Research Article

Effect of Antioxidants, Amino Acids and Plant Growth Regulators on *in vitro* Propagation of *Rosa centifolia*

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Background: *Rosa centifolia*is commercially propagated by asexual means but *in vitro* propagation ensure the production of disease free and healthy plants and browning of explants creates hurdle in their multiplication.
Objectives: The aim was to reduce oxidative browning of shoots of *R. centifolia* in MS medium during *in vitro* propagation.
Materials and Methods: Axillary buds of *R. centifolia* were sterilized with 70% ethyl alcohol for 4 min and 5% sodium hypochlorite for 2 min followed by three washing with sterilized double distilled water. In order to control oxidative browning, Ascorbic acid (100 $mg.L^{-1}$), citric acid (100 $mg.L^{-1}$) and activated charcoal (3 $g.L^{-1}$) were used while to control withering of shoots, different concentrations (3.0 $mg.L^{-1}$, 6.0 $mg.L^{-1}$, 9.0 $mg.L^{-1}$) of either glutamine, asparagine and proline were put into trial. Different concentrations of Benzyl aminopurine (BAP) and naphthalene acetic acid (NAA) were used for *in vitro* shoot and root formation.
Results: Minimum browning percentage (20%) was achieved in the presence of activated charcoal (3.0 $g.L^{-1}$) and pretreatment of explants with running tap water. Asparagin (9.0 $mg.L^{-1}$) produced maximum shooting (93%), minimum withering (6.67%), and it took longer period (27 days) for shoots to wither. BAP (3.0 $mg.L^{-1}$) + NAA (0.5 $mg.L^{-1}$) was produced the highest number of shoots (1.63), in a shortest periods (9 days). For root production, NAA (1.5 $mg.L^{-1}$) + BAP (0.5 $mg.L^{-1}$) reduced the time to 11 days with maximum number of roots (4.33) and root length (4.20 cm).
Conclusions: The supplement of activated charcoal (3.0 $g.L^{-1}$), a sparagin (9.0 $mg.L^{-1}$) and combination of BAP and NAA in the MS medium is effective for *in vitro* propagation of *R. centifolia*.

Keywords: Activated charcoal; Browning; *In vitro* Propagation; *Rosa centifolia*

1. Background

Rose, a member of *Rosaceae*, has more than 150 species and 1400 cultivars (1). *R. centifolia* is famous among oil producing species of roses with 4.25 tons per year around the globe (2). *R. centifolia* is commercially propagated by asexual methods that are sucker, hard wood cutting, semi hard wood cutting, budding and grafting. Propagation by vegetative methods does not ensure healthy and disease-free plants, as opposed to tissue culture (3). Furthermore, Propagation of *R. centifolia* through cutting is not very successful. This is considered as the main hurdle in oil industry that requires a huge amount of flowers. In roses, micro propagation of axillary buds is the method of choice, using varieties of growth regulators (4, 5).

All woody plants including roses excretes phenolic compounds from their tissues which may cause browning of the media (6) that can result in cessation of growth or development, and ultimately death of the explants. Browning is one of the important factors, which limits the growth of rose's *in vitro* culture. Browning can be controlled by adding substances such as pharmaceutical grade polyvinyl pyrolidone (PVP), ascorbic acid and activated charcoal (7).

Here, antioxidants, amino acids and plant growth regulators were used to address tissue browning of *R. centifolia* and to develop a protocol of its *in vitro* propagation. Eventually, oil industry based on *R. centifolia* will benefit from such attempts.

2. Objectives

Focusing on problems related to propagation of *R.*

centifolia, the present study was conducted to overcome problems of oxidative browning and withering that occurred during *in vitro* propagation by using antioxidants and amino acids.

