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In vitro Propagation of Critically Endangered Endemic *Rhaponticoides mykalea* (Hub.-Mor.) by Axillary Shoot Proliferation

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1. Introduction

Turkey is one of the richest countries in variability of flora. It has nearly 9000 plant species about 3000 of which are endemic [1]. *Asteraceae*, is represented by 50 species in Turkey with an endemism of nearly 54% [2]. *Rhaponticoides mykalea* (Hub.-Mor.) M.V. Agab. & Greuter which belongs to the *Asteraceae* family, falls within the CR (Critically Endangered) category in the Red Data Book of Turkey [1]. While *R. mykalea* (Hub.-Mor.) was classified under the section *Centaurea* as *Centaurea mykalea* (Hub.-Mor.) before now. Today it has been separated from the section *Centaurea* [3]. It spreads very scarce in Kuşadası (Aydın), Muğla and Isparta, and faces with the danger of extinction. *R. mykalea* that has very limited number of individuals is under strong anthropogenic pressure such as the gradually increase in ongoing urbanization due to rapid developments of tourism sector, the conversion of natural habitats into human dominated lands, the over-grazing and collecting capitula of *R. mykalea* by local people for food. The species has already been under the threat of extinction and the situation above will increase the risk of extinction of this species even more [4]. For this reason, local protection measures and global conservation strategies are necessary [5].

Nowadays, the conservation of wild plant genetic resources is very important for preventing a decrease in genetic variability. Conservation of the endemic or threatened plants is carried out using different strategies. *In vitro* culture is an efficient method for *ex situ* conservation of plant diversity [6,7], because many endangered species can be quickly propagated and preserved from a minimum of plant material with low impact on wild populations with this technology [8]. In recent years, there has been an increased interest in *in vitro* techniques that offer powerful tools for germplasm conservation and the mass multiplication of many

threatened plant species [9]. Especially *in vitro* propagation of endangered plants can offer considerable benefits for the rapid cultivation of at risk species that have a limited reproductive capacity and exist in threatened habitats [5].

Micropropagation constitutes a powerful tool for *ex situ* conservation programs of threatened plants, especially for species with very reduced populations or low seed production [6,7]. This technique facilitates the rapid establishment of a large number of stock plants, from a minimum of original plant material, thus imposing minimum impact on the endangered wild populations. Axillary shoot proliferation typically results in average tenfold increase in shoot number per monthly culture passage. In a period of 6 months, it is feasible to obtain as many as 1 000 000 propagules or plants, starting from a single explant [10]. With this technology various endemic and endangered species have been successfully propagated; such as and *Centaurea paui* [8], *Anthemis xylopoda* O.Schwarz [11], *Centaurea spachii* [12], *Centaurea zeybekii* [13], *Centaurea junoniana* [14], *Astragalus chrysochlorus* [15], *Centaureum rigualii* [16] and *Syzygium alternifolium* [17].

However, during our literature search, no report concerning *in vitro* regeneration of *R. mykalea* by axillary shoot proliferation was found.

The objective of the present study was to establish an efficient *in vitro* method for the rapid propagation via axillary shoot propagation of *R. mykalea*, a critically endangered endemic plant species. The shoots that were obtained from *in vitro* germinated mature embryos were used for axillary shoot proliferation. For that reason, the most appropriate cytokinin type and concentration were determined.

2. Material and methods

2.1. Plant material and explant source

Capitula of *Rhaponticoides mykalea* were collected from a wild population in Aydın-Turkey (Samsun mountain, localities: N 37° 47.01 " ; E 027° 19.16 ") during summer period (July and August -2008) before seed dormancy period (Figure 1).

R. mykalea has been propagated from seed in the past [18]. However, researchers have explained that the seed is not suitable explant for *in vitro* propagation of *R. mykalea* due to strong seed dormancy and low germination frequency even after dormancy period. Therefore, embryos isolated from achenes which have not yet crossed dormancy periods were used as initial explant.

The achenes isolated from capitula were sterilised, and mature zygotic embryos that were dissected out from achene were used as initial explant. Mature zygotic embryos were dissected out from achenes and cultured on Murashige and Skoog (MS) [19] basal medium for germination. The shoots that were obtained from *in vitro* germinated mature embryos were used for axillary shoot proliferation.



