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Induction of bulblets on leaf and bulb explants of endangered *Lilium bosniacum* (G. Beck) G. Beck ex Fritsch

Adisa Parić[1*], Jasmina Čakar[2], Edina Muratović[1] and Erna Karalija[1]

1 Faculty of Science, Department of Biology, Zmaja od Bosne 33-35, Sarajevo, Bosnia and Herzegovina

2 Institute for Genetic Engineering and Biotechnology, Gajev Trg 4, Sarajevo, Bosnia and Herzegovina

ABSTRACT: Organogenic capacity of leaves and bulb explants of *Lilum bosniacum* was tested. For direct shoot formation MS medium with combinations of 0,5 mg/L BA and 0,2 mg/L IBA as well as 0,5 mg/L TDZ and 0,2 mg/L IBA was used. Both combinations supported shoot regeneration from bulb explants, but only the BA + IBA combination encouraged shoot regeneration from leaf explants. Rhizogenesis was induced on MS basal medium with 0,2 mg/L IBA. Plantlets were successfully acclimated under greenhouse conditions.

Key words: Regeneration, *Lilum bosniacum*, bulb and leaves explants

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INTRODUCTION

Nowadays, many plant species are endangered in their natural habitats and they may soon become extinct. However, techniques of *in vitro* culture offer means for efficient salvation of endangered species. These techniques may be used not only for *in vitro* conservation but also for species rapid propagation. *In vitro* culture comprise methods of cell, tissue and organ cultivation using artificial nutrient media under, controlled, aseptic conditions. Methods are reliable, easy, efficient, and require only a small amount of plant material for initial explants. Plant tissue culture techniques can also provide facilities for rapid biomass production giving additional aid in the conservation of rare and endemic plant species.

Lily species have been used as ornamental plants for centuries. The most important reasons for their cultivation is their abundant, large and colourful flowers often with recurved perigon. Although the very first lilies hybrids originated from the 19th century (Woodcock & Stearn 1950; Jefferson-Brown & Howland 1995), the systematic breeding of new cultivars started in the 1950's. At present, there are several thousand registered hybrid lily cultivars (Pelkonen 2005). Also, there are many studies dedicated to their tissue culture propagation (Sheridan 1968; Simmonds & Cumming 1976; Stimart & Ascher 1978).

In spite of the large number of registered cultivars, genus *Lilium* in Europe incorporates only 10 recognised wildlife species (Matthews 1980). Additionally, a number of European lilies are with problematic taxonomical status (Matthews 1980). One of them is *L. bosniacum,* endemic species from Central Dinaric Alps with extremely attractive Turk's-cap flowers.

L. bosniacum is a rare and vulnerable taxon, included in the list for the "Red book" of the Flora of Bosnia and Herzegovina (Šilić 2000). The main data given until now for *L. bosniacum* concern its morphological (Beck 1903; Košanin 1926; Matthews 1984), ecological (Lakušić & Kutleša 1971; Lakušić 1987) or molecular cytogenetic

*correspondence: adisacausevic@hotmail.com

characteristics (Muratović *et al*. 2005, Muratović *et al*. 2010). This is the only European lily (including *L. cattaniae* /Vis./ Vis.) that has not been cultivated for commercial purpose or for specialist collections so far (Matthews 1984). The main intention of the study presented here was to develop a shoot regeneration protocol for *L. bosniacum* which can be used as a tool to aid its protection and preservation.

MATERIAL AND METHODS

L. bosniacum seeds were collected from a natural population located at the Mt. Crepoljsko, Bosnia and Herzegovina, at 1200m altitude on carbonate geological substrate. Surface sterilization and germination of seeds were done as previously described (Parić *et al.* 2008). Seeds were soaked in 0,3 M NaOH for 30 minutes at room temperature, followed by overnight treatment with 0,2% (v/v) sodium hypochlorite at 4°C. The next day seeds were submerged in 2% (v/v) sodium hypochlorite for 2 hours and washed in autoclaved water. After surface sterilization, the testa was carefully removed and embryos were placed on the MS (Murashige & Skoog 1962) medium supplemented with 0.15 mg/L gibberrelic acid (GA_3).

