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Influence of Different *Agrobacterium rhizogenes* Strains on Hairy Roots Induction in *Eustoma grandiflorum*

G. POPA*, C. P. CORNEA **, A. BREZEANU ***
**Research and Development Station for Fruit Trees – Băneasa, Bd. Ion Ionescu de la Brad, No.4 Bucharest, Romania*
*** Faculty of Biotechnology, Bd. Mărăşti No.59, 71331 Bucharest,*
**** Romanian Academy - Institute of Biology, Bucharest*

Abstract

Leaf fragments from three genotypes (Pink Sahin, Blue-Sakata Yodel and White Yodel) of Lisianthus (L. russellianus Hook syn. Eustoma grandiflorum Griseb) were used as explants to initiate in vitro culture. Within two weeks of in vitro culture on MS [8], with 3.0 mg/l BAP, callus initiation occurred at the cut end of leaf sections of Lisianthus spp. adventitious buds developed from callus developed on the cut end of leaflets within 20 days of culture. Elongation of shoot buds occurred on MS basal media in the presence of BAP (1.0 mg/l) and GA_3 (5.0 mg/l). The induction and improvement of in vitro rhizogenesis of microshoots in Lisianthus spp. were performed using the genetic transformation system with different wild strains of Agrobacterium rhizogenes: LBA 9402, 8196, NCPPB 2659, in the presence of indole-3-butyric acid (IBA) in the culture media. The genetic transformation of plant material with different A.rhizogenes strains was used as an alternative method for inducing the rhizogenesis process in Lisianthus spp.

Keywords: lisianthus genotypes, morphogenesis, adventitious roots, rhizogenesis, *Agrobacterium rhizogenes*, genetic transformation

Introduction

Lisianthus (*L .russellianus* Hook syn. *Eustoma grandiflorum* Grise.) belongs to the family Gentianaceae and is native to the American prairies ranging from South Nebraska to Louisiana and Texas. It is known by its local names of the Texas Bluebell or the Prairie Gentian. Due to variable colour of flowers (pink, white, purple, plum) Lisianthus is now very popular as a cut flower or pot plant in many countries [5]. A problem in seedlings of lisianthus is that after rising, the plant has tendency to remain in the rosette stage because of inadequate conditions in the plant exigency (temperature, light etc) [9,10]. In this case, the development of the stem is not happen, the flowering time is delayed. Inducing of *in vitro* shoot development in various explants types could overcome the rosette stage [7]. Micropropagation protocols and organogenesis in *Eustoma* cultivars were reported by different authors, with slightly variations [4,1,2].

Many attempts to overcome the problem of rooting and shooting have been carried out on various plant species using *Agrobacterium rhizogenes*-mediated transformation [3]. The Gram negative soil bacterium *Agrobacterium rhizogenes* has the ability to infect plants through wounds and to induce abundant adventitious roots (hairy roots) after the transfer of T-DNA in the plant genome [13,11]. In ornamentals, the most successful method of DNA delivery from bacteria has been the co-cultivation of regenerable explants with *A. tumefaciens*

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and, in a few cases, with *A. rhizogenes* [12]. In lisianthus (*Eustoma grandiflorum*), hairy roots were obtained after the infection with *A. rhizogenes* strain A13 [6].

The aim of this work is to compare the ability of different wild type strains of *Agrobacterium rhizogenes* (LBA9402, 8196 and NCPPB 2659) to induce and improve the *in vitro* rhizogenesis from microshoots of lisianthus in the presence of indole-3-butyric acid (IBA) in the culture media.

Materials and Methods

Plant material Leaf fragments (10x10mm) of lisianthus collected from three cultivars: Pink Sahin, Blue Sakata Yodel and White-Yodel grown in the greenhouse before flowering were used as explants for culture establishment.

