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Adventitious Rooting and Proliferation from Different Explants of *Citrullus colocynthis* (L.) Schrad an Endangered Medicinally Important Cucurbit

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ABSTRACT

An efficient procedure has been developed for induction of adventitious rooting and proliferation from different explants of *Citrullus colocynthis*. Leaf, stem, cotyledon and hypocotyledon explants cultured on different concentration of auxins and cytokinins, were supplemented on MS medium to test their ability to induce rhizogenesis. Murashige and Skoog (MS) medium fortified with 2,4-dichlorophenoxyacetic (2,4-D), indoleacetic acid (IAA), Indole-Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA). Each at the concentrations of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹ were evaluated for their effects on adventitious root induction in the present study. High concentration of IAA (2.0 mg L⁻¹) and low concentration of IBA (1.5 mg L⁻¹) is best for induction of rhizogenesis. High concentration of IBA favored formation of malformed and thick roots. The NAA in combination with IBA is most potential in inducing high percentage of rooting (50%) and IAA in combination with IBA induced maximum rooting 62% with highest number of roots per shoot (3.80±0.2) and root length (3.8±0.1 cm) were obtained from leaf, cotyledon and stem than hypocotyls. Results revealed that, the best rooting ability was observed from in stem explants cultured on MS medium supplemented with 2.0 mg L⁻¹ IAA and 1.5 mg L⁻¹ IBA, multiple roots fibrous were produced from cotyledon explants on MS fortified with 2.0 mg L⁻¹ 2,4-d and multiple roots cluster produced from leaf explants 1.5 mg L⁻¹ IBA and 2.0 mg L⁻¹ IAA. A distinct feature of this study is the adventitious rooting and proliferations from different explants of *Citrullus colocynthis* which has not been reported previously.

Key words: *Citrullus colocynthis*, plant growth regulators, rhizogenesis, auxins, cytokinin, adventitious roots, endangered and explants

INTRODUCTION

The *Citrullus colocynthis*, also known as bitter apple, bitter cucumber, egusi, is a vine plant mainly found in Mediterranean Europe, Asia, Turkey, Nubia, Trieste, Egypt, Iran, Pakistan, Afghanistan, India and North Africa. It mainly contains glycosides, cucurbitacins (colocynthin and colocynthetin), cucurbitacins A, B, C, D and E (á-elaterin) (Adam *et al.*, 2001), cucurbitacins E, I, J, K and L (Sturm *et al.*, 2009), cucurbitacin glycosides (Hatam *et al.*, 1981; Seger *et al.*, 2005; Abbas *et al.*, 2006), flavonoids and flavone glycosides (Maatooq *et al.*, 1997; Abbas *et al.*, 2006). It is a proven antioxidant, antimicrobial, antimalarial, hepatoprotective, antispermatogenic and carcinogenic. The fruits are traditionally used against poisonous bites of dogs, snake bites and also used as an enema (Warrier, 1997). Tannin-Spitz *et al.* (2007) and others studied the effects of

cucurbitacin glucosides extracted from *Citrullus colocynthis* leaves and roots on human breast cancer cell growth. Leaves and roots were extracted, resulting in the identification of cucurbitacin B/E glucosides. The cucurbitacin glucoside combination (1:1) inhibited growth of ER(+) MCF-7 and ER(-) MDA-MB-231 human breast cancer cell lines. The aqueous roots extract of *Citrullus colocynthis* showed significant reduction in blood sugar level when compared with standard groups ($p < 0.01$) (Agarwal *et al.*, 2012).

The phenomenon of root formation is called rhizogenesis. Root initiation is a type of organogenesis (Tabei *et al.*, 1991). Root growth does not always occur in the earlier stages in plant cell culture and is of course a requirement for successful plant growth after the micropropagation procedure. The ability of shoots to initiate root or plants to survive acclimatization was dependent on the concentration of cytokinins and auxins on MS medium (Gamborg *et al.*, 1974; Chlyah and Van, 1975; Dudits *et al.*, 1975). The developments of *in vitro* rooting have three stages (a) Induction, (b) Initiation and (c) Elongation. The MS media supplemented with IBA, NAA, IAA and 2,4-D are the best medium for both shoot and root proliferation with no verification. Combination of cytokinins and auxins in higher concentrations can induce adventitious shoot formation but root formation is generally inhibited.

