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Effect of Plant Growth Regulators on Callus Induction and Plantlet Regeneration of Bitter Apple (*Citrullus colocynthis*) from Stem Explant

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ABSTRACT

A protocol was developed for *in vitro* plantlet regeneration of *Citrullus colocynthis* using stem explants which was selected after screening of different seedling explants. Contamination free culture was established treating stem explants with 0.1% $HgCl_2$ for 3 min. Optimum sucrose concentration for callus formation was 3%. The role of different growth additives like pyridoxine and thiamine HCl was assessed in enhancing callus number. The maximum number of callus induction was achieved from stem explants on MS medium enriched with 0.5 mg L^{-1} IAA, 2,4-D and 1 ppm of 6-BA which yielded morphogenic compact hard greenish white calli at a frequency of 80% than compared to MS medium enriched with other combination of auxin and cytokinin. Compact, hard, white/greenish white callus was formed in different amount at all concentration after 4 weeks interval. Then the callus was transferred to the shooting medium containing different concentration of auxin and cytokinin (MS medium with 1.5 mg L^{-1} 6-BA and 0.5 mg L^{-1} NAA) showed better shooting. The regenerated shoots were further elongated on same medium. In vitro shoots were excised from shoot clumps and transferred to rooting medium containing 6-benzyl adenine (6-BA, 3.0 mg L^{-1}) with 0.2% activated charcoal. The rooted plants were hardened in polycups containing sterile soil and vermiculite and finally well established in the field.

Key words: Bitter apple, sand dune, greenish callus, stem, IAA

INTRODUCTION

Coastal sand dunes are formed by the sand deposited from sub tidal and intertidal regions. Wind is one of the most important factors, which help in formation, movements and distribution of sand dunes (Untawale and Nair, 1974). The sand dune vegetation has play a significant in coastal region (Barson and Calder, 1981). It helps in prevention of sand erosion by decreasing wind speed at ground level. The sand dune vegetation is totally a different plant community with remarkable ability to locate hostile environment of drought, nutrient deficiency high winds, salts sprays and sand blast (Desai, 1995). *Citrullus colocynthis* is cultivated for its edible fruits and seeds which are rich in oil and proteins (Esquinas-Alcazar and Gulik, 1983). The shoots developed from *Salvia canariensis* explants formed roots when transferred to half strength MS medium supplemented with lAA, NAA or IBA. Shoots were rooted most effectively in 1/2 MS medium supplemented with 1.0 mg L^{-1} IBA (Mederos-Molina, 2004). Among the different basal media (MS, B5, LS and Wl1ite's) and hormones (NAA, BA, KN, TDZ, Zeatin) tried in different combinations only MS media

supplemented with 2 mg L^{-1} BA and 2 mg L^{-1} IAA responded best for plant regeneration of cotton (Tripathy and Reddy, 2002). High frequencies of multiple shoot regeneration were achieved from nodal explants of *Zehneria scabra* on MS fortified with 5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA. The elongated shoots were subcultured for rooting on MS supplemented with 2 mg L^{-1} NAA (Anand and Jeyachandran, 2004). The excised shoot tips of *Cucurbita maxima* were cultured on MS medium containing Kn, BA, NAA at various levels of concentration and combination for shoot induction and proliferation and best response was found at 3.0 mg L^{-1} of BA (Mahzabin *et al*., 2008). The maximum frequency of adventitious shoot regeneration was obtained when cotyledon explants of bottle groud were cultured onto MS medium with 10iM BA (Han *et al*., 2004). The sand dune plant *Citrullus colocynthis* belong to the family of cucurbitaceae are purgative and used for the treating mamilities, jaundice and urinary disease. Furthermore, in several accessions of this species resistance to various diseases has been identified (Hassan *et al*., 1991). Antimicrobial and anti inflammatory effect of *Citrullus colocynthis* showed potential therapeutic herbs reported in our laboratory (Gurudeeban *et al*., 2010; Rajamanickam *et al*., 2010). The antidiabetic effect of *Citrullus colocynthis* had countless possibilities for investigation and searching for noval and more effective therapeutic compounds (Gurudeeban and Ramanathan, 2010). The callus induction of cucurbitaceous crop (*Momordica dioica* Roxb.) was most suitable in combination 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA followed by 0.2 mg L^{-1} NAA in Tease gourd. In these out of 4 types of explants such as node, shoot tip, leaf and the cotyledon, the cotyledon showed the best performance (Nabi *et al*., 2002). Now several important advances have been made within the past several years. Auxiliary buds have been induced to produce multiple shoots in cucumbers (*Cucumis sativus*) and watermelons (*Citrullus lanatus*) and both organogenesis and embryogenesis have been accomplished from several tissue sources of cucurbits. The effects of various concentration and combinations of 2, 4-dichlorophenoxyacetic acid (2, 4-D), 6-benzylaminopurine (6-BA) and alpha-naphthalene Acetic Acid (NAA) on regeneration of rough lemon (*Citrus jambhiri* Lush.). The importance of developing tissue culture methods for *Citrullus colocynthis* to facilitate large scale production of true type plants and for therapeutics potential.