3. Materials and Methods

3.1. Preparation of Explants and MS Medium

All three steps of this study were conducted in Plant Tissue Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. The axillary buds were used as the explants and were collected from field grown healthy plants of *R. centifolia*. The explants were first washed with tap water and disinfected sequentially with 70% (v/v) ethyl alcohol for 4 min and 5% (v/v) sodium hypochlorite for 2 min followed by three times rinsing with sterilized double distilled autoclaved water. The explants were cultured on MS medium (8), containing 8 $mg.L^{-1}$ agar and 30 $g.L^{-1}$ sucrose with vitamins (Inositol (100 $mg.L^{-1}$, Nicotinic acid (0.5 $mg.L^{-1}$) and Thiamine HCl (0.5 $mg.L^{-1}$). Test tubes were maintained at 25ºC with 16 h photoperiod.

3.2. Treatments for Control of Browning

Ascorbic acid, citric acid and activated charcoal were used to control browning. Explants were divided in three sets, the first set of explants was washed with running tap water for 1 h and placed in medium containing activated charcoal (3 $g.L^{-1}$) while second and third sets of explant were pretreated by stirring in ascorbic acid (100 $mg.L^{-1}$) and citric acid (100 $mg.L^{-1}$) respectively for 1hand placed in simple medium (without activated charcoal).

3.3. Treatments for Control of Withering

Axillary buds were placed under running tap water for 1 h, cultured in the MS medium containing 3.0 $g.L^{-1}$ activated charcoal, supplemented with different concentrations (3.0 $mg.L^{-1}$, 6.0 $mg.L^{-1}$, 9.0 $mg.L^{-1}$) of glutamine, asparagin or proline to control withering of explants after shoot formation. When shoots reached to the height of about 2 cm, numbers of healthy shoots/withering% were recorded.

3.4. Shoot and Root Induction

Axillary buds were washed under running tap water for 1h and cultured on the MS medium containing activated charcoal (3 $g.L^{-1}$), 0.09 $mg.L^{-1}$ of asparagin, BAP (1.5 or 3.0 mgL^{-1}) alone and in combination with NAA (0.5 $mg.L^{-1}$) for shoot formation. Data of number of days to initiate shoot, number of shoots, number of leaves and shoot length were recorded before transferring the shoots to rooting media.

After reaching 2 cm height, shoots were transferred into the MS medium supplemented with different concentrations of NAA (1.5 or 3.0 $mg.L^{-1}$) alone or in combination with BAP (0.5 $mg.L^{-1}$) for root induction. Number of days to induce roots, number of roots and root length were noted before shifting plantlets in small pots.

3.5. Acclimatization of Plantlets

Well-developed rooted shoots (15-20 cm in height) were transferred to square plastic pot containing peat moss, covered with polythene and placed in hardening room for acclimatization (27ºC,12 h photoperiod duration, 3000 foot-candles of light intensity). Plantlets were gradually acclimatized and exposed to greenhouse conditions.

All the experiments were conducted in completely randomized design (CRD). Each treatment had three replications and each replication had 12 explants. The treatment means were compared by applying the Least Significant Difference (LSD) test at 5% probability level.

4. Results

The lowest browning (20%) was observed in the explants were washed under running tap water for 1 h and cultured in MS medium supplemented with activated charcoal (Figures 1 and 2). Pre-treatment of explants with ascorbic acid and citric acid solutions produced 40% and 33.33% browning, respectively. Maximum browning (93.33%) was noticed in control (Figure 1). Withering of explants started after 27 days in medium supplemented with 9.0 $mg.L^{-1}$ asparagin (Table 1 and Figure 2). MS media without any amino acid was the least effective to stop withering; it took10