Leaves and bulbs from one-month-old seedlings were used as initial plant explants for *in vitro* propagation. Leaf segments, 1 cm in length and whole bulbs, 0.5 cm in diameter, were inoculated in Petri dishes on MS medium with two combinations of plant growth regulators. First contains 0.5 mg/L 6-benzilaminopurine (BA) and 0.2 mg/L indol-3-buteric acid (IBA) and second 0.5 mg/L thidiazuron (TDZ) and 0.2 mg/L IBA. There were five explants per plate and 25 explants per treatment. Four weeks later all explants were transferred to medium, supplemented with 0.5 mg/L BA and 0.2 mg/L IBA. Healthy, well developed shoots were rooted on MS medium containing 0.2 mg/L IBA. Regenerated plantlets were acclimated in greenhouse conditions at 25°C and 60-90% relative humidity.

All media were adjusted to a pH 6.5 and 0.8% (w/v) agar was used as the gelling agent before autoclaving 20 min at 120 °C. The day/night temperature in the growth chamber was maintained at 21/19°C, with a 16h photoperiod under continuous illumination (35μmol m^{-2} s^{-1}) provided by fluorescent lights.

RESULTS

Surface sterilized seeds with testa removed were cultured on MS medium supplemented with 0.15 mg/L GA_3 to improve their elongation. This treatment enabled majority seeds (77%) to germinate. Seeds with intact testa did not germinated under same experimental conditions. It is obvious that the presence of the seed coat prevented germination, although the way in which the testa causes seeds dormancy is not yet clear. According to literature data testa could inhibit germination through preventing imbibition (Ballard 1973) or regular gas exchange (Brown 1940).

Leaf segments and whole bulbs were inoculated on MS medium with 0.5 mg/L BA + 0.2 mg/L IBA and 0.5 mg/L TDZ + 0.2 mg/L IBA. After four weeks of culture, bulblet regeneration occurred directly from bulb explants. *Lilium* bulbs naturally divide to form new bulbs. Removal of individual bulb scales can induce adventitious bulblet formation, a process known as scaling, which is commonly used to propagate lilies (McRae 1998). Whole bulbs used in our experiment gave rise to small bulblets consisted from at least two bulb scales and shoot regeneration arising directly from bulb explants with no callus formation. Regeneration varied considerably between explants and type of cytokinin.

Bulb explants manifested a very high regeneration ability producing 100% regeneration on BA supplemented medium (66 shoots in total) and 96% regeneration on TDZ supplemented medium (51 regenerated shoots).

The regeneration ability of leaf explants was much lower. On the BA supplemented medium only 16% explants regenerated bulblets and shoots while on the TDZ supplemented medium regeneration was not registered. A total of 35 shoots were produced from leaf explants on medium with 0.5 mg/L BA and 0.2 mg/L IBA. Regeneration from various explants type is presented in Fig. 1 A, B and C.

Regenerated shoots were removed and cultured on multiplication medium. Visual examination of the cultures after four weeks indicated that shoot induction occurred principally as axillary or adventitious shoots (Fig. 1 D).

Healthy well developed shoots were rooted on MS basal medium supplemented with 0.2 mg/L IBA. First roots appeared after 5 days and their subsequent development was fast. It is interesting to note that the root length depended on the type of cytokinin used in the previous subculture. Thus, explants from the BA supplemented medium showed a higher number of roots per explants while the explants from TDZ supplemented medium showed higher length (Fig. 1 E and F). Well-rooted plantlets were acclimated *ex vitro* (Fig. 1 G).