Culture establishment and shoot multiplication. Explants were surface sterilized under the laminar flow hood with a solutions of 0.5 % mercury chloride for 3 min. followed by three rinses in sterile distilled water then were inoculated, in aseptic conditions, on the shoot proliferation medium. Leaf fragments of lisianthus cultivars were cultured on MS [8] basal media with 3.0 mg l^{-1} BAP (6- benzyl-aminopurine) and subcultured on MS media supplemented with 1.0 mg l^{-1} BAP and 5.0 mg l^{-1} GA_3 (gibberellic acid), 3% sucrose and 0.8% agar. All media components were mixed and adjusted to an appropriate pH (5.7) before autoclaving at 121^{o}C for 20 min. Cultures were incubated at 23± 2^{o}C in a growth room under a 16 h light photoperiod. Sub-culturing was carried out every three weeks. A total of 30 explants with three replicates were used for each cultivar.

Bacterial strains. Three wild strains of *Agrobacterium rhizogenes* were used in the experiments*: A.rhizogenes* 8196 and *A.rhizogenes* LBA 9402 were kindly provided by dr. David Tepfer from I.N.R.A., Versailles, and *A.rhizogenes* NCPPB 2659 were obtained from dr.Simon Weller. The bacteria were maintained on solid M16 media [14] at 28 ^{0}C for 48 h, in darkness. Bacterial inoculum was obtained by cultivation of each bacterial strain in liquid M16 medium for 48 hrs until an optical density of 0.4-0.5 at 600nm was obtained.

Rooting of Lisianthus shoots. Shoots containing 2-4 leaves were selected from *in vitro* culture for the following experimental variants:

1) Shoots were cultivated on half-strength MS medium (control);

2) Shoots were cultivated on half-strength MS medium with IBA (indolyl - butyric acid);

3) Shoots were infected, by co-cultivation, with the specific bacterial inoculum from *A.rhizogenes* LBA9402, *A.rhizogenes* 8196 or *A.rhizogenes* NCPPB 2659. Before the infection with *A.rhizogenes* strains, shoots were wounded with a sterile scalpel and immersed in the bacterial suspension for 30 min. The resulting plant materials were incubated at 25 ± 2^{o}C in darkness for 48 hrs prior to cultivation on MS solid basal medium with 500 mg l^{-1} cefotaxime, without phytohormones. The cultures were then incubated on the same medium at 25^{o}C, under constant illumination. After 8 weeks, rooting percentage, roots number and their length were measured. A total of 24 explants per treatment and two replicates for each experiment were used.

Data were evaluated using Student's *t* test at 0.5 % level.

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Results and Discussion

Ten days of *in vitro* culture were sufficient to allow the callus initiation that occurred at the cut end of leaflet (Figure 1).

Figure 1.Callus initiation at the cut end of leaf segments on MS media supplemented with BAP (3 mg/l)

Adventitious buds developed from callus formation on the cut end of leaf segments were appeared after 15 days of culture. The morphogenic response (number of shoots and shoots length) was appreciated six weeks after the initiation of cultures. Maximum percentage of shoot regeneration was achieved with white cultivars (92 %) and the minimum with the blue cultivars (table 1).

Table 1. Morphogenic response of *in vitro* culture six weeks after initiation

Lisianthus variety	% of regeneration	Shoots number/ explant	Shoots length (cm)
Pink	84	19.75 a ± 2.92	2.20 a ± 0.35
White	92	31.10 b ± 1.44	3.12 b ± 0.33
Blue	69	22.05 a ± 5.18	2.64 a ± 0.46

Values with same letter showed no significant differences and represent the means ± standard error of three independent measurements 6 weeks after culture initiation.

Within six weeks of cultivation numerous shoots become visible. Elongation of shoot buds occurred rapidly followed by initiation and development of new buds when gibberellic acid at high concentration (5.0 mg l^{-1}) was added in the media (Figure 2).