In most of the cucurbits, the root induction was achieved on either basal MS medium alone or with very low level of auxin (Mythili and Thomas, 1999). The IBA is widely used plant growth regulator for root induction in cucurbits (Sarowar *et al.*, 2003; Krug *et al.*, 2005) while, NAA is also used (Kathiravan *et al.*, 2006). A previous study with watermelon shoots from tissue culture demonstrated that only shoots longer than 1.6 cm were capable of efficient rhizogenesis and acclimatization (90-100%) (Compton *et al.*, 1993).

The difference in rooting response may be a result of genotype or cultural conditions. Rooting response variation may be affected by different conditions of the shoots used for root induction, (1) Variation in the medium used for multiplication before root induction, (2) The number of subcultures before root induction and (3) The culture period on multiplication medium before transfer to root induction medium. Other factors that affect rooting rates include the seed source and year the seed was collected, water source and different technique.

MATERIALS AND METHODS

Seeds of *Citrullus colocynthis* (L.) Schrad were collected from koonoor river valleys at the end of summer season, Warangal, Telangana State. The collected plants were maintained in the department green house. The young healthy plants were raised from *in vitro* condition and different explants like leaf, stem and cotyledon were washed with 1% laboline and kept under running tap water for 30 min and subsequently surface sterilized 0.1% Mercuric chloride for 3-5 min and immediately washed the explants with double sterilized distilled water. Then the pH of the medium was adjusted to 5.6-5.8 by using either 0.1 N NaOH or 0.1 N HCl before autoclaving. About 10 mL of the medium was dispensed in each culture tube and sealed with nonabsorbent cotton plugs prior to autoclaving at 121°C for 15 min under 15 psi. Sterilized cotyledon and leaf explants were cultured on MS sterilized medium supplemented with various concentrations of auxins and cytokinin and incubated at 25±1°C under a 16/8-h (light/dark) photoperiod provided by cool white fluorescent tubes (Crompton India Ltd.) with light intensity of 2,000 lux. The medium was supplemented with various growth hormones at different levels such as IAA 1.0-2.5 mg L⁻¹, IBA 0.5-2.0 mg L⁻¹, NAA 0.5-2.0 mg L⁻¹, 2, 4-D 0.5-2.5 mg L⁻¹ and combination with auxin to auxin

IAA 1.0-2.5 mg L⁻¹+IBA 0.5-2.0 mg L⁻¹, IAA 1.0-2.5 mg L⁻¹+NAA 0.5-2.0 mg L⁻¹ and cytokinin combination with auxin BAP 0.5-1.5 mg L⁻¹+IAA 1.0-2.5 mg L⁻¹, BAP 0.5-1.5 mg L⁻¹+IAA 1.0-2.0 mg L⁻¹.

RESULTS

The type and concentration of auxin and cytokinin, either alone or in combination, has been known to strongly influence growth as well as the secondary metabolites in tissue culture. Auxins concentrations 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹ were evaluated for their effects on adventitious root induction in the present study. High concentration of IAA (2.0 mg L⁻¹) and low concentration of IBA (1.5 mg L⁻¹) is best for induction of rhizogenesis from leaf, stem, hypo cotyledon and cotyledon explants of *Citrullus colocynthis* (L.) Schrad.

Rhizogenesis from stem and leaf explant culture: Callus was initiated from stem explants cultured on MS medium fortified with 1.0 mg L⁻¹ 2, 4-d and 1.5 mg L⁻¹ IBA (Fig. 1a). The same callus was sub cultured on MS medium with 2.0 mg L⁻¹ NAA and 1.0 mg L⁻¹ IBA rhizogenesis occurred (Fig. 1b). The low concentration of auxins facilitated better rooting on 2.0 mg L⁻¹ IBA and 1.0 mg L⁻¹ IAA. The direct multiple roots were produced from callus after subcultures on the same medium (Fig. 1c, d).

