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Efficient Regeneration of 'Caralis' *Alstroemeria* Cultivar from Rhizome Explants

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Abstract

In this paper, the effects of a number of growth regulators as well as supplements to the Murashige and Skoog (MS) basal medium were evaluated on the regeneration of *Alstroemeria* rhizome explants. In the first experiment the effects of three cytokinins (BA, TDZ and 2IP each at 0.5, 1 and 2 mg/l) in combination with NAA (0.2 mg/l), followed by another PGR combination of 2IP (at 0.5, 1 and 2 mg/l) with NAA (0 and 0.2 mg/l), on regeneration of rhizome-derived explants, was investigated. Through the second experiment, the effects of a number of supplements, including glucose (30 g/l as the alternative for sucrose), casein hydrolysate (1 g/l), asparagine and glutamine, (each at 30 mg/l) added to MS medium, containing 1 mg/l BA and 0.2 mg/l NAA, was examined on rhizome explants' regeneration. Among the tested cytokinins, BA induced better regeneration of rhizome explants, resulting in a higher number of shoots compared to the other cytokinins. A medium supplemented with 1 mg/l BA and 0.2 mg/l NAA proved to be the most effective, with an average of 4.16 regenerated shoots per explant. In the second PGR combination, addition of NAA at 0.2 mg/l improved regeneration, compared to NAA-free treatments. In the second experiment, glucose substitution for sucrose improved regeneration with an average of 5.10 regenerated shoots per explant, compared to 4.16 shoots in sucrose-containing medium; whereas glutamine and asparagine (with 2.66 shoots) and casein hydrolysate (with 3.80 shoots) showed a negative influence on rhizome explants' regeneration.

Keywords: 6-benzylaminopurine; glucose; *in vitro* culture; naphthalene acetic acid; N-phenyl-N-1,2,3

Abbreviations: TDZ= N-phenyl-N-1,2,3, thidiazol 5-yl-urea; BA= 6-benzylaminopurine; MS= Murashige and Skoog; NAA= a-Naphthalene acetic acid; PGRs= Plant growth regulators; 2IP= N6-isopentenyl-adenine

Introduction

Alstroemeria is a cross-pollinating plant of *Alstroemeriaceae* Family, originated in South America (Khaleghi *et al*., 2008). The genus comprises numerous species, a number being also cultivated as ornamental cut, garden and pot flowers (Bond and Alderson, 1993). This plant is currently considered one of the most important and popular cut flowers on global market indebt to its beautiful and durable flowers (Khaleghi *et al*., 2008). Low temperature requirement has resulted in increasing global production of the plant, especially in North America (Lin *et al*., 2000). *Alstroemeria* is traditionally propagated by rhizome splitting, but this method is constrained by limitations such as viral contamination of the propagated plants, low propagation rate, and seasonal limitation. *In vitro* culture methods have attracted considerable interest as a means to overcome these limitations and for producing virus-free plants (Khaleghi *et al*., 2008). Apical shoot bud (Pedraz Santos *et al*., 2005), lateral shoot bud (Lin *et al*., 1997) and rhizome (Bond and Alderson, 1993; Gabryszewska, 1995; Gabryszewska and Hempel, 1985; Hakkaart and Versluijs, 1988; Lin *et al*., 2000; Lin and Monette, 1987) explants have been used for *in vitro* propagation of *Alstroemeria*. Somatic embryogenesis is another propagation route, but the occurrence of somaclonal variation and the heterozygosity of regenerated plants have limited its application mainly to genetic transformation and embryo rescue practices (Akutsu and Sato, 2002).

