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Original paper

Alleviation of shoot tip necrosis in in vitro propagation of Salvia santolinifolia, Boiss

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Abstract Shoots of Salvia santolinifolia can be regenerated and multiplied on MS medium containing 3.0 mg/l of BA. Shoot tip necrosis has been noticed to be the main preventive factor in *in vitro* shoots regeneration. The necrosis of shoots appeared with the yellowing of top leaves following burn of leaf and finally death of whole shoots. The intensity of shoots tip necrosis were found more in elongated shoots (5-7 cm) whereas shoots less than 5-7 cm the frequency of shoots tip necrosis were low. Various factors such as strength of the media, charcoal and different combination of nutrients have been investigated for reducing the occurrence of shoot necrosis. Shoots tip necrosis can be reduced (0-10 %) by increasing the level of calcium in combination with increased concentrations of Magnesium (Mg) Manganese (Mn) and Iron (Fe) of MS medium containing 3.0 mg/l of BA. Apart from calcium, subculture interval also reduced shoot tip necrosis still at low level. Transfer of shoots showing easily signs of necrosis to half strength MS medium supplemented with BA (3.0 mg/l). All the additive including Activity charcoal promoted shoot tip necrosis and inhibited shoots multiplication.

Keywords micropropagation, mineral salts, *Salvia*, Lamiaceae.

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Introduction

Family Lamiaceae is one of the most diverse and wide spread plant family in term of ethnomedicine and its medicinal value is mainly base on volatile oil concentration (N SARAC & A UGUR [1]. Many species of this family are used for the treatment of guina worm, itch, cough and are applied to wounds as poultice (SJ DENTALI & JJ HOFFMANN [2]). Species of Lamiaceae are well known for their antitumor phytochemicals (H GINDA & H KAKISAWA [3]). The genus *Salvia* is rich source of terpens and steroids (R MURDAD & al [4]). *Salvia* species have been widely use in traditional medicine as antibacterial and antituercular remedy (H GINDA & al [5]). Some of them have also been used as antitumor agent (H GINDA & H KAKISAWA [3]). *Salvia* is immensely important genus of the family Lamiaceae as for as medicinal significance is concerned. Among species of the genus, *Salvia cavaleriei* is used for the treatment of dysentery, haemoptysis, boils and fall injuries (H-J ZHANG & L-N LI [6]). *Salvia apiana* called white sage, is used as a diaphoretic and diuretic and externally as a skin wash. The leaves are burnt as an aromatic smudge (SJ DENTALI & JJ HOFFMANN [2]). Extract of the intact plant of *Salvia santolinifolia* showed anticancer activities (Z AMIRGHOFRAN & al [7]). *Salvia multiorrhiza* is used for the cure of coronary heart diseases, myocardial infraction and hypertension [JMF WAN & al [8]).

The success of plant tissue culture is highly influenced by the growth regulators and nutrition supplied in the media (R MURDAD & al [4]; FA OZDEMIR [9]). Best shoot proliferation of *Covolvulus galaticus* was obtained with TDZ and IAA (AU TURKER & YB ARZU [10]). Various kinds of organic additives have been used in plant tissue culture to promote the growth of the plants including coconut water, banana pulp, potato homogenate and juice, honey, date palm syrup, papaya extract and also beef extract (R MURDAD & al [4]). Organic additives help in producing more protocorm like bodies, shoots and leaves (S AKTER & al [11]). Shoot tip necrosis is the main problem in the effective propagation of certain species by tissue culture. The first signs of shoot tip necrosis are browning of buds and the youngest leaves. The first possibility of shoot tip necrosis is that it is produced by nutrient shortage. The symptoms of nutrient deficiency of less mobile elements such as calcium and boron (JA RAVEN [12]) first appear in the meistematic regions and young leaves whereas symptoms of excessive amounts of these minerals are first observed on the older leaves (M BARGHCHI & PG ALDERSON [13]).