Figure 1. Achene containing mature embryo (before dormancy).

2.2. Achene viability

Achene viability was subjected to tetrazolium test. Tetrazolium test is based on reduction of colourless solution 2,3,5-triphenyltetrazolium chloride or bromide into insoluble 2,3,5-triphenylformazan red in colour. This solution acts as an indicator for detection of reduction processes that take place in living parts of the seed. Inside the seed, tetrazolium intakes hydrogen from dehydrogenase. By hydrogenization of tetrazolium a red, stable substance called formazan, which dyes living parts of the seed, is formed in the living cells. Tissue of many plant species must be removed to introduce the dye into the tissue. Tissue removal can be done by pilling the seed coat off, punching, and longitudinal or cross-cutting of unessential seed parts. Prepared seed is submerged into 0,5 – 1% tetrazolium solution. Seed must be completely covered with solution, and not exposed to direct light. After the time needed for dyeing expires (it depends on plant species) the estimation of dyeing is approached. All tissue (necessary for normal seedling development) of a viable seed should be dyed. Except completely dyed, viable seeds, and completely undyed, unviable seeds, a partly dyed seeds may also be found. Depending on the species, small undyed spots of some parts of these tissues may be accepted. Location, size of undyed areas, and sometimes intensity of dyeing, determine whether some seed is considered as viable or not [20].

To determine achene viability of *R. mykalea*, longitudinally-halved seeds were treated in tetrazolium solution (TTC, 1%) for 2 h at room temperature. After that time, red staining embryos were evaluated as alive.

2.3. Seed sterilisation, media preparation and culture conditions

In order to determine proper sterilisation procedure, achenes isolated from capitula were washed thoroughly under running tap water for 30 mins. Subsequently at various times, achenes were put in 70% (w/v) ethanol and 4.5% (w/v) sodium hypochlorite containing 2 drops

of wetting agent (Tween-80); afterwards, the achenes were rinsed three times (5 mins each) with sterile distilled water in a laminar flow hood. After sterilisation, zygotic embryos were isolated from achenes and cultured on PDA (Potato Dextrose Agar) to determine early contamination. PDA cultures were maintained at 24 ± 2 °C for 3 days. At the end of this period, observations were made in order to determine the appropriate sterilisation time.

All the experiments were maintained on semi-solid basal medium supplemented with or without various concentration of plant growth regulators. Basal medium contained Murashige and Skoog (MS) [19] mineral salts, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine-HCl, 0.1 mg l^{-1} thiamine-HCl, 3% (w/v) sucrose, 8 g l^{-1} agar (Agar-agar), various concentration of plant growth regulators ¹N⁶- Benzyladenine (BA) and Kinetin (KIN), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or naphthalene acetic acid (NAA) were used in experiments depending on experimental objectives.

The pH of media was adjusted to 5.8 with 1M NaOH or HCl prior to autoclaving at 105 kPa and 121° C for 15 min. Culture vessels were 190 ml glass jars containing 30 ml of medium.

2.4. Axillary shoot proliferation

Mature embryos that were isolated from achenes were cultured on MS basal medium to obtain sterile seedlings (unpublished data). After eight weeks, seedlings (~2-3 cm), were separated from primary roots and transferred to MS medium containing different concentrations of BA or KIN (0.1, 0.5, 1.0 and 2.0 mg l^{-1}) for axillary shoot propagation. A control treatment without cytokinins was also included. At the end of the 3 subculture, the number of shoots per explant and average shoot length was evaluated for each cytokinin type and concentration.

Axillary shoot proliferation experiments were conducted with 15 replications consisting of one explant per jar and were repeated three times. Cultures were incubated at 24 ± 2 °C under a light regime of 16 h photoperiod by cool-white fluorescent lamps. The cultures were subcultured to fresh medium of the same composition at an interval of 4 weeks.

2.5. Shoot rooting and acclimatization of plantlets

After three subcultures, elongated shoots (~4 cm) were excised stock cultures and transferred to MS and half strength MS medium ($\frac{1}{2}$ MS) with or without different concentrations (0.5, 1.0, 2.0 and 5.0 mg l^{-1}) of auxins (IBA, IAA or NAA) for rooting. The results of rooting experiments were expressed in percentage after 6 weeks of culture initiation.