DISCUSSION

Bulb scales are most commonly used explants type both for vegetative and *in vitro* culture propagation of *Lilium* since they have a high capacity for adventitious bulb regeneration (Takayama & Misawa 1979). Unfortunately, underground parts of plants are difficult to use as explants

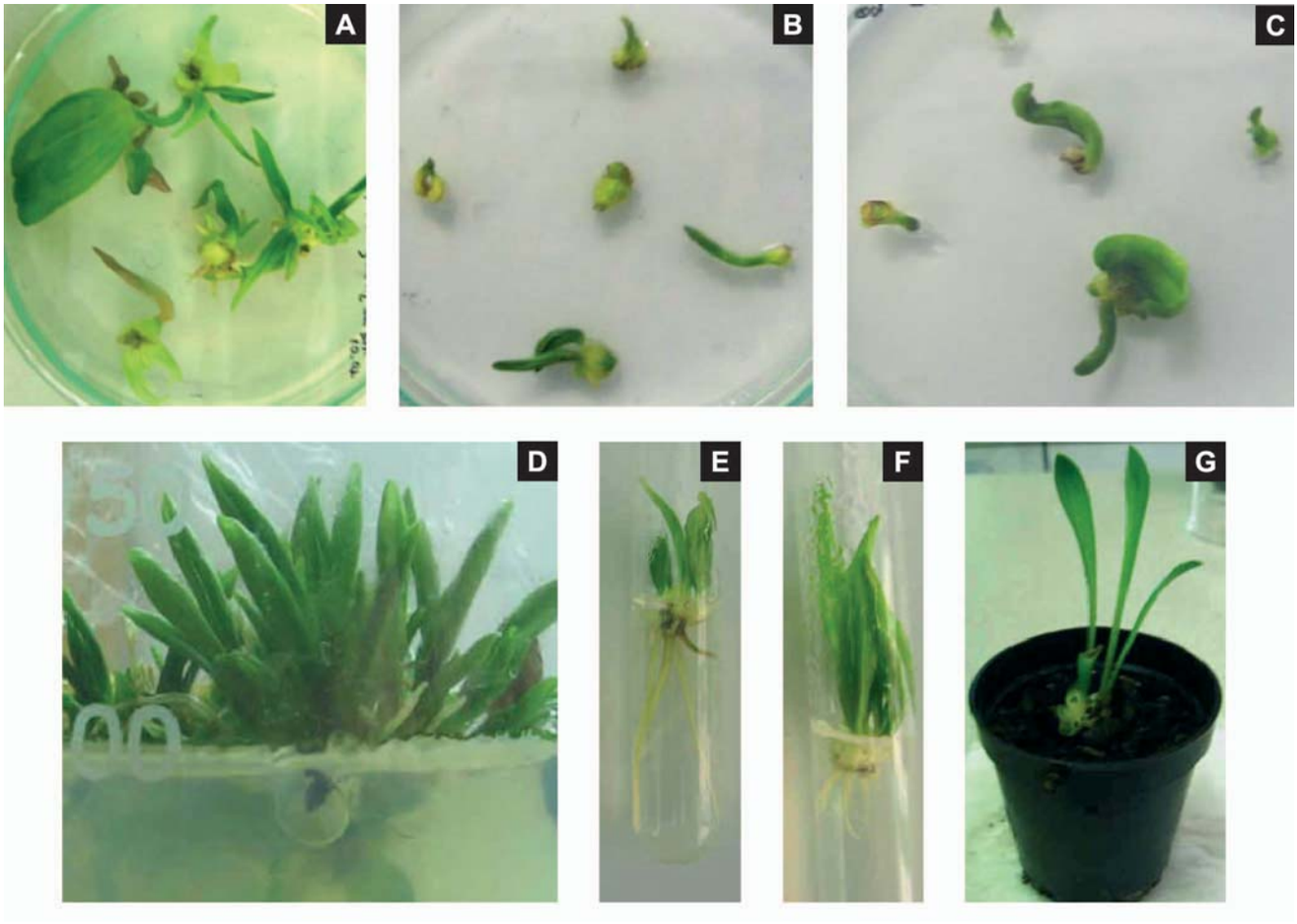

Fig1. Plant regeneration of *L. bosniacum*. **A**. Regeneration from bulbs on MS basal medium supplemented with 0.5 mgL^{-1} BA and 0.2 mgL^{-1} IBA. **B**. Regeneration from bulbs on MS basal medium supplemented with 0.5 mgL^{-1} TDZ and 0.2 mgL^{-1} IBA. **C**. Plant regeneration from leaves on MS basal medium supplemented with 0.5 mgL^{-1} BAP and 0.2 mgL^{-1} IBA. **D.** Multiplication of *L. bosniacum* on MS basal medium supplemented with 0.5 mgL^{-1} BAP and 0.2 mgL^{-1} IBA **E**. Root formation on MS basal medium supplemented with 0.2 mgL^{-1} IBA on regenerated shoots from MS basal medium supplemented with TDZ. **F**. Root formation on MS basal medium supplemented with 0.2 mgL^{-1} IBA on regenerated shoots from MS basal medium supplemented with BAP. **G**. Adapted plants under *ex vitro* conditions.

for *in vitro* culture propagation since there is high contamination risks. We therefore used seeds as a far better choice for *in vitro* culture establishment. It is also well known that seeds of many *Lilium* species are good starting experimental material for obtaining virus-free plants (Pelkonen 1997).

We tried to optimize the conditions for *in vitro* regeneration of *L. bosniacum* leaves and bulbs from *in vitro* growing plants. High bulblet and shoot regeneration was obtained from bulb explants on MS medium with 0.5 mg/L BA and 0.2 mg/L IBA (100%) as well as on MS medium with 0.5 mg/L TDZ and 0.2 mg/L IBA (96%). Although a good number of shoots were regenerated from bulb explants in TDZ comprising media, most of the shoots were poorly elongated and remained stunted with poorly developed leaves, comparing to BAP (Fig. 1 A and B).