The main problem with this species in the *in vitro* culture is the browning of tip of the longest shoots. The aim of this study was to investigate the effect of frequent subculture, Ca, Mg, Mn & Fe and different additives on shoot tip necrosis to facilitate rapid multiplication and the *in vitro* propagation of *Salvia santolinifolia*. This is the first study to the best of our knowledge on controlling of shoot tip necrosis in *Salvia santolinifolia*.

Material and Methods

Healthy branches (5-12 cm long) were excised from mature plant of *Salvia santolinifolia* and sterilized with 0.05% Mercuric Chloride solution containing few drops of Tween-20 for 10-13 minutes. Murashige and Skoog medium was used for the induction of shoots supplemented with N^6benxyladenin (BA) (3.0 mg/l). pH was adjusted to 5.5 to 5.55 and 0.6% agar (agar-agar Mikrobiologie, Merck, USA) was used as solidifying agent.

Modified media: Morashige and Skoog's (MS) medium was modified by adding increased level of different salts;

MS_1 medium contained 2 time of Calcium chloride, Magnesium (Mg) Manganese (Mn) and Iron (Fe) of MS medium with 3.0 mg/l of BA.

MS_2 medium contained 2 time of Calcium chloride of MS medium with 3.0 mg/l of BA.

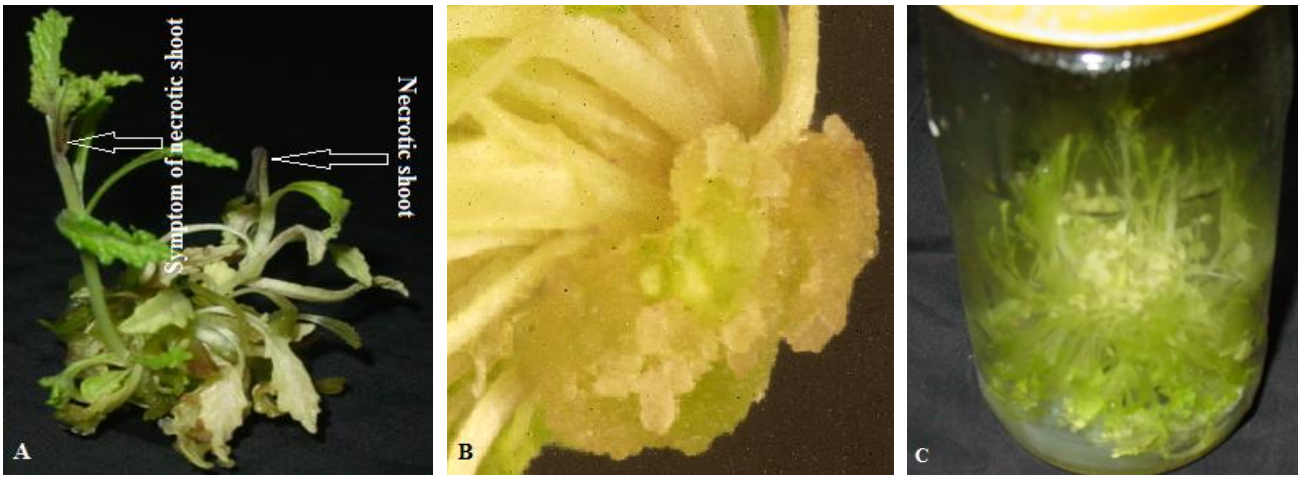
MS_3 medium contained 2 time of Magnesium (Mg) Manganese (Mn) and Iron (Fe) of MS medium with 3.0 mg/l of BA. The controlled plants were cultured on full strength MS medium.

The effect of the addition of additives on browning and multiplication of shoots: In order to enhance multiplication and control browning of shoots tips, different additives Casein Hydrolysate (200, 500 and 1000 mg/l), Adenine (50, 100 and 150 mg/l) and Coconut Milk (2.5%, 5.0% and 10%) were added to the MS medium containing BA (3.0 mg/l).

Culture condition: All cultures were maintained at 26±2 C°, under a light regime of 16 hrs day and 8 hrs nights. In the growth chamber light was provided from cool white fluorescent tubes.

