

Somatic embryogenesis and *in vitro* plantlet regeneration of *Lilium martagon* L. var. *cattaniae* Vis.

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Abstract:

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In this study organogenic capacity of two different explants type (leaves and whole bulbs) of *Lilium martagon* L. var. *cattaniae* Vis. was examined. For induction of *in vitro* somatic embryogenesis and adventitive regeneration different concentrations of 2,4-dichlorophenoxyacetic acid and 6-benzilaminopurine (from 0,25 mg/l to 8,00 mg/l) added to MS basal medium were used. Our results indicate that concentration of 0,5 mg/l 2,4-dichlorophenoxyacetic acid and 4 mg/l 6- benzilaminopurine promoted somatic embryogenesis from leaves of *Lilium martagon* var. *cattaniae*, while all other concentrations promoted direct shoot regeneration from bulb explants. Root formation was induced on MS basal medium with 0,2 mg/l indole butyric acid. These plantlets were acclimatized well in a greenhouse conditions.

Key words: *Lilium martagon* var. *cattaniae*, somatic embryogenesis, regeneration

Introduction

Lilium martagon L. var. *cattaniae* Vis. is a monocotyledonous plant from the Liliaceae family. This taxon is a vulnerable endemic taxon of Central Dinarids distributed in warmer areas of Mediterranean and Submediterranean. In Bosnia and Herzegovina this taxon is also categorised as rare and endangered, and as such is listed in the checklist of plant species for the Red book of Bosnia and Herzegovina (Šilić, 1996). In recent years, there is an increasing interest in somatic embryogenesis as a method for *in vitro* propagation of lilies and other plant species. This is probably because somatic embryogenesis produces a large number of individuals and can be used for production of artificial seeds and for the purposes of transformation as well. The method for induction of somatic embryogenesis has been established for many lilies including *Lilium martagon* (Kedra & Bach, 2005), but such methods are unknown for *Lilium martagon* var. *cattaniae*. Therefore, the aim

of this study was to investigate the regeneration potential and possibility of somatic embryogenesis induction in this taxon.

Material and methods

Plant material and *in vitro* conditions

Leaves and bulbs of *in vitro* grown *Lilium martagon* var. *cattaniae* (Hindija et al., 2007) were used for induction of somatic embryogenesis and plantlet regeneration. All media were adjusted to pH 5.7-5.8 using 1 N KOH/HCl and 0.8% agar was used for solidification of media. Media were autoclaved at 120°C for 20 min. All cultures were kept at 24°C (±2) and relative humidity 70%. During the first 15 days of the treatment the cultures were kept in the dark while for the remainder of the experiment cultures were kept in conditions of 16 hour photoperiod provided by cool, white, fluorescent tubes (3000 lux).

In vitro treatments and acclimatisation of plantlets

For induction of somatic embryogenesis and plantlet regeneration, bulbs and leaves were cultivated on MS (Murashige & Skoog, 1962) medium containing different concentrations of 2,4-D and BAP (Tab. 1), including control, with no plant growth regulators (PGR) added. Embryogenic nature of cultures was confirmed by visual identification. After 30 days of PGR treatment all cultures were then transferred to PGR free MS basal medium.

Regenerated shoots from bulb explants were multiplied on the MS medium containing 0,5 mg/l BAP, 0,2 mg/l IBA and 0,1 mg/l GA₃, and they were subsequently cultivated on MS medium containing 0,2 mg/l IBA for induction of rhizogenesis.

Acclimatisation was conducted in pots containing commercial soil for plant cultivation, under greenhouse conditions, with the temperature of 25°C and relative humidity of 70%.

Three replicates were taken for each treatment. Data from all of the replicates was statistically analyzed using statistics program SPSS version 7.

Results and discussion

Induction of somatic embryogenesis

Out of all of the tested concentrations of 2,4-D and BAP only 0,5 mg/l 2,4-D and 4 mg/l BAP mg/l was successful in inducing somatic embryogenesis from leaf explants. The primary sign of induction of somatic embryogenesis was swelling of explants tissue (George et al., 2008), which in our experiment was evident on the basal parts of leaf explants (Fig. 1a), 15 days after inoculation. Globular embryos appeared directly from the surface of the explants (Fig. 1b). It seems that this combination provides suitable conditions for progression of totipotent somatic cells into somatic embryos. All other applied concentrations showed a reverse effect on embryogenesis.