Rhizome-tip (apical meristem) has been reported to feature a higher growth rate compared to other types of explant mentioned above (Lin *et al*., 2000). Chiari and Bridgen (2000) examined the effect of rhizome excision (non-excised *vs*. excised lengthwise or crosswise) on rhizome multiplication of the *in vitro* cultured explants, and reported the lengthwise excision as the most effective treatment. Among cytokinins, BA has been used to increase the number of regenerated shoots in rhizome-tip explants (Hamidoghli *et al*., 2007; Khaleghi *et al*., 2008; Pierik *et al*., 1988). Zeatin together with kinetin (in a 4:1 ratio) has resulted in the initiation of growth in the same explant of rhizome-tip in cv. 'Alsaan' (Lin and Monette, 1987). MS basal medium, containing low concentrations of NAA (2 μM) (Pedersen *et al*., 1996), has resulted in improved regeneration of *Alstroemeria*; whereas other different or contrasting results have been reported as well (Pierik *et al*., 1988). PGR treatments can exhibit different impacts, depending on plant genotype, and no such study has been

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reported for cv. 'Caralis'. To the authors' knowledge, there is also a lack of comparative studies which evaluate several different cytokinins in this plant. Carbon-(Shigetaa *et al*., 1996) and nitrogen-bearing (Olsen, 1987) organic compounds (such as asparagine, proline and alanine) have been reported to increase growth of different plant species' *in vitro* cultured tissues , but such an evaluation has not yet been published for *Alstroemeria*.

This work was aimed at evaluating the effects of a number of plant growth regulators (BA, TDZ, 2IP and NAA) and a number of culture medium supplements including glucose (substituting sucrose), glutamine, asparagine and casein hydrolysate, on the regeneration of rhizome-tip derived explants of *Alstroemeria*. Optimization of a culture medium with the best PGR as well as supplement composition for cv. 'Caralis' micropropagation was the object of this study.

Materials and methods

Plant material and explant preparation

Rhizomes of pale orange-colored *Alstroemeria* plants (cv. 'Caralis') were used as the source of explants. According to the fact that *in vitro* contamination of explant is influenced by growth conditions of the donor plant, the plants were maintained under optimal conditions, and gradually used for explant preparation during the experiment. For explant preparation, plants were removed from growth bed and the rhizomes excised with a sharp blade up to the first node (Fig. 1 A). Apical buds (rhizome tips) were then washed under tap water for half an hour (with several drops of detergent), then surface sterilized by shaking in 70% ethanol (for one minute) and 3% sodium hypochlorite solution, containing a few drops of Tween 20 (for 20 minutes), respectively (Pedersen and Brandt, 1992) with slight modifications. Rhizomes were then rinsed three times with sterilized distilled water, aseptically under a laminar flow cabinet in order to remove the remaining sodium hypochlorite. After drying with filter paper, rhizome tips were finally placed onto the culture medium.

Rhizome tips were cultured on MS medium (Murashige and Skoog, 1962), containing different levels of BAP and TDZ (0.5, 1 and 2 mg/l) in combination with 0.2 mg/l NAA, or, separately, in a medium containing different levels of 2IO (0.5, 1 and 2 mg/l) alone or in combination with 0.2 mg/l NAA. All the media contained 30 g/l sucrose and were solidified with 0.8% agar. Moreover, the effects of glucose substitution for sucrose (at the same concentration of 30 g/l) and supplementation of the medium with casein hydrolysate (1 g/l) and glutamine plus asparagine (each at 30 mg/l) were studied on growth parameters in MS basal medium supplemented with 1 mg/l BA and 0.2 mg/l NAA. Supplement concentrations were chosen according to previous reports on other plant species (Anis *et al*., 2003; Narula *et al*., 2003). Culture tubes were incubated at 25 ± 2°C under a 16/8-h (day/night) photoperiod. Subcultures were made at 3-week intervals, and shoot number, bud number, shoot height, root number, chlorophyll content and shoot diameter were recorded at the 4th subculture.

For acclimatization, rooted plantlets were transferred to pots with sand, peat and perlite, being kept under high moisture, low light conditions in greenhouse. The moisture was later decreased and the light was increased gradually to acclimatize the plants in glasshouse.