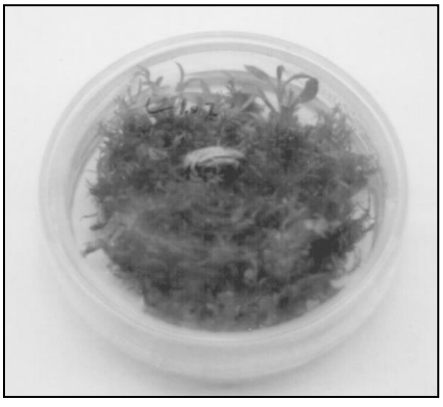

Figure 2. Multiple shoot proliferation on MS media supplemented with BAP (1 mg/l) and GA_3 (5 mg/l).

Regarding the number of shoots per explant and shoots length, the results showed no significant differences between Pink (19.75± 2.92; 2.20±0.35) and Blue cultivars

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(22.05±5.18; 2.64±0.46), while maximum shoots per explant and shoots length (31.10± 1.44; 3.12± 0.33) were noted for White cultivar (Table 1).

In vitro propagation of various plant species is often inefficient because of the difficulty to root. In some cases it was possible to improve *in vitro* rooting with hormone application or using of polyamines [3].

In our experiments, in a control variant, shoots were cultivated on half-strength MS free phytohormones media and the root development was examined during two months. No roots were appeared in this case. In order to induce the root formation and to improve the process, two experimental variants were applied: the exogenic application of an auxin (IBA) in the culture media and the using of *A. rhizogenes* transformation system.

The ability of root formation after infection with *A. rhizogenes* strains LBA 9402, 8196 and NCPPB 2659 or by IBA treatment was recorded within 8 weeks. Hairy roots developed three weeks after inoculation. The average of rooting percentage, root number per explant and root length were significantly higher, for all genotypes tested, when *A. rhizogenes* LBA 9402 was used: the Pink and the Blue cultivars gave 100% rooting efficiency, and the number and the length of the roots were also the highest (table 2).

Table 2. *In vitro* rooting of lisianthus shoots with IBA and different strains of *Agrobacterium rhizogenes*

Genotype	*A.rhizogenes* strain or IBA treatment	Rooting percentage (%)	Root number / explant	Root length (cm)
PINK	8196	71	4.00 ± 2.12	2.23 ± 1.36
	2659	50	2.25 ± 1.99	0.93 ± 0.95
	9402	100	11.0 ± 4.50	6.60 ± 4.72
	IBA	78	5.03 ± 1.35	2.78 ± 0.65
WHITE	8196	36	0.71 ± 0.65	0.43 ± 0.39
	2659	25	0.75 ± 1.23	0.44 ± 0.69
	9402	86	8.85 ± 6.78	3.50 ± 4.25
	IBA	77	4.03 ± 1.04	2.43 ± 0.62
BLUE	8196	45	1.18 ± 1.40	1.36 ± 1.14
	2659	13	0.33 ± 0.49	0.47 ± 0.69
	9402	100	10.5 ± 11.43	6.33 ± 3.90
	IBA	65	2.90 ± 0.88	3.04 ± 1.00

Values represent the means ± standard error of two independent measurements. A total of 24 explants per treatment and two replicates for each experiment were used. No root formation was observed on control shoots.

When the rooting was induced by adding IBA in the cultivation medium of untransformed shoots, data recorded showed no significant differences between Pink (78%) and White cultivars (77 %) (table 2). However, some changes in the root number and root length occurred between genotypes tested in our experiments: the best results were found in Pink and White cultivars in the presence of IBA. All the analysis was performed versus control (normal shoots placed on half strength MS solid medium).

Comparative analyses of the results showed variations in the rooting ability among bacterial strains used for lisianthus infection: the most efficient proved to be *A. rhizogenes* LBA 9402, followed by *A. rhizogenes* 8196, but as a general conclusion the infection always had a positive effect for all cultivars tested (figure 3).