MATERIALS AND METHODS

Source of explants: *Citrullus colocynthis* were freshly collected from the southeast coast of Parangipettai (Tamil Nadu) India during November 2009 and kept under shade net (50%) house environment. The specimen was certified by Botanical Survey of India (BSI) Coimbatore and by the Herbaria of C.A.S. in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu and India.

Surface sterilization: The stem was used as explants material for initiating the organogenic calli. The explants were surface sterilized with 70% (v/v) ethyl alcohol for 1-5 min followed by 0.1% $HgCl_2$ for 3 min. The explants were then washed 4 times with sterile distilled water to remove traces of $HgCl_2$. The experimental chemicals were purchased from (Hi-Media, Mumbai).

Plantlet regeneration: The stem (10 mm) explants were cultured for callus induction in 250 mL conical flask and 25 mL Petri plates containing Murashige and Skoog medium (MS) supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar and various concentration of cytokinin such as 6-BA and auxin such as 2,4-D and IAA (Table 2). Control cultures initiated on MS medium with growth regulators. The pH was adjusted to 5.8 prior to the addition of agar. Media were autoclaved at 15 lbs for

15 min. Culture was incubated at 25±2°C and relative humidity of 60±10%. An eight hour photoperiod (16 h dark) with light intensity of 20-30 μmol/m/sec was provided by cool day light fluorescent tubes. The maximum number of callus induction was achieved from stem explants on MS medium enriched with 0.5 mg L^{-1} IAA, 2,4-D and 1 ppm of 6-BA which yielded morphogenic compact hard greenish white calli and transferred to the shoot induction medium. Multiple shoots obtained were transferred to elongation medium supplemented with different growth regulators. The cultures were regularly subcultured on fresh medium at four week intervals and observation was recorded. Elongated and healthy plantlets were transferred to rooting medium containing 3.0 mg L^{-1} concentration of 6-benzyl adenine with activated charcoal (0.2%). Regeneration plantlets were transferred to pots containing sterilized soil and vermiculture (3:1). The plantlets after 10-15 days in green house; they were then placed in the normal environment for 1 h and assessed for sighs of wilting. The exposure was increased daily until the plants were established fully under normal environmental conditions; they were then transferred into the field for normal growth. Experiments were set-up in completely randomized design. Each treatment had 10 replicates. Significance of the treatment effects was determined using analysis of variance (ANOVA, $p \leq 0.05$) and comparison between mean values of treatments were made by Tukey's test. All statistical analysis were performed using the software SPSS (version 14, USA).

RESULTS

Sterilization procedure for explants: To overcome contamination problem surface sterilization of explants was done with 0.1% $HgCl_2$ for different duration to assess the contamination percentages and viability of the explants used for *in vitro* culture. Contamination free cultures with elegant survivability (100%) were achieved by treatment the explants with 0.1% $HgCl_2$ for 3 min (Table 1).

