifolia* (SANCHES-GRAS & CALVO 1996), *Ocimum basilicum* (SINGH & SEHGAL 1999) and *Melissa officinalis* (MESZAROS & al. 1999) from fam. *Lamiaceae*, the length of *N. rtanjensis* shoots was reduced in the presence of growth regulators (Fig. 4), and this inhibition was stronger as BAP and Kn concentrations increased.

*N. rtanjensis* shoots spontaneously rooted in vitro on hormone-free ½ MS medium. This aspect is beneficial for preservation, as it results in less manipulation and avoids the use of growth regulators in additional operations for rooting. All tested concentrations of BAP and Kn negatively

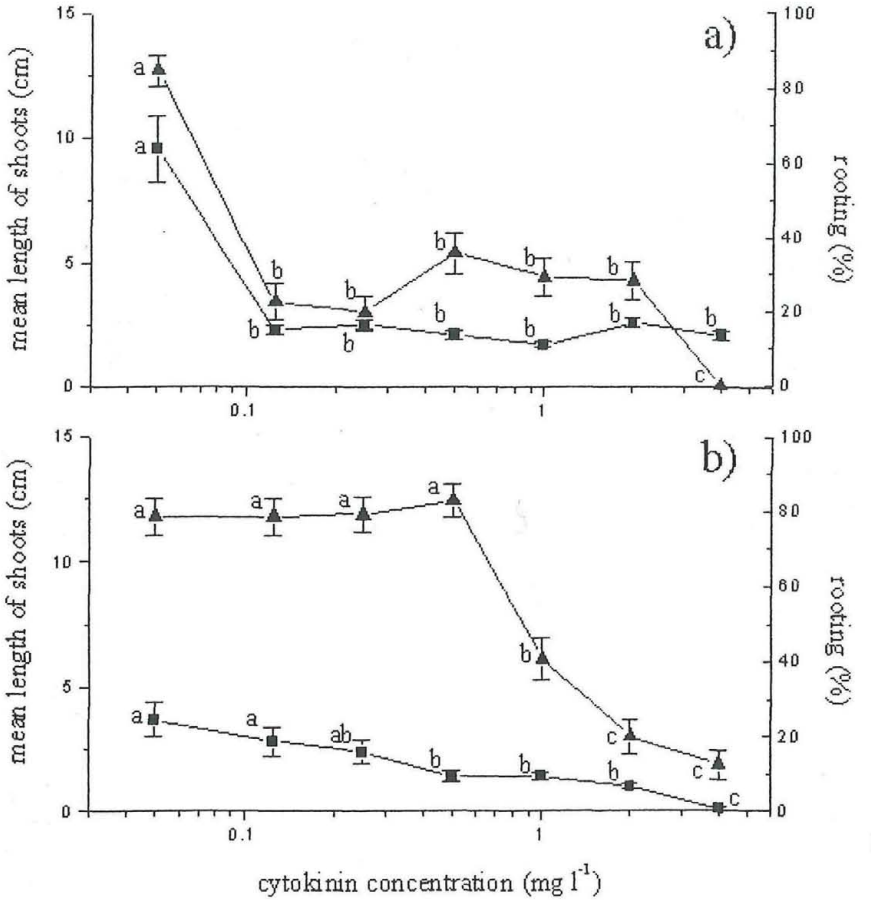


Fig. 4. The mean length (cm) of the highest shoot (■) and rooting percentage (▲) of *N. rtanjensis* shoots on media containing combinations of different BAP (a) or Kn (b) concentrations with 0.1 mg $l^{-1}$ IAA. Untreated cultures exibited ~100% rooting and the mean length of 9.0 ± 0.9 cm.

affected in vitro rooting of *N. rtanjensis* shoots. The percent of rooting decreased with the increase of cytokinin concentrations (Fig. 4). Nevertheless BAP showed stronger inhibitory effect compared to Kn. Similarly to the results reported for *Melissa officinalis* (MESZAROS & al. 1999), high levels of both cytokinins in the multiplication media resulted in the formation of only short and thick roots. The poor shoot elongation and rooting of shoots on media supplemented with cytokinin concentrations higher than 0.5 mg $l^{-1}$ were accompanied by hyperhydricity.

Therefore, the hormone-free media, or media supplemented with low levels of BAP (0.05–0.5 mg $l^{-1}$) and 0.1 mg $l^{-1}$ IAA were the best combina-

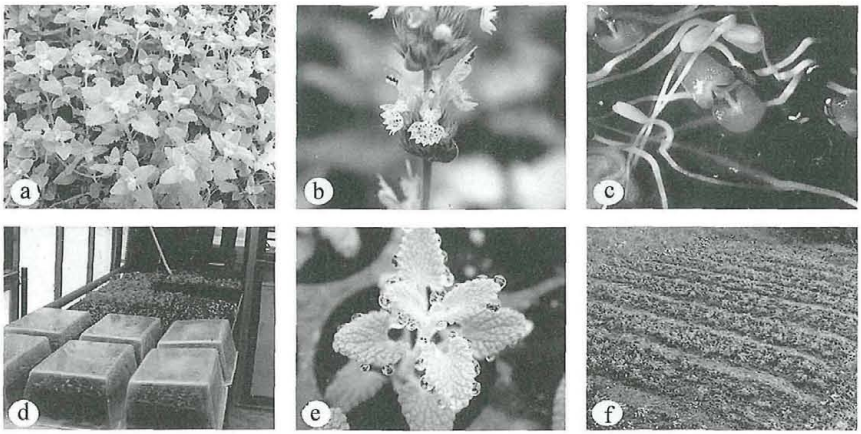


Fig. 5. Plants of *N. rtanjensis* acclimatizatized in greenhouse (a) flowered (b) and produced viable and disease-free seeds (c); Vegetative propagation in greenhouse conditions (d and e); Acclimatized plants grown in open field conditions (f).