Bulblet regeneration has been used previously (Lian *et al.* 2003) in *Lilium* oriental hybrid Casablanca. Similar results (Varshney *et al*. 2000) were obtained from *in vitro* micropropagation of *Lilium* Asiatic Hybrid, *L. japonicum* (Maesato *et al.* 1994) and *L. longiflorium* (Stimart & Ascher 1978; Lesham *et al.* 1982) as well as in other species of the same genus (Chang *et al.* 2000).

Regeneration capacity of leaf explants was significantly lower which is in accordance with other reports. Still leaf explants have been successfully used as starting material (Niimi 1986, 1995).

In vitro culture regeneration protocol, that we elaborated, enables maintenance and propagation of *L.*

bosniacum. Therefore developed tool can be used as an aid for conservation and protection of this endangered plant species.

Our results showed that *in vitro* propagation using bulbs as a starting material is practical for the production of *L. bosniacum* plantlets. Having in mind that this endemic species is endangered, conservation of its populations is of extremely importance.

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REZIME

Indukcija regeneracije iz listova i lukovica ugrožene vrste *Lilium bosniacum* (G. Beck) G. Beck Ex Fritsch

Adisa Parić, Jasmina Čakar, Edina Muratović, Erna Karalija

Ispitivan je organogeni kapacitet listova i lukovica bosanskog ljiljana (*L. bosniacum*). Za direktno formiranje izdanaka korištćena je MS osnovna podloga uz dodatak 0.5 mg/L BA i 0.2 mg/L IBA, kao i 0.5 mg/L TDZ i 0.2 mg/L IBA. Obe kombinacije auksina i citokinina su potstakle direktno formiranje izdanaka iz lukovica, dok je samo kombinacija BA + IBA imala pozitivno delovanje na formiranje izdanaka iz listova. Formiranje korenova je indukovano na MS podlozi uz dodatak 0.2 mg/L IBA. Ovako dobijene biljke su aklimatizovane na uslove spoljašnje sredine.

Ključne reči: Regeneracija, *Lilum bosniacum*, lukovice i list