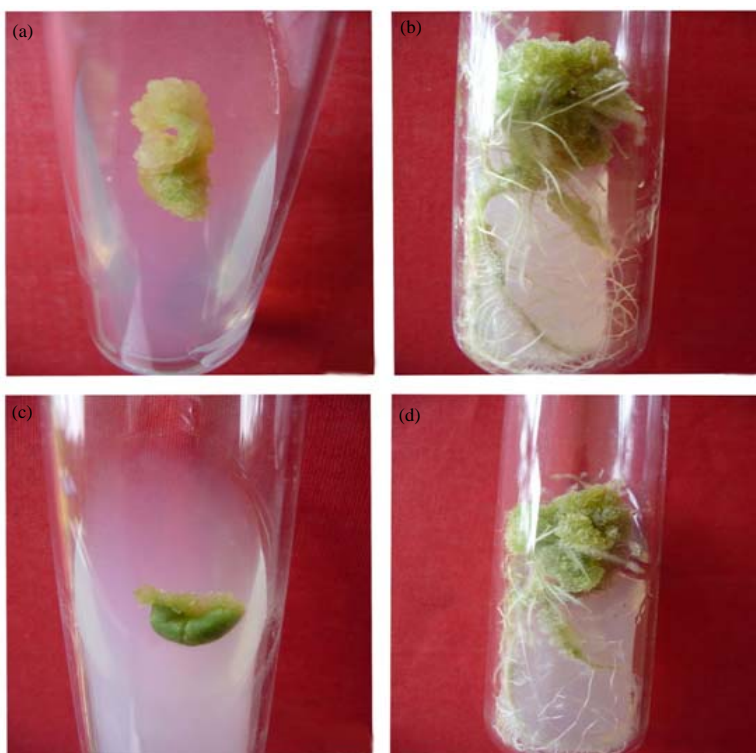


Fig. 1(a-d): Rhizogenesis from stem and leaf explants of *Citrullus colocynthis* (L.) (a) Initiation of callus from stem explant on MS+2.0 mg L⁻¹ IBA+0.5 mg L⁻¹ BAP, (b) Rhizogenesis from stem explant on MS+2.0 mg L⁻¹ IBA+1.0 mg L⁻¹ IAA, (c) Initiation of callus from leaf explant on MS+2.0 mg L⁻¹ IBA+1.0 mg L⁻¹ IAA and (d) Rhizogenesis from leaf explant on MS+2.0 mg L⁻¹ IBA+0.5 mg L⁻¹ IAA

Roots can be directly produced from leaf explants before callus is induced completely on 2.0 mg L⁻¹ IBA and 1.0 mg L⁻¹ IAA. Green callus was initiated on MS medium with 2.0 mg L⁻¹ NAA alone. Multiple fibrous roots with root caps were produced on the same medium from callus (Fig. 1d).

Observation on the experiment proved that NAA alone or in combination with IBA and IAA induced the rhizogenesis. The percentage of calli forming rooting, root length and number of roots per shoot were noticed by applying various concentrations of auxins as shown in (Table 1, Fig. 2).

Rhizogenesis from cotyledons: During the present studies it was observed that rhizogenesis occurs in different way i.e., either directly from explant or from callus. Callus was initiated on 1.0 mg L⁻¹ IAA and 1.0 mg L⁻¹ NAA (Fig. 3d). White friable callus was seen on MS medium with 2.0 mg L⁻¹ IAA. Multiple hairy roots were induced directly from cotyledon derived callus sub cultured on MS media with 1.0 mg L⁻¹ IAA and 1.0 mg L⁻¹ IBA (Fig. 3e). Multiple roots and shoots cluster were produced on MS fortified with 2.0 mg L⁻¹ IAA and 1.0 mg L⁻¹ IBA+1.5 mg BAP (Fig. 3f).