Results

The symptoms of shoots tip necrosis appeared when the regenerated shoots reached to a height of 4-5 cm and increased in severity when reached to a height of 5-7 cm. The first visible symptom was the appearance of pale yellow colour in the apical region of terminal leaves after two weeks. The yellow (chlorotic) leaves of the apical region became brown/black and the browning/blackening gradually increased downward (basipetal) and finally died the apical shoots after three weeks of inoculation (Fig 1A). Subsequently by four weeks of culture the necrotic area enlarged and finally death of the whole shoots occurred. An enhance shoot tip necrosis was observed with the formation of callus tissues at the base of cultured explants (Fig. 1B). The restoration of normal tip is not possible once the shoots became necrotic they kill the whole explants even transferred on to fresh media.



Figue 1. *In vitro* growth of *Salvia santolinifolia*: A) Necrotic shoot & necorsis of apical shoot, B) Callus development at the base of explants and C) STN recovered in MS_2 medium.

Subculture and the intensity of shoot tip necrosis: Subsequent sub-culturing of the explants derived from the *in vitro* regenerated shoots increased the number of shoots and reduced the intensity of the shoots tip necrosis. Subculture at 2 weeks interval had the minimum (28.57%) incident of shoots tip necrosis compared to those sub-cultured at 4 weeks interval (57.50%). Subculture at 2 weeks interval reduced shoots tip necrosis along with increased in the multiplication of shoots (Table 1). The initial symptom of shoot tip necrosis give an indication of deficiency of immobile nutrients such as calcium which is further substantiated by our findings. Our results show the influence of calcium chloride on alleviating shoots necrosis. The increased concentration of calcium chloride effectively controlled the problem of necrosis and more than 85% shoots produced were without shoot tip necrosis (Table 2). Furthermore the multiplication of shoots index remained unaffected with increased concentration of calcium (Table 2).

Table 1. Shoots tips browning and the multiplication of shoots in 2-week and 4-week cultures under the influence of BA

Growth regulators	% of tip browning after		Mean numbers of shoots ±SE after		Mean length of shoots (cm) ±SE after	
	2-week	4-week	2-week	4-week	2-week	4-week
BA						
3.0	28.57	57.50	13.16±4.31	12.66± 3.85	3.41±1.73	4.33±1.58
3.5	31.66	55.55	10.25±2.45	11.85±4.21	3.18±0.89	3.05±1.34

Symbol; SE: Standard Error

Modified MS medium and Shoots tip necrosis: Regenerated shoots were transferred to fresh shoot multiplication media containing different strength (full and half) of MS medium, different modified MS media (MS_1, MS_2 and MS_3), activated charcoal and different additives. Recovery of necrotic shoots were not possible when transferred of shoots were done with sign of necrotic shoots.

Subculture of regenerated shoots to shoots multiplication media containing different strength of mineral nutrients tested for the inhibition of shoot tip necrosis half strength medium proved to be unsuccessful compared with full strength MS (Table 2).

To overcome the problem of tip necrosis, MS medium was modified by adding increased concentration of Calcium chloride individually (MS_1) or in combination with increased concentration of Magnesium (Mg) Manganese (Mn) and Iron (Fe) (MS_2) of MS medium plus 3.0 mg/l of BA. In an another experiment increased concentrations of Magnesium (Mg) Manganese (Mn) and Iron (Fe) (MS_3) of MS medium plus 3.0 mg/l of BA but Calcium chloride concentration was kept as in MS medium. High concentration of Calcium chloride individually (MS_1) was not suitable although they inhibiting shoot tip necrosis but the synergistic effect of Calcium chloride in combination with Magnesium (Mg) Manganese (Mn) and Iron (Fe) (MS_2) of MS medium with 3.0 mg/l of BA in the same medium facilitated 90% healthy shoots regeneration without shoot tip necrosis. The shoots that have been regenerated on increased level of Calcium chloride in combination with increased level of Magnesium (Mg) Manganese (Mn) and Iron (Fe) (MS_2) of MS medium plus 3.0 mg/l of BA in the same medium were again subculture

on their respective media. In the 2nd subculture multiplication of shoots index declined but length of shoot increased whereas shoot tip necrosis was controlled effectively (Table 2).The increased concentration of Magnesium (Mg) Manganese (Mn) and Iron (Fe) (MS_3) of MS medium with 3.0 mg/l of BA was not suitable an inhibiting shoot tip necrosis (Table 2).