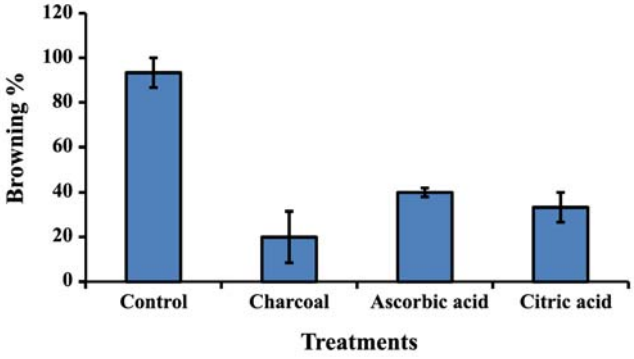

Figure 1. Effect of different treatments on browning of the MS medium

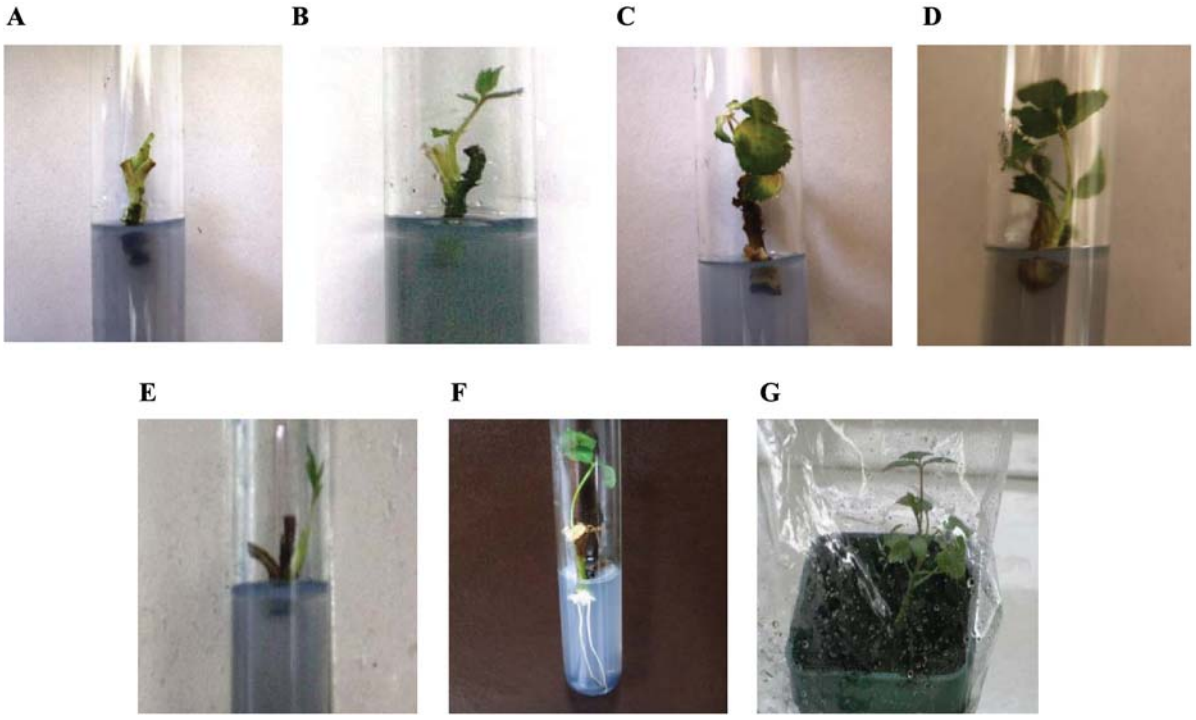

Figure 2. A: Browning of axillary buds in control MS medium, B: Browning of axillary buds in 3 g.L^{-1} activated charcoal, C: Withering of *in vitro* shoot in control, D: Withering of in vitro shoot in 0.09 mg.L^{-1} Asparagin, E: *In vitro* Shoot formation, F: *In vitro* Root induction, G: Acclimatization

days. After which, glutamine (6.0 mg.L^{-1}) ranked second least effective (12 days). The least withering (6.67%) was observed in the presence of 9.0 mg.L^{-1} asparagin (Table 1). The highest withering (93.33%) was observed in media with no amino acid (Table 1).