Embryo development reached cotyledon state (Fig. 1b) a month after inoculation, and 20 embryos per explant were formed. Further development and germination of embryos was accomplished after 75 days of culture, with subsequent transfer of globular embryos on PGR free medium (Fig. 1c). Similar results were also described for somatic embryogenesis in other Lilies as well as other species, where somatic embryos developed into plantlets when plated onto MS basal medium without PGRs (Tabira, 1994; Tribulato et al.,

1997; Ho et al., 2006; George et al., 2008). Plantlets were well acclimatised in greenhouse conditions. No induction of somatic embryogenesis was noticed from bulb explants.

Induction of plantlet regeneration from bulb explants

Regeneration was successful for all tested combinations of concentrations of 2,4-D and BAP but showed different rate of regeneration (Tab. 1). Rate of regeneration was ranging from 50 to 100%, depending on PGRs applied. Bulblets regeneration varied from 7, on medium containing 1 mg/l 2,4-D + 1 mg/l BAP, up to 16 bulblets per explant, on medium containing 2 mg/l 2,4-D + 1 mg/l BAP (Tab. 1). However, the differences were not significant. Regeneration of leaves also varied, from 2 to 20 leaves per explant (Tab. 1). The highest number of regenerated leaves was on the MS medium containing 0,25 mg/l 2,4-D and 8 mg/l BAP with 20 regenerated leaves on average (Fig. 1d). Statistically significant differences on $p < 0,05$ level (LSD) were noticed for treatment containing 2 mg/l 2,4-D and 1 mg/l BAP in comparison with results obtained on MS medium containing 0,25 mg/l 2,4-D and 8 mg/l BAP and in comparison with control treatment. Bulbs are the most commonly used explants in lily cultures as they show the highest organogenic potential (Takayama & Misawa, 1979). Numerous authors studied the importance of interaction between auxins and cytokinins on formation of bulbs and shoots *in vitro* with higher concentrations of cytokinins promoting a higher regeneration of bulblets (Takayama & Misawa, 1979, 1982; Niimi, 1995). Results of our research also show that with the rise in the concentration of cytokinins there is an increase in the regeneration of leaves with regeneration rate peaking on the medium with the highest concentration of BAP (8 mg/ml), but for best regeneration of bulblets auxin/cytokinin ratio was in favour of auxine (treatment with 2 mg/l 2,4-D + 1 mg/l BAP).

In our research 2,4 D induced callus formation in only one case, specifically on the medium containing 4 mg/l BAP and 0,5 mg/l 2,4 D, which produced leaves and bulbs after being transferred onto a PGR free medium. Okazaki & Koizumi (1995) reported that for shoot production from callus it is necessary to cultivate callus on PGR free medium for a period of six months or by lowering the sucrose concentration from 3% to 1%.

Mori et al (2005) later showed that for a large number of lily species shoot induction

Table 1. Effect of 2,4-D and BAP on leaf and bulblet regeneration from bulb explants of *L. martagon* var. *cattaniae*

PGR treatment: 2,4 D + BAP (mg/l)	Rate of regeneration (%)	No. of regenerated leaves	No. of regenerated bulblets
0,25 + 8,00	90	20 ^a	14
0,50 + 4,00	70	12	13
1,00 + 2,00	80	11	12
2,00 + 1,00	100	2 ^{a,b}	16
1,00 + 1,00	50	5	7
0,00 + 0,00	50	18 ^b	15

Values are mean of three replicates per each treatment. Same letter within the column indicates a significant differences at $p < 0,05$ by LSD test. Values with no indicators (a or b) show no significant difference.



Figure 1. Induction of somatic embryogenesis on MS basal medium containing 0,5 mg/l 2,4-D and 4 mg/l BAP; a) globular embryos; b) cotyledon phase; c) germinated embryo d) regeneration from bulb explant on MS basal medium containing 0,25 mg/l 2,4-D and 8 mg/l BAP.

could be successful with only two months of cultivation on a PGR free medium. In our research differentiation of shoots was successful after only a month of callus cultivation on PGR free medium.

Regenerated shoots were cultivated on multiplication medium containing 0,5 mg/l BAP, 0,2 mg/l IBA and 0,1 mg/l GA₃. Multiplication was successful producing up to 30 shoots per explant. After induction of rhizogenesis on the MS medium

containing 0,2 mg/l IBA, plants were acclimatized in the greenhouse conditions.

Conclusion

Our research showed that induction of somatic embryogenesis could be successfully induced from leaf explants by cultivation on MS medium containing 0,5 mg/l 2,4-D + 4 mg/l BAP. Regeneration from bulb explants was successful

with differences in bulblet and leaf regeneration. Best treatment for leaf regeneration contained high cytokinins level (8 mg/l), while treatment with best results for bulblet regeneration contained more auxin than cytokinin in ratio 2:1. Multiplication was very successful with production of large number of shoots with good acclimatisation rate.

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