Statistical analysis

A completely randomized design with 8 replications was used to study the effect of the three cytokinins on growth parameters. In order to separately examine the impacts on growth parameters of 2IP and NAA and their interaction, a factorial experiment (with three 2IP and 2 NAA levels), in completely randomized design with 8 replications, was conducted. A completely randomized design

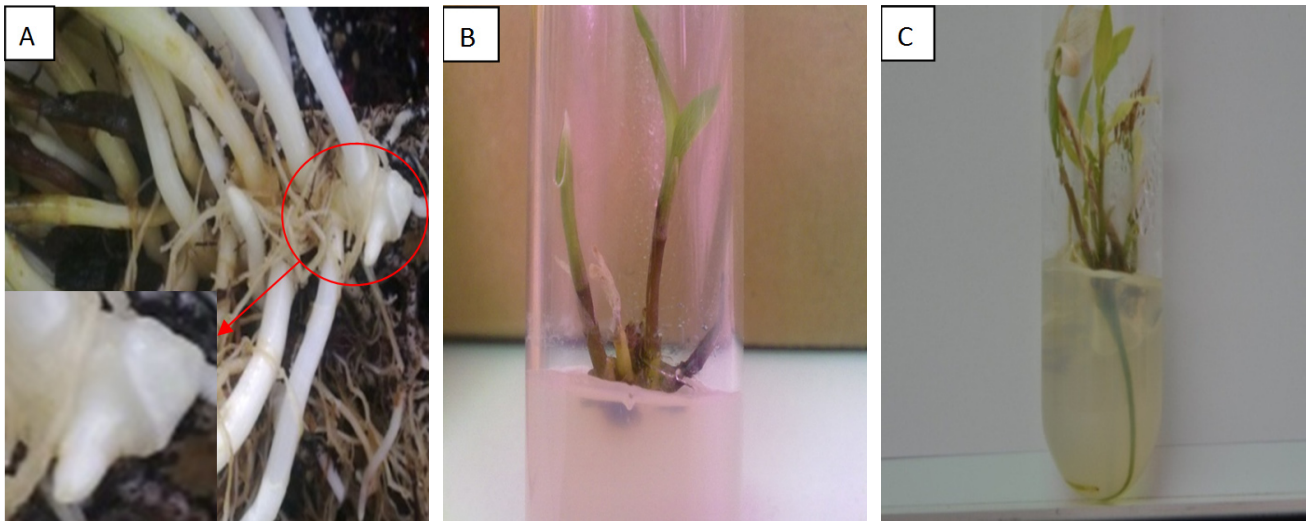

Fig. 1. Explants preparation and *in vitro* growth (A) preparation of rhizome-tip explants; (B) explants growth and shoot emergence in the 5th week; (C) rooting in the 7th week

with 10 replications was used for the effects of hydrocarbons and amino acids. Analysis of variance was performed by the SPSS software package 17 and comparison of means was performed using the Duncan's multiple range tests at 5% level of significance.

Results and discussion

Effects of different cytokinins on rhizome explant regeneration

The explants produced shoots after about 5 weeks of culture (Fig. 1 B) and started rooting in the 7th week (Fig. 1 C). Analysis of variance revealed a significant difference ($p<0.05$) among BA, TDZ and 2IP treatments with respect to the number and diameter of the regenerated shoots (Tab. 1). Using BA and 2IP resulted in the highest (4.16) and lowest (2.60) shoot numbers, respectively. Cytokinin levels also showed different impacts. Increasing BA concentration from 0.5 to 1 mg/l increased the number of regenerated shoots but further increments of BA concentration reduced shoot numbers. In case of TDZ, the highest shoot number (4.00) was reported at the 0.5 mg/l treatment. For 2IP, a continuously increasing shoot number was observed up to the highest tested concentration of 2 mg/l. BA treatments resulted in shoots with higher diameters compared to the other PGRs, with the highest shoot diameter (3.20 mm) observed at the 0.5 mg/l BA treatment; whereas the average shoot diameter at 2IP and TDZ treatments was 1.7 mm (Tab. 1). Cytokinin levels also showed a significant effect on shoot height ($p<0.01$); increasing cytokinin levels resulting in reduced shoot heights (Tab. 1). 2IP treatments produced higher shoots compared to BA and TDZ treatments. Generally, BA resulted in regeneration of a higher number of shoots with higher diameters and lower heights; whereas 2IP resulted in a lower number of shoots with lower diameter and more significant heights. Cytokinins induce shoot formation and reduce shoot height through induction of cell growth and reduction of the apical dominance (Ranjan *et al*., 2003). Hamidoghli *et al*. (2007) and Khaleghi *et al*. (2008) employed BA for shoot induction in *Alstroemeria*, and suggested the positive impact of BA compared to PGR-free treatments. The number of buds and roots, regenerated from the rhizome explant, was not influenced by the type of cytokinin ($p<0.05$). Rooting is primarily influenced by the type and concentration of auxins (Khaleghi *et al*., 2008), and cytokinins have shown little impact on explant rooting (Hamidoghli *et al*., 2007).