Asparagin at 9.0 mg.L^{-1} produced maximum healthy shoot (93.33%) (Table 1). Minimum healthy shooting (6.67%) was observed in the MS medium without amino acid followed by glutamine 3.0 mg.L^{-1} (20.00%) and 6.0 mg.L^{-1} (26.67%).

BAP in combination with NAA had significant effect on the presented variable results for shoot initiation (Table 2 and Figure 2). BAP (3.0 mg.L^{-1}) in combination with (0.5 mg.L^{-1}) NAA took minimum days (9) to produce shoots, while the MS medium without growth regulators took maximum days (17) to emerge shoots.

BAP (3.0 mg.L^{-1}) along with 0.5 mg.L^{-1} NAA formed maximum (1.63) number of shoots followed by BAP 3.0 mg.L^{-1} which produced 1.30 numbers of shoot (Table 2). MS medium without any plant growth regulator produced minimum (0.80) number of shoots. The highest number of leaves (2.63) was produced in the MS medium supplemented with BAP 3.0 mg.L^{-1} in combination with 0.5 mg.L^{-1} NAA. The MS medium without any growth regulator produced minimum (1.8) number of leaves. Maximum shoot length (5.9 cm) was recorded in the MS medium containing BAP 3.0 mg.L^{-1} in combination with 0.5 mg.L^{-1} NAA (Table 2). While BAP (3.0 mg.L^{-1}) alone and BAP (1.5 mgL^{-1}) along with 0.5 mg.L^{-1} NAA produced 4.07 cm and 4.27 cm shoot length, respectively. The MS without

Table 1. Effect of amino acids on withering control of explants of *Rosa centifolia*

Treatments (mg.L^{-1})		Withering (%)	No. of days to start withering	Healthy shooting (%)
Control	0	93.33 A	10.11 E	6.67 D
Glutamine	3.0	80.00 AB	25.11 AB	20.00 CD
	6.0	73.33 AB	11.56 DE	26.67 CD
	9.0	66.67 AB	22.22 ABC	33.33 CD
Asparagin	3.0	66.67 AB	20.11 ABC	33.33 CD
	6.0	33.33 CD	22.56 ABC	66.67 AB
	9.0	6.667 D	27.44 A	93.33 A
Proline	3.0	60.00 BC	17.00 CDE	40.00 BC
	6.0	53.33 BC	18.89 BCD	46.67 BC
	9.0	34.40 BC	18.90 BCD	47.20 BC
LSD value P≤0.05		30.16	7.85	30.16

Means with same letters are considered as non-significant

Table 2. Effect of plant growth regulators for *in vitro* shoot and root induction of *Rosa centifolia*

Treatments (mg.L^{-1})		No. of days to initiate shoots	No. of shoots	No. of leaves	Shoot length (cm)	Root length (cm)	No. of roots	No. of days to initiate roots	Treatments (mg.L^{-1})	
BAP	NAA								NAA	BAP
Control									Control	
1.5		17.03 A	0.80 C	1.80 B	3.83 D	3.26 C	2.00 C	17.10 A	1.5	
3.0		16.53 A	1.07 BC	2.03 B	3.97 CD	3.27 C	2.03 C	17.07 A	3.0	
1.5	0.5	15.47 B	1.30 AB	2.00 B	4.07 BC	3.43 BC	2.13 C	16.40 A	1.5	0.5
3.0	0.5	16.30 AB	1.00 BC	2.23 AB	4.27 B	4.20 A	2.63 B	11.73 C	3.0	0.5
LSD value		9.23 C	1.63 A	2.63 A	5.90 A	3.70 B	4.43 A	14.87 B		
P≤0.05		1.57	0.35	0.34	0.47	0.24	0.37	1.84		

Means with same letters are considered as non-significant

any growth regulator produced minimum (3.83 cm) shoot length. BAP (1.5 mg.L^{-1}) produced least (3.97 cm) shoot length.