Effect of 2, 4-D and IAA together with 6, BA on callus induction and plantlet regeneration: The cumulative work on plant tissue culture revealed that the ability of callus formation depends on donor tissue and influenced by type of growth regulator and their concentration in the nutrient medium. Similarly in *Citrullus colocynthis*, the formation of callus depending upon the type of combination and concentration of auxin and cytokinin (Table 2). Addition of high level of 6-BA (1 ppm) in combination with Auxin (2, 4-D and IAA) induced callus formation from the cut end of the explants. In the second and third week of culture entire explants was covered with callus and the size of the callus increased with time surrounded by a creamy friable mass of cells was formed. Different forms of calli such as compact, hard greenish white, yellowish green and green callus shows in (Fig. 1a-e), respectively. Among different concentrations used, best response towards shoot

Table 1: Standardization of $HgCl_2$ treatment period for surface sterilization of the explants

Treatment duration (min) with 0.1% $HgCl_2$	No. of explants	Role of contamination (after days of treatment) 3	6	9	12	15	% of contamination free explants after 15 days
1	10	-	5	7	8	10	-
2	10	1	4	6	6	7	30
4	10	-	1	1	3	3	80
6	10	-	-	-	-	-	100
8	10	-	-	-	-	-	100
10	10	-	-	-	-	-	100

-: Indicates no contamination

Table 2: Effect of different concentration of 6-BA in the combination with IAA, 2, 4-D for callus induction from *Citrullus colocynthis* stem explants. There were ten explants for each treatment and data were taken after three weeks of culture

Growth hormones concentration (mg L^{-1}) (IAA+ 2,4-D+6-BA)	Degree of callus formation	Callus character
0.5+0.25+0.25	+	Compact, loose, green
0.5+0.25+0.5	++	Compact, loose, white
0.5+0.5+0.25	++	Compact, loose, yellowish green
0.5+1.0+0.5	++	Compact, hard ,white
0.5+0.5+1.0	+++	Compact, hard, greenish white

+++: Indicates excellent degree of callus, ++: Indicates good degree of callus, +: Indicates poor degree of callus

Table 3: Effect of plant growth regulators on shoot formation from stem explants of *Citrullus colocynthis* on MS medium

	Plant growth regulators (mg L^{-1})			
S. No.	6-BA	Kn	NAA	No. of shoots per explants
1	0.5			6.5±0.226
2	1.0			7.56±0.021
3	1.5			9.38±0.246
4	2.0			11.9±0.412
5		0.5		1.9±0.359
6		1.0		5.8±0.672
7		1.5		2.2±0.180
8		2.0		5.6±0.336
9	0.5		0.5	16.3±0.276
10	1.0		1.5	12.6±0.756
11	1.5		0.5	20.0±0.180
12	2.0		2.0	8.7±0.549

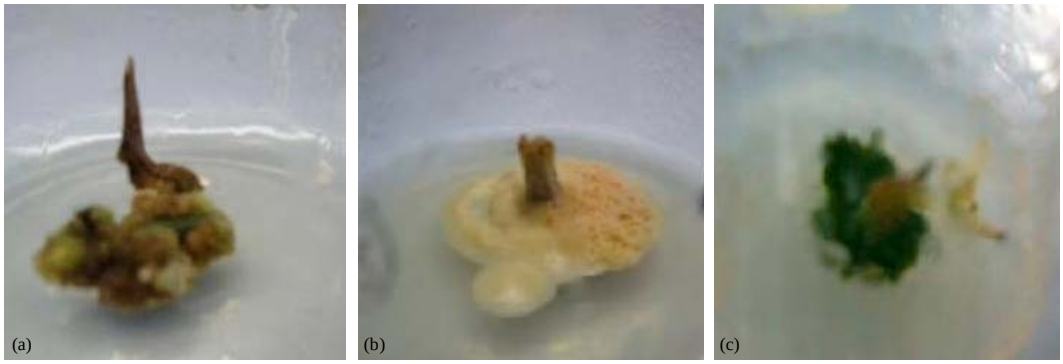

Fig. 1: Different forms of calli from stem explants of *Citrullus colocynthis* (a) compact, hard greenish white, (b) yellowish green and (c) green callus