Table 2. The effect of media composition on shoot necrosis of *Salvia santolinifolia*

Medium	Number of subculture	Growth regulator	Mean number of shoots ±SE	Mean length of shoots (cm) ±SE	Percentage of extent of necrosis (%)
MS_1	1s	BA 3.0	11.42±2.19	2.97±1.82	10-15
	2nd	BA **3.0**	9.28±1.79	3.24±2.34	5-15
MS_2	1st	BA 3.0	14.42±3.99	3.24±0.51	0-10
	2nd	BA 3.0	13.57±2.64	4.47±2.45	5-10
MS_3	1st	BA 3.0	12.85±2.79	3.66±1.31	30-40
$MS_{1/2}$	1st	BA 3.0	9.36±2.73	2.89±1.31	30-40
MS Control	1st	BA 3.0	17.62±5.08	4.81±1.86	30-40

Effect of additives and activated charcoal: The influence of different concentrations of additives (Casein Hydrolysate, Coconut Milk and Adenine) and activated charcoal was investigated in MS medium containing 3.0 mg/l of BA in order to alleviate shoot tip necrosis and enhance shoots multiplication. The addition of coconut milk at different concentrations (2.5, 5.0 and 10.0%) influence shoot tip necrosis and multiplication differently (Table 3). Lower concentration slightly inhibited shoot tip necrosis and increased shoot multiplication whereas higher concentrations proved unsuccessful and stimulated callus formation at the base of explants (Fig. 1B). The supplementation of Casein Hydrolysate and Adenine to the medium with 3.0 mg/l of BA inhibited shoots multiplication and promote shoot tip necrosis at all concentrations (Table 3). The transferred of shoots to the medium that contained activated charcoal (0.3 %) with BA (3.0 mg/l) completely inhibited shoots multiplication and elongation. After remaining for 15-20 days on the activated charcoal supplemented medium, stem and leaves of the transferred shoots gradually turned light green and then turned white and finally became dead Table 3).

Table 3. The effect of different concentrations of addition on necrosis and multiplication of shoots in MS media containing 3.0 mg/l of BA

Additive	Concentration of additive (mg/l)	Percentage of necrosis (%)	Mean number of shoots ± SD	Mean length of shoots (cm)±SD
Casein hydrolysate	200	90	5.0±2.65	2.17±1.34
	500	95	4.0±3.77	2.7±1.5
	1000	100	0.0	0.0
Coconut milk	2.5%	27	16.75±6.99	6.32±1.75
	5%	40	11.16±4.49	4.03±0.71
	10%	47	11.0±3.57	4.7±1.52
Adenine	50	85	4.0±2.1	3.77±1.75
	100	90	1.25±0.57	2.7±0.28
	150	92	2.0±0.15	3.5±0.14
BA+AC	0.3%	100	0.0	0.0

Discussion

In the present study induction and multiplication of shoots occurred rapidly in the presence of cytokinins (BA 3.0 mg/l) but tips of the elongated shoots become browned. Leaf burn was followed by the necrotic shoot tip and finally death of the shoots during shoot multiplication stage. Different workers instructed diverse explanations as the cause for shoot tip necrosis in another plant species: exudation of phenolics, prolong subculture (PG