Effect of different BA and TDZ levels on the SPAD number

The SPAD number is a measure of the leaf's greenness, and higher SPAD number equals a darker leaf color due to higher chlorophyll content, thus a higher light absorption capacity (Netto *et al*., 2005). Measurements of the SPAD number showed a significant difference ($p<0.05$) between BA and TDZ treatments regarding chlorophyll content of the leaves (Fig. 2). Plantlets, regenerated in TDZ treatments, had a higher SPAD number than those regenerated in BA treatment. SPAD number was raised by increasing BA levels, whereas in case of TDZ it showed an initial rise and then declined. The highest and the lowest SPAD numbers (22.8 and 15.6 respectively) were observed in 1 mg/l TDZ and 2 mg/l BA treatments, respectively. According to the positive relationship of chlorophyll content with light absorption and photosynthesis rate, the higher SPAD number of TDZ-treated plantlets can be considered a positive factor toward increased photosynthesis and better growth of the regenerated plants.

Examining the influence of NAA presence vs. absence

The combined influence of NAA and 2IP on plantlet regeneration from rhizome-tip explants

The results showed that NAA combined with 2IP increased the number of regenerated shoots and roots (to

Tab. 1. Effects of the three cytokinins on shoot number, shoot height, bud number, root number, and shoot diameter (regenerated from rhizome-tip derived explants of *Alstroemeria*)

PGR treatments*	Shoot number	Shoot length (cm)	Bud number	Root number	Shoot diameter (mm)
BA					
0.5	3.60 bc	5.35 abc	1.20 a	3.80 a	3.20 a
1	4.16 a	3.50 bcd	1.66 a	4.60 a	2.70 b
2	4.00 ab	3.16 cd	1.66 a	4.00 a	2.50 b
TDZ					
0.5	4.00 ab	5.73 ab	1.33 a	4.16 a	1.70 c
1	3.50 c	2.75 d	1.50 a	4.50 a	1.70 c
2	3.30 cd	2.16 d	1.33 a	4.50 a	1.69 c
2IP					
0.5	2.60 e	7.33 a	1.66 a	3.66 a	1.73 c
1	3.00 de	4.33 bcd	1.33 a	4.33 a	1.73 c
2	3.30 cd	3.10 cd	1.50 a	4.00 a	1.72 c

Note: Means with the same letters, based on the Duncan's test, showed no significant differences. * Each PGR treatment contained 0.2 mg/l NAA in addition to one of the cytokinin levels listed above

Tab. 2. Effect of different concentrations of 2IP and NAA on rhizome regeneration

PGR treatments (mg/l)		Shoot number	Shoot height	Bud number	Root number
NAA	2IP				
0	0.5	1.33 b	3.00 c	1.33 a	1.00 c
0	1.0	1.25 b	2.22 d	1.50 a	1.50 c
0	2.0	1.20 b	1.04 e	1.40 a	1.80 c
0.2	0.5	2.60 ab	7.33 a	1.66 a	3.66 b
0.2	1.0	3.00 ab	4.33 b	1.33 a	4.33 a
0.2	2.0	3.30 a	3.10 c	1.50 a	4.00 ab

Note: Means with the same letters are not significantly different based on Duncan's test

Tab. 3. Effects of glucose, asparagine, glutamine and casein hydrolysate on shoot number, shoot height, root number and bud number

Supplements	Shoot number	Shoot height	Root number	Bud number
Control	4.16 b	3.50 ab	4.60 b	1.66 a
30 mg/l asparagine +30 mg.l^{-1} glutamine	2.66 d	4.00 a	3.40 c	1.90 a
1 g/l casein hydrolysate	3.80 bc	2.91 b	3.60 c	1.40 a
30 g/l glucose	5.10 a	3.51 ab	5.50 a	1.77 a

Note: Means with the same letters are not significantly different based on Duncan's test