proliferation from stem explants was obtained on MS medium with 6-BA (1.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) (Table 3). Shoots elongated on the same medium, from the results it is clear that a combination of 6-BA and NAA was suitable for shoot multiplication as well as shoot elongation. Root formation is an energy demanding process and thus, exogenous supply of carbohydrates is required. In vitro shoots were excised from shoot clumps and transferred to rooting medium containing 6-benzyl adenine (6-BA, 3.0 mg L^{-1}) with 0.2% activated charcoal (Fig. 2a-c; Table 4).

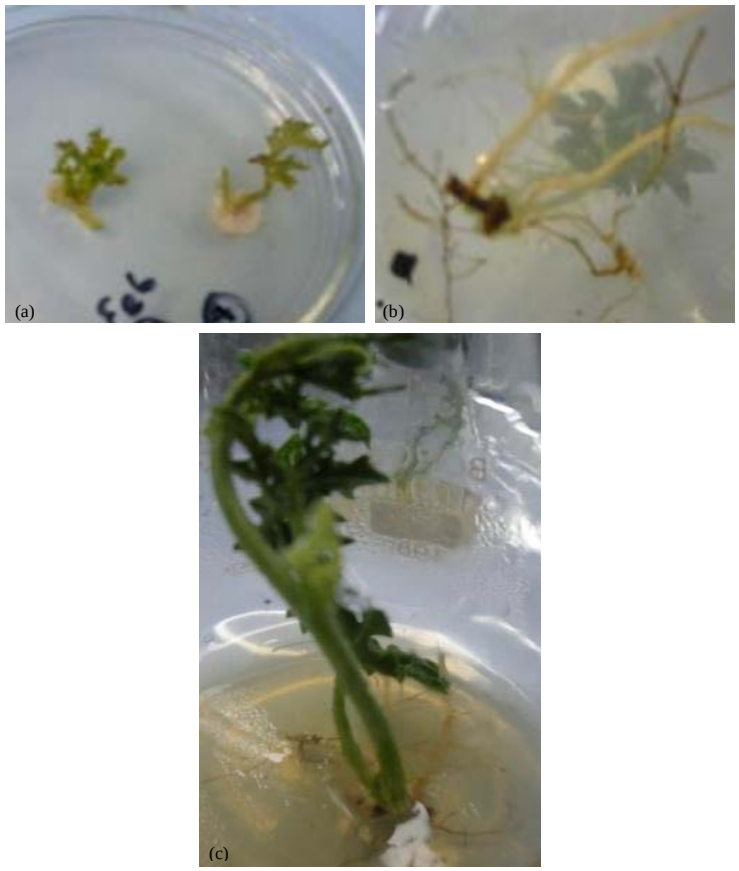

Fig. 2: *In vitro* plant regeneration of *Citrullus colocynthis* from stem eplant (a) shoot induction (MS medium with 1.5 mg L^{-1} 6-BA and 0.5 mg L^{-1} NAA), (b) root induction (MS medium with 6-benzyl adenine 3.0 mg L^{-1} and 0.2% activated charcol) and (c) plantlet regeneration

Table 4: Effect of auxin (6-BA) along with 0.2% of activated charcoal on root induction shoots of *Citrullus colocynthis* after 4 week of culture

6-BA (mg L^{-1})	% of cutting of rooted	No. of shoots per roots
1	75	4.62±0.23
2	65	7.5±0.860
3	70	15.08±0.12
4	85	11.32±0.15
5	80	10.26±0.34

Values are Mean±SE from 5 replicates in each treatment

However, this being the last stage of *invitro* culture, it is important to transform the plant from heterotrophic to autotrophic mode of nutrition. Thus, the supply of exogenous sugar should be reduced at this time. The rooting response differed according to different concentration and combinations of hormones.