ALDERSON & al [14]), drop of pH of the medium and deficiency of calcium (L. SHA & al [15]). The problem of shoot tip necrosis has also been reported in banana and Pistacia vera, (K.P. MARTIN & al [16]; A. ABAOU-SALIM & S. H. MARTELL [17]). Frequent sub-culturing at 2 weeks interval is more beneficial in the reduction of shoot tip necrosis than subculture at 4 four weeks interval in this study. Decline of necrosis by more frequent subculture has been demonstrated in *Prunus tenella* (PG ALDERSON & al [14]). In the present study shoot tip necrosis appeared due to nutrients deficiency. The increased concentration of Calcium chloride only or in mixture with other nutrients (Megnacium Manganese and Iron) in the media considerably reduced the problem of shoot tip browning. The use of increased concentration of Calcium chloride alone did not diminished shoot tip necrosis, but the percentage of necrosis was reduced to great extent. This may be due to chloride toxicity caused by the supraoptimal level of calcium chloride in the medium. Excess calcium may also produce deficiencies in magnesium and potassium. Although calcium can be present in mill-molar concentrations within the plants as a whole, calcium ions are pumped out of the cytoplasm of cells to maintain the concentration at around only 0.11 M. This removal of Calcium ion from the protoplasm is necessary to prevent the precipitation of phosphate and interference with the function of Magnesium ion (EF GEORGE [18]). Calcium as a component of cell walls, membranes and lignin, protects membranes from damage and thus maintaining cell integrity. Shoot tip necrosis by calcium deficiency has also been demonstrated in *Amelanchier, Betula, Populus, Sequoia and Ulmus* (L. SHA [15]). Mitigation of necrosis by increasing calcium in the medium as in the present study has been reported by (BH MCCOWN & JC SELLMER [19]) in tree species. The symptoms of hyperhidricy and necrosis in leaf of *Calathea ornate* was reduced with raising the level of Ca, Mg, Fe and Mn. Study has shown that increased level of Ca, Mg, Fe and Mn decreased the rate of multiplication (M PODWYSXYNSKA [20]).

In view of the beneficial effect of different additives on morphogenesis in different plant species, a range of concentrations of selected additives were incorporated in the media containing 3.0 mg/l of BA which was normally used for the multiplication of shoots of *Salvia santolinifolia*. Results of additives show that multiplication of shoots was more or less the same under the influence of most of the additives except Coconut Milk. The highest mean number of shoots produced per explants was 16.75±6.99 in the medium containing 2.5% Coconut Milk with 3.0 mg/l BA. Among the three concentrations of Coconut Milk, applied in combination with 3.0 mg/l of BA, the multiplication of shoots was highest in the presence of low concentration of Coconut Milk (2.5%). When concentration of Coconut Milk was increased shoots number was slightly decreased. The amount of the callus induced on Coconut Milk and BA supplemented medium was 2 fold greater than the one produced in the control. Callus was yellowish-white and was looking healthy. Coconut milk contains cytokinins as growth factors. The addition of Coconut Milk at higher concentrations might have rendered cytokinin concentration unsuitable for rapid multiplication of shoots.

Casein Hydrolysate is an undefined complex mixture of amino acids and serves as a rich source of organic nitrogen of amino acid origin. An enhanced multiplication of shoots was observed by (S. RAY & S. JHA [21]) by the addition of Casein Hydrolysate in *Withania somnifera*, however, in our study with *Salvia santolinifolia* Casein Hydrolysate decreased shoot multiplication. An overall depressed morphogenetic expression by the application of the entire additive may be due to an increase in osmotic pressure of the medium. The addition of Activated Charcoal to the shoot regeneration medium completely inhibited the formation of shoots (Table 3). It not only adsorbs toxic substances which are deleterious for the growth of culture but may also adsorb growth regulators. In this study Activated Charcoal might have adsorbed phytohormones making culture condition unsuitable for any morphogenesis to occur.

Conclusion

From the present study it is concluded that shoot tip necrosis of *Salvia santolinifolia* is due to nutrients (particularly calcium) deficiency. The increased level of calcium in combination with increased level of Mg, Mn and Fe of MS medium have been recommended for the *in vitro* multiplication and elongation of shoots of this species.

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