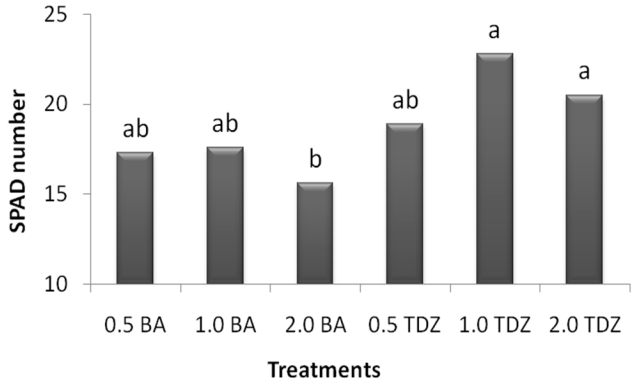

Fig. 2. Effect of different BA and TDZ levels on the SPAD number (all treatments also contained 0.2 mg/l NAA). Means with the same letters are not significantly different based on Duncan's test (p<0.05)

1.73 and 2.55, respectively) as well as shoot heights (to 2.84 cm) compared to 2IP alone (Tab. 2).

In these experiments, the highest number of shoots and roots were obtained in a culture medium containing 2 mg/l 2IP and 0.2 mg/l NAA, and the most considerable shoot height was observed in the medium with 0.5 mg/l 2IP and 0.2 mg/l NAA. These results are consistent with those reported by Han *et al*. (1994), Hamidoghli *et al*. (2007) and Khaleghi *et al*. (2008) concerning the positive effect of low NAA concentrations combined with a cytokinin on shoot induction in rhizome explants. Cytokinins plus a low concentration of NAA can induce rhizome shooting. In contrast however, Pierik *et al*. (1988) reported that the auxin had no effect on shooting. Podwyszynnska *et al*. (1998), Hamidoghli *et al*. (2007) and Khaleghi *et al*. (2008) also reported that a low concentration of NAA in a cytokinin-containing culture medium can induce the rooting of shooting rhizomes. The interaction of 2IP and NAA was significant on shoot height (p<0.01), with the highest shoots being observed in the medium containing 0.5 mg/l 2IP and 0.2 mg/l NAA. This is consistent with Khaleghi *et al*. (2008), Hamidoghli *et al*. (2007) and Podwyszynnska *et al*. (1998) who reported the influence of NAA on increasing shoot height in some other cultivars of *Alstroemeria*. 2IP also showed a significant impact on shoot height (p<0.01), while increasing 2IP concentrations resulted in low height shoots. The effect of 2IP on the number of regenerated buds and roots was not significant (p<0.05, Tab. 2).

Effects of glucose, asparagine, glutamine and casein hydrolysate on regeneration of rhizome explants

The results showed that the employed medium supplements had different effects on regeneration of shoots and roots. Glucose, as a replacement for sucrose, significantly increased the number of regenerated shoots and roots (5.10 and 5.50, respectively) compared to the control (4.16 and 4.60, respectively) (p<0.05) (Tab. 3). In contrast, addition of glutamine, asparagine and casein hydrolysate had a negative influence on the number of regenerated shoots and roots compared to the control (Tab. 3). Glutamine and asparagine supplementation increased the height of regenerated shoots. Glucose, at a concentration of 30 g/l, increased the frequency of rooting at *Asparagus officinalis* (L.) (Shigetaa *et al*., 1996). The present results also suggest a positive impact of glucose on the number of regenerated *Alstroemeria* roots. Chalupai and Durzan (1973), supplementing the culture medium of conifers with glutamine and asparagine, reported no significant increase in fresh weight or axis' length. Addition of organic nitrogen sources such as casein hydrolysate and glutamine, reduced the number of regenerated roots and shoots, and

increased shoot length which was consistent with the results reported by Anis *et al*. (2003) in mulberry tissue culture experiments.

Conclusions

A main objective of commercial plant micropropagation is rapid regeneration with a higher number of shoots and roots. In these experiments, the best medium for regeneration of rhizome tip explants of 'Caralis' *Alstroemeria* proved to be MS with 1 mg/l BA, 0.2 mg/l NAA and 30 g/l glucose (substituting sucrose).

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