Rooting experiments were conducted with 15 replications consisting of one explant per jar and were repeated three times. Cultures were incubated at 24 ± 2 °C under a light regime of 16 h photoperiod by cool-white fluorescent lamps. The cultures were subcultured to fresh medium of the same composition at an interval of 4 weeks.

After 8 weeks of rooting *in vitro*, the plantlets were removed from the culture jars then the agar was carefully washed off the rooted plantlets to minimize pathogen attack. The plantlets were planted into 10 cm diameter plastic pots containing garden soil and kept in the growth chamber

under $24 \pm 2^\circ\text{C}$ and 16-h light photoperiod. After 4 weeks the plantlets kept at normal laboratory conditions.

2.6. Statistical analyses

Means of shoot number per explant, shoot length and frequency of rooting were analyzed by one-way analysis of variance (ANOVA, SPSS for Windows v.9., SPSS, USA). Differences were analyzed by analysis of variance and the means compared using Duncan's multiple range test at $p < 0.05$. Data giving in percentages were subjected to $x' = \arcsin \sqrt{(x/100)}$ transformation [21].

3. Results and discussion

The viability percentage of achenes was 80% according to Tetrazolium test. According to our results, the most suitable sterilisation procedure of achenes is as follows: The achenes are washed thoroughly under running tap water for 30 mins. After this process, seeds must be exposed to 70% (w/v) ethanol for five mins and then to 4.5% (w/v) sodium hypochlorite containing 2 drops wetting agent (Tween-80) for eight mins. Finally, seeds are rinsed three times with sterilised distilled water (5 mins each). Sterile cultures are then obtained in high proportion (100%).

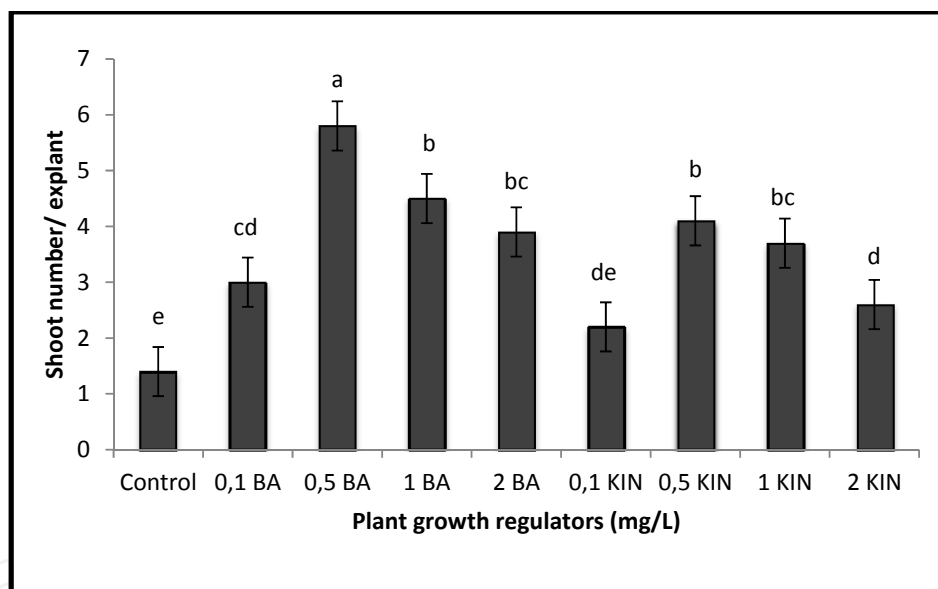
Four-week-old sterile seedlings obtained from mature zygotic embryos were used as explant for axillary shoot proliferation experiments. At the end of the experiments, the most appropriate cytokinin type and concentration were determined (Figure 2). Axillary shoot propagation of *R. mykalea* was obtained in all media without or with cytokinin. Cytokinins are generally recognized as critical for the production of shoot primordia under *in vitro* conditions. Both cytokinins induced healthy shoots in our study. However, it is shown that BA is more effective cytokinin than KIN. The maximum shoot number per explant were obtained in 0.5 mg l^{-1} BA added MS medium (5.8 shoot/explant) (Figure 2 and 3).