tions for shoot multiplication. They yielded a satisfactory number of axillary buds (~7–10 per explant), as well as rooted shoots longer than 20 mm, which produce the greatest number of stem nodes. It was estimated that the micropropagation system described herein potentially enables the production of more than 100,000 plants per explant per year. This number is several hundred-fold higher than the estimated number of wild population specimens.

## Acclimatization and Seed Germination

Survival of plants depends on their ability to carry out photosynthesis and withstand water loss. The survival rate of in vitro-rooted *N. rtanjensis* plants, placed in pots and acclimatized over a 6-week period under misty conditions, was 98%. The result of successful acclimatization was the production of more than 7,000 plantlets with normal sexual reproduction (Fig. 5a). Plants transferred to the greenhouse in March 2002 flowered in September 2002 (Fig. 5b). Seeds, collected from the fruits, had normal morphology and exhibited 47% germination after storage of 6 months at room temperature (Fig. 5c). The germination percentage of *N. rtanjensis* seeds collected from the wild was significantly lower (~1%). Due to low seed viability, which is probably the consequence of fungal infection (RANČIĆ & al. 2002), the sexual reproduction, as well as the dispersion of this species is limited. However, this micropropagation system enables the production of disease-free seeds that can be used as propagation material, as well as for conservation in seed banks. To preserve the greatest genetic diversity within plant populations, seeds are normally the preferred

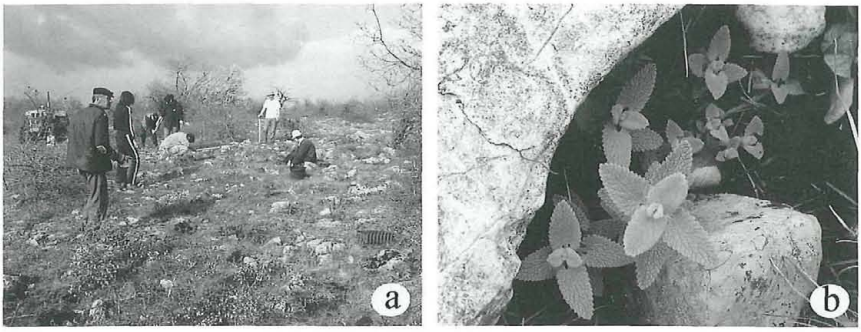


Fig. 6. Reintroduction of *N. rtanjensis*: (a) April 2004 – planting of plantlets on calcareous stony ground, in the area of Javor, on the southern slopes of Mt Rtanj in SE Serbia; (b) May 2004 – acclimatized *N. rtanjensis* (survival rate was 99%).

propagation material (FAY & al. 1999). The production of viable and disease-free seeds enhances the germination success, and thus the dispersion and perpetuation of species.

Plants which were obtained by conventional cutting propagation in greenhouse conditions also exhibited 98% survival (Fig. 5d and 5e). The survival rate of plants transferred to open field conditions for acclimatization was 100% (Fig. 5f). On the second year of cultivation in open field, plants flowered, fruited and subsequently produced seeds. This confirmed the possibility of successful large-scale cultivation of *N. rtanjensis*.

Rapid and efficient multiplication rate, rooting, and successful transfer of plantlets to the greenhouse, as well as to the field conditions, make this protocol suitable for large scale multiplication of *N. rtanjensis*. Therefore, the micropropagation system described enables the production of plantlets for the population reinforcement within a short span of time, and thus offers a large scope for the further work in preventing this species from becoming extinct. The production of viable and disease-free seeds, which can also be used as propagation material further, justifies our selection of methods for ex situ conservation. Raising the plantation of this potentially medicinal plant is another approach that would further lower the pressure on the critically endangered wild populations.

## Reintroduction

Reintroduction was performed in order to test the possibility to increase natural local populations with plants obtained by non-conventional in vitro techniques for the propagation. One thousand plantlets were planted within the historic range of *Nepeta rtanjensis*, what thereabout tripled the number of individuals on the southern slopes of Mt Rtanj (Fig. 6a).

Although some measures of viability indicate that the *Nepeta rtanjensis* reintroduction has been successful (survival rate, observed one month after planting, was 99%), long-term persistence of the population is still to be bear out (Fig. 6b). However, our results in planting *N. rtanjensis* in an experimental field in Belgrade are encouraging and are a good indication of long-term viability of reintroduced population. The true success would be establishment of a viable, self-sustaining population that would be stable or increasing in size. Therefore, a long term biological monitoring, what is the course of our further work, would be useful to evaluate the effects of conservation efforts and evolutionary potential of this population.

The large-scale reintroduction of plantlets is certainly raising hopes for the recovery of these spectacular endemic species, although serious challenges remain. It also highlights both the opportunities and challenges for recovery of other endangered plant species in Serbia by using the same or similar strategies.

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