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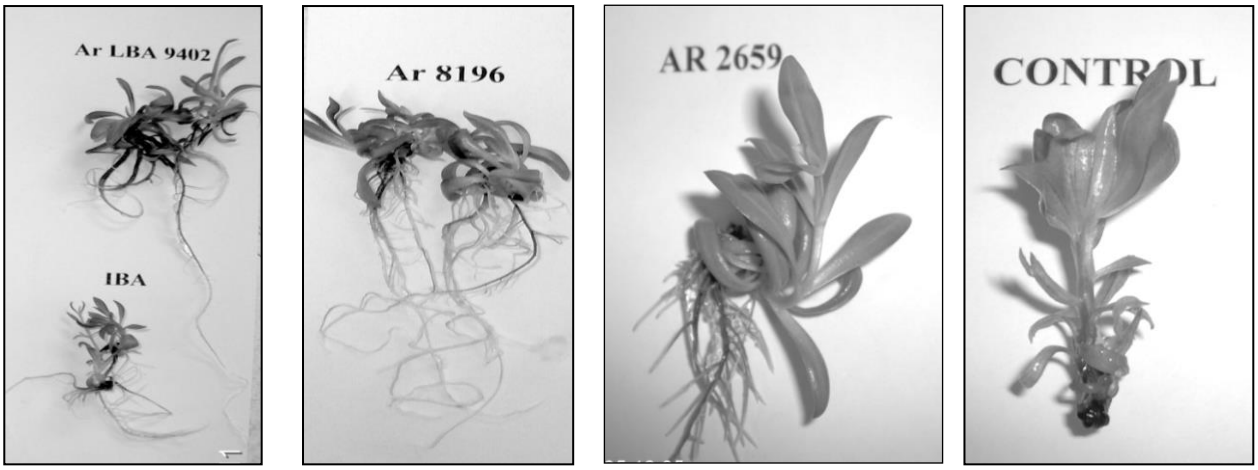

A **B** **C** **D**

Figure 3. Rooting plantlets of Lisianthus. A. Plantlet rooted after *A. rhizogenes* LBA 9402 infection comparing with the planted rooted on half-strength MS medium containing IBA (arrow); B. Plantlet rooted after *A. rhizogenes* 8196 infection; C. Plantlet rooted after *A. rhizogenes* NCPPB 2659 infection; D. Control (untransformed) shoot.

However, variations in susceptibility to bacterial infection of the lisianthus genotypes were detected but these were correlated with the bacterial strain used for treatment. It seems that the most reactive were Pink and Blue cultivars for *A. rhizogenes* LBA9402 and *A. rhizogenes* 8196 (table 2).

Conclusions

The results presented in this paper indicate that the percentage of regenerating, shoot number per explant, shoots length were significantly affected by genotype and the interaction of genotype with the culture media. An efficient micropropagation protocol using leaf fragments as explants was described for lisianthus. Between White, Pink and Blue cultivars tested, the best results as well as per cent of regeneration (92 %), number of shoots per explant (31.10) and shoot length (3.12) were recorded for White cultivar. Addition of high concentration of GA_3 (5 mg/l) in the media containing 1.0 mg/l BAP enhanced the shoot elongation process. *In vitro* rooting of lisianthus shoots in medium supplemented with IBA and different strains of *A.rhizogenes* depended on the plant genotype and the *A. rhizogenes* strain. The study of adventitious root induction in lisianthus cultivars by *A. rhizogenes* strains LBA 9402, 8196, NCPPB 2659 and IBA treatments and comparative analyses of results after rooting, were showed that among each cultivar the best results were found in the treatments including LBA 9402 strain and IBA. The three bacterial strains and IBA treatment differed significantly in the ability of rooting percentage, roots number and roots length using the Lisianthus genotypes. Maximum response in all treatments used, was recorded for Pink cultivar six weeks after infection or cultivation in the presence of IBA. No root formation was observed on control shoots.

The results obtained in our investigations proved that *A.rhizogenes* transformation system could be used as an efficient alternative method for inducing the rhizogenesis process in *Eustoma grandiflorum*.

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