Table 1: Effect of auxins treatment on *in vitro* rooting of cultured shoots of *Citrullus colocynthis* (L.) Schrad after 48 days of culture

Auxins (mg L ⁻¹)	Shoots rooted (%)	Mean no. of roots/shoot	Mean length of longest root (cm)
IBA			
MS+0.5	39	2.00±0.0	1.53±0.1
MS+1.0	48	2.75±0.2	2.47±0.1
MS+1.5	66	3.33±0.3	3.83±0.1
MS+2.0	80	3.00±0.2	3.40±0.1
IAA			
MS+1.0	46	2.80±0.3	2.08±0.2
MS+1.5	70	3.14±0.2	2.64±0.2
MS+2.0	78	3.80±0.2	3.11±0.1
MS+2.5	50	2.40±0.2	1.80±0.0
NAA			
MS+0.5	20	2.00±0.0	1.92±0.1
MS+1.0	32	2.33±0.1	1.80±0.1
MS+1.5	44	2.75±0.2	2.26±0.1
MS+2.0	50	2.25±0.2	1.70±0.1

±: Standard error, IBA: Indole butyric acid, MS: Murashige and Skog, IAA: Indoleacetic acid, NAA: Naphthalene acetic acid

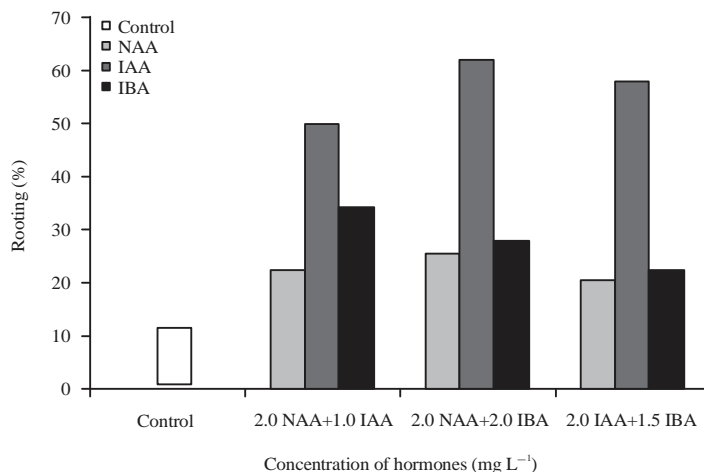


Fig. 2: Root initiation from various explants after 4 weeks of culture with IBA, IAA and NAA at different concentrations in *Citrullus colocynthis* (L.) Schrad



Fig. 3(a-f): Rhizogenesis from hypocotyl and cotyledon explant of *Citrullus colocynthis* (L.) Schrad, (a) Initiation of hairy roots from hypo cotyledon derived callus on MS+1.0 mg L⁻¹ 2,4-D+1.0 mg L⁻¹ IBA, (b) Formation of secondary roots from hypocotyledon explant on MS+2.0 mg L⁻¹ 2,4-D+1.5 mg L⁻¹ IBA, (c) Cluster of hairy roots and fibrous roots developed from hypocotyledon explant on MS+2.0 mg L⁻¹ NAA+0.5 mg L⁻¹ IAA, (d) Induction of massive hairy roots developed from cotyledon derived callus culture on MS+1.0 mg L⁻¹ IAA and 1.0 mg L⁻¹ IBA and (e) Induced multiple roots and shoots from cotyledon derived callus subculture on MS+2.0 mg L⁻¹ IAA+1.0 mg L⁻¹ IBA+1.5 mg L⁻¹ BAP

Rhizogenesis from hypocotyl explant cultures: Rooting was occurred directly from explant before callus is completely induced on MS medium with 2.0 mg L⁻¹ 2,4-D and 1.5 mg L⁻¹ IBA (Fig. 3b). Hypocotyl explants cultured on MS medium with 2.0 mg L⁻¹ NAA produced brown callus (Fig. 3c). After five weeks of culture, brown callus was subcultured on MS medium fortified with 2.0 mg L⁻¹ NAA and 1.0 mg L⁻¹ IBA rhizogenesis occurred (Fig. 3d). Auxins in different combinations induced direct rooting from explants (Table 1 and Fig. 2).