DISCUSSION

Citrullus colocynthis the maximum number of callus induction was achieved from stem explants on MS medium enriched with 0.5 g L^{-1} IAA, 2, 4-D and 1 ppm of 6-BA which yielded

organogenic calli than compared to MS medium enriched various concentration of hormones. But in *Momordica cymbalaria* plant the inclusion of higher concentration of IAA, NAA and 2,4-D (5.0 μM) together with BA (2.5 μM) or KN (10.0 μM) placed an important role in the proliferation of callus from the leaf explants (Nikam *et al*., 2009). In compared to the cotyledon explants of *Citrullus colocynthis* the addition of NAA caused a significant decrease in the frequencies of organogenic calli and calli with shoots compared to the media without auxin. The best results were obtained in the medium containing only 25 μM 6-BA where 81.7% of the calli were organogenic. The effective role of NAA in combination with BAP for the induction of multiple shoots has been reported in *Basilicum polystachyon* (Chakraborty and Roy, 2006), *Musa sapientum* L. (Kalimutha *et al*., 2007), *Rauwolfia serpentine* (Baksha *et al*., 2007), *Citrullus colocynthis* (Meena and Patni, 2007). The effect of NAA on the regeneration efficiency from cotyledon explants of *Citrullus colocynthis* was detrimental as also described for the watermelon cultivar Melitopolski by Srivastava *et al*. (1989). According to Compton and Gray (1993) that the addition of IAA (0.5-5 μM) to the media with 6BA (5-20 μM) inhibited shoot formation and increased callus production from cotyledons of diploid, triploid and tetraploid watermelon. The effect of 6-benzylaminopurine (6-BA) alone or in combination with naphthalene acetic acid or indoleacetic acid on the morphogenetic response of cotyledon explants of *Citrullus colocynthis* was tested by Dabauza *et al*. (1997). The best results were obtained with a medium containing 25 μm 6-BA, which yielded organogenic calli at a frequency of 81.8%. When these organogenic calli were transferred to elongation medium (basal medium supplemented with 0.5 μm 6-BA), 80% produced well-developed shoots. A high frequency and rapid regeneration protocol was developed from shoot tip explants of *Citrullus colocynthis* on Murashige and Skoog (1962) (MS) medium supplemented with N6-benzylamino-purine (BAP, 0.5 mg L^{-1}) and α-naphthalene acetic acid (NAA, 0.5 mg L^{-1}) (Meena and Patni, 2007). In contrast to the above mentioned results, some researchers observed that the combination of BAP and IAA on MS-medium favoured multiple shoot buds in *Capsicum annuum* (Sobhakumari and Lalithakumari, 2003) and *Acalypha wilkesiana* (Sharma *et al*., 2007). Combination of cytokinins also favoured multiple shoot proliferation in *Ocimum sanctum* (Girija *et al*., 2006) *C. annuum* (Rao *et al*., 2006) and *Amygdalus communis* (Akbas *et al*., 2009).

CONCLUSION

In conclusion, we have demonstrated a simple and efficient protocol for calli induction and plantlet regeneration of *Citrullus colocynthis* from stem explants with different combinations of auxin and cytokinin. The maximum number of calli induction was achieved from stem explants on MS medium enriched with 0.5 g L^{-1} IAA, 2,4-D and 4 mg L^{-1} of 6-BA which yielded organogenic compact hard greenish white calli at a frequency of 80% than compared to MS medium enriched with other combination of auxin and cytokinin. MS medium with 1.5 mg L^{-1} 6-BA and 0.5 mg L^{-1} NAA showed better shooting. The regenerated shoots were further elongated on same medium. In vitro shoots were excised from shoot clumps and transferred to rooting medium containing 6-benzyl adenine (6-BA, 3.0 mg L^{-1}) with 0.2% activated charcoal. Using this protocol, it is possible to clonally produce viable, uniform and healthy plants with maximal survival rate that can be used for large scale cultivation, genetic transformation studies, nanotechnology and pharmacological applications.

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