A decrease in the number of shoots were observed at both higher (1 and 2 mg l^{-1}) and lower concentrations of BA (0.1 mg l^{-1}). Similar results were also reported for axillary shoot proliferation of *Centaurea spachii* [12] and *Centaurea zeybekii* [13]. BA was also reported as an effective cytokinin for other endemic and threatened *Centaurea* species [14, 16]. However, BA was evaluated as an effective cytokinin for shoot multiplication in many species of *Asteraceae*; such as *Centaurea junoniana* [14], *Gerbera jamesonii* hybrida [22], *Centaureum rigualii* [16], *Syzygium alternifolium* [17] and *Anthemis xylopoda* [11].

MS medium supplemented with 0.1 mg l^{-1} Kinetin (KIN) was determined as the most suitable medium for the maximum shoot length (7.35 cm) (Figure 4). While BA is more effective cytokinin on shoot multiplication, KIN is more effective on shoot length. In spite of the increased number of shoots on media containing cytokinin, the shoot length is decreased. A negative correlation between the shoot number and their length has been observed. This kind of negative correlation was reported in *Centaurea paua* by using inflorescence stalk as explant [8] and *C. zeybekii* by axillary shoot proliferation [13].



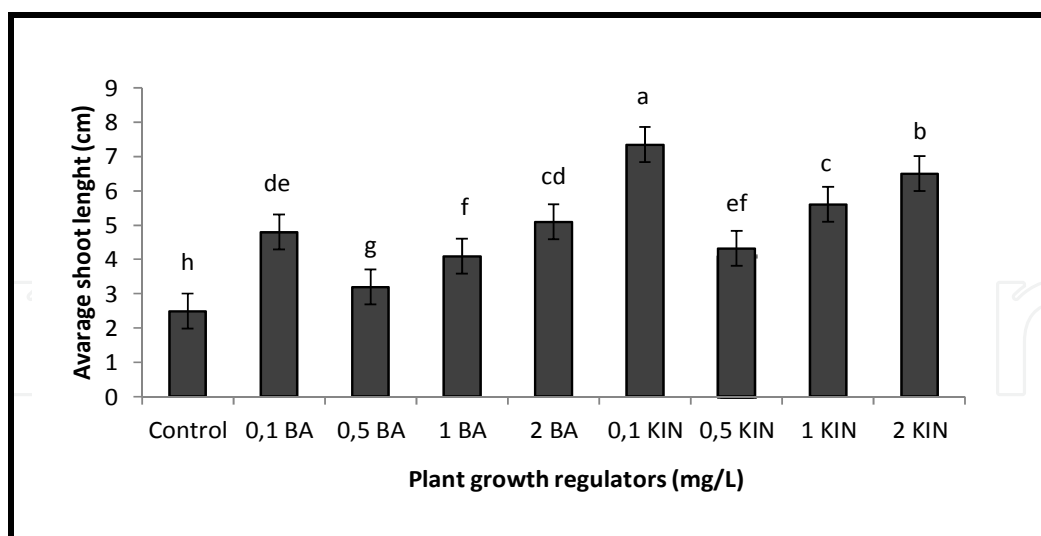
Figure 2. Axillary shoot proliferation on MS medium added 0.5 mgL⁻¹BA.



Means by different letters in each column and capital letters in each row are significantly different ($p < 0.05$), according to Duncan Multiple Range Test.

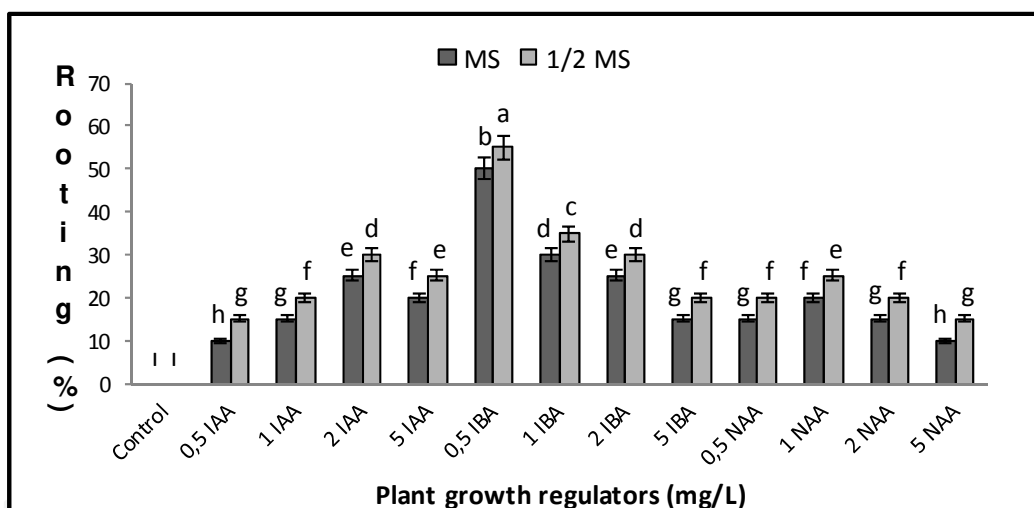
Figure 3. Cytokinin effects on axillary shoot multiplication of *R.mykalea*.