NAA (1.5 mg.L^{-1}) along with BAP (0.5 mg.L^{-1}) induced roots within minimum 12 numbers of days (Table 2), while the MS medium without growth regulator took maximum numbers of days (17). Maximum numbers of roots (4.43) were produced in the MS medium containing 3.0 mg.L^{-1} NAA in combination with 0.5 mgL^{-1} BAP. Minimum root number (2)was obtained in the MS medium without any growth regulator (Table 2). NAA alone at concentration of 1.5 and 3.0 mg.L^{-1} produced 2.03 and 2.13 number of roots, respectively. The MS medium supplemented with 1.5 mg.L^{-1} NAA in combination with 0.5 mg.L^{-1} BAP produced maximum root length (4.20 cm). Minimum root length 3.26 cm was observed in the MS medium without growth regulator followed by root length of 3.27cm in the MS medium supplemented with 1.5 mg.L^{-1} NAA.

5. Discussion

Browning of explant and withering of shoots are among the prominent problems faced during *in vitro* propagation of *R. centifolia* which were addressed successfully in this study.

MS medium supplemented with activated charcoal and explants placed in running water for 1 h was found effective for decreasing the browning percentage. Activated charcoal has very fine pores, larger surface area and volume to absorb the phenolic compounds, secondary metabolites that modulate plant development. Sharada *et al*. (9) found activated charcoal effective against oxidative browning in *Celastrus paniculatus* and *C. orchioides*, respectively.

Stirring of explants in solutions of ascorbic acid or citric acid was also found to be effective in the present study and results are supported by Wu and du Toit, (10) who successfully controlled browning by stirring of explants of *Proteacynaroides* in ascorbic acid and citric acid solution for 1 h.

We tested effect of different amino acids on axillary bud of *R. centifolia*. Asparagin at 0.09 mg.L^{-1} produced minimum withering percentage, maximum healthy shooting and took maximum days to show withering of shoots. Haroun *et al*. (11) noticed an increase in growth with the lower concentrations of asparagine and glutamine in the MS medium in *Phaseolus vulgaris*. Amino acids are very effective in plant growth because most of the nitrogen is bound up to them. During *in vitro* propagation, addition of amino acids provides primary fast source of nitrogen supply to plants as compared to inorganic nitrogen. Baker *et al*. (12) also observed the increase in formation and elongation of cell wall, and cell division due to the addition of amino acids.

Explant that took minimum days to produce shoot, produced more number of laterals shoots and leaves. These results are consistent with the findings of Carelli and Echeverrigaray, (4) who observed that BAP in combination with NAA is very effective for shoot initiation in roses.

Maximum numbers of shoots were produced in the MS medium supplemented with BAP along with NAA. Nak-Udom *et al*. (13) noted higher shoot multiplication in the MS medium supplemented with 3.0 mg.L^{-1} of BAP with lower concentration of NAA. Explants, which had more number of shoot, showed good growth in the rooting and potting media. Shoots with less number of laterals had maximum chance to die.

Higher concentration of BAP with lower concentration of NAA produced maximum number of leaves. The leaves that were remained in the shooting media

for a long period of time became dry and fell down on the media. Chu *et al*. (14) also observed that plantlets began to exhibit chlorosis in the 6th week of culture. Shoots with only one or two leaves had maximum chance to die. MS medium without any growth regulator proved the least effective for *in vitro* shoot formation of *R. centifolia*. Rout *et al*. (15) noticed that the addition of cytokinin into the MS medium improved the *in vitro* shoot multiplication of hybrid roses.

NAA in combination with BAP was found very effective for *in vitro* rooting of *R. centifolia*. These results are related with Sajid *et al*. (16) who found that NAA along with BAP is more effective for root formation. Explant cultured on the MS medium supplemented with higher concentrations of NAA alone and in combination with BAP also produced callus at lower end of explants. Baig *et al*. (17) observed callus on the explants cultured on the MS medium containing higher cytokinin concentrations.

Acknowledgements

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