After three subculturing, solitary shoots excised from multiple shoot cultures were transferred to MS and $\frac{1}{2}$ MS media containing IAA, IBA and NAA at various concentrations for rooting. Rhizogenesis was not occurred MS and $\frac{1}{2}$ MS medium without plant growth regulators. Auxin is necessary for *in vitro* rooting of *R.mykalea* axillary shoots. Generally, $\frac{1}{2}$ MS medium added auxin is more effective than MS medium added auxin for rooting. The maximum rooting rate was obtained with half-strength MS medium supplemented with 0.5 mgL⁻¹ IBA (55%) (Figure 5 and 6). There are many of reports about IBA is



Means by different letters in each column and capital letters in each row are significantly different ($p < 0.05$), according to Duncan Multiple Range Test.

Figure 4. Average shoot lengths of axillary shoots dependent on cytokinin type and concentration.



Data were subjected to $x' = \arcsin \sqrt{(x/100)}$ transformation and used analysis. Means by different letters in each column and capital letters in each row are significantly different ($p < 0.05$), according to Duncan Multiple Range Test.

Figure 5. Rooting of *R. mykalea* axillary shoots.

more effective than other auxins on rooting for Asteraceae such as *Anthemis xylopoda* [11, 23], *Centaurea spachii* [12], *Centaurea ragusina* [24], *Centaurea zeybekii* [25] and *Saussurea obvallata* [26].

There was a statistically significant difference between MS and 1/2 MS medium on rooting. 1/2 MS medium is more effective than MS medium on rooting in all experiments. Also, there was a statistically significant difference on rooting of *R. mykalea* between auxin type and concentration.



Figure 6. Rooting plantlets on $\frac{1}{2}$ MS medium added 0.5 mg l^{-1} IBA.

In this study, we described a successful and rapid propagation techniques to regenerate critically endangered *R.mykalea* the first time by *in vitro* tissue culture techniques. Mature zygotic embryos isolated from achenes were used as starting material. The shoots that were obtained from *in vitro* germinated mature embryos were separated from primary roots and used for axillary shoot propagation. The highest axillary shoot number per explant was obtained on MS medium supplemented with 0.5 mg l^{-1} BA (5.8 shoot/explant). MS medium supplemented with 0.1 mg l^{-1} KIN was determined as the most suitable medium for the maximum shoot length (7.35 cm). Solitary shoots, removed from stock cultures, were transferred onto half-strength MS ($\frac{1}{2}$ MS) or MS media supplemented with various concentrations of auxins. The maximum rooting rate was obtained with half-strength MS medium supplemented with 0.5 mg l^{-1} IBA (55%). Rooted plantlets were transferred to external environment step by step.

The plantlets with well developed root were transferred to *ex vitro* conditions (Figure 7). Percentage of survival of shoots was approximately 60%. The appearance and growth of these plantlet were also normal.



Figure 7. Acclimatized plantlets.

4. Conclusions

In conclusion, the present work presents a simple and successful procedure for the *in vitro* propagation of *Rhaponticoides mykalea* (Hub.-Mor.) M. V. Agab. & Greuter, a critically endangered endemic plant species.

To date there is no report on micropropagation of *R. mykalea*. This study is the first report on micropropagation of this species using seedlings from *in vitro* germinated embryos and aims to contribute ongoing *ex situ* conservation programs. Additionally, this outlined protocol can be utilized as an aid in the local conservation programs to preserve this species, and it will lead for further studies on conservation and propagation of this rare and critically endangered endemic plant.

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