Effect of auxin-auxin and auxin-cytokinin combination on rhizogenesis: In the present study different concentration of auxins and cytokinins were supplemented to MS medium to test their ability to induce rhizogenesis from different explants. The percentage of rooting number per shoot, root length and number of roots were increased with increase in the concentration of auxins IAA, NAA and IBA. NAA alone has more effect in decreasing percentage of rooting 44% with highest number of roots per shoot (2.75±0.2) and root length (2.26±0.1 cm) when compared to other auxins viz., IBA and IAA were obtained (Table 1).

Effect of auxin and auxin- auxin on different explants of *Citrullus colocynthis* (L.) Schrad for induction of rhizogenesis. Results revealed that, the best rooting ability was observed from in stem explants cultured on MS medium supplemented with 2.0 mg L⁻¹ IAA and 1.5 mg L⁻¹ IBA, multiple fibrous roots were produced from cotyledon explants on MS fortified with 2.0 mg L⁻¹ IAA and multiple roots clusters produced from leaf explants 1.5 mg L⁻¹ IBA and 2.0 mg L⁻¹ IAA. The IAA in combination with IBA is most potential in inducing high percentage of rooting (62%) and NAA in combination with IBA induced maximum rooting 50% (Fig. 2). Moreover, single auxin IBA supplement on MS medium 1.5-2.0 mg L⁻¹ produce highest percentage of roots for shoots (80%), same way single IAA supplement on MS medium 1.5-2.0 mg L⁻¹ produce highest percentage of roots for shoots (78%), mean no. of roots and shoots (3.33±0.3) and mean of root length (3.83±0.1 cm) were obtained (Table 1).

DISCUSSION

Induction and development of roots at the base of *in vitro* grown shoots is an indispensable step to establish tissue culture derived plantlets on the soil. In the present investigation, roots were produced from various explants of *Citrullus colocynthis*, among all highest percentage of root formation occurred from leaf, cotyledon and stem explant. For root induction, MS medium supplemented with various concentrations of IAA, 2,4-D, NAA, IBA and BAP are most suitable auxins and cytokinins (Bagadekar and Jayaraj, 2011; Shasthree *et al.*, 2009).

In the present investigation, rooting occurred in all concentrations but with different rooting percentages. Among the media tested for root induction a very low concentration of IBA and IAA produced significantly 66-80% of root formation. *In vitro* grown multiple shoots were cultured on half strength of MS supplemented with NAA individually. Highest numbers of roots were produced at 2.0 mg L⁻¹ NAA. These results were supported by Hoque *et al.* (1998) in *Momordica dioica* MS supplemented with 0.2 mg L⁻¹ IBA, 0.2 mg L⁻¹ NAA and maximum root induction and proliferation was found in *Stevia rebaudiana*, when the medium is supplemented with 0.5 mg L⁻¹ NAA (Rafiq *et al.*, 2007). In bottle gourd (*Lagenaria siceraria*) elongated shoots were successfully rooted on MS media with 0.1 mg L⁻¹ IAA. In *Citrullus lanatus*, MS media supplemented with 0.5 mg L⁻¹ IBA is the best medium for root proliferation with no vitrification, so it was selected as best media for *in vitro* studies (Barnes, 1979). The IBA was found to be the best auxin for inducing rooting also in *Pisum sativum* (Ozcan *et al.*, 1992) and *Prosopis tamarugo* (Nandwani and Ramawat, 1992).

In the present study, high concentration of IAA (2.0 mg L⁻¹) and low concentration of IBA (1.0 mg L⁻¹) is best for induction of rhizogenesis. High concentration of IBA favoured formation of malformed and thick roots. The NAA in combination with IBA is also best suitable for callus induction than 2,4-D and also favoured direct rooting from stem and leaf explant of *Heliotropium indicum* (Bagadekar and Jayaraj, 2011). Efficient rooting was achieved in *Trichosanthes dioica* at different concentration of IBA (0.5 mg L⁻¹) and NAA (2.0 mg L⁻¹), (Kumar *et al.*, 2003). Lower concentration of these two 2 mg L⁻¹ 2,4-D and 1 mg L⁻¹ NAA facilitated better rooting without any callus formation in *Erythrina variegata* (Shasthree *et al.*, 2009). The IBA is better than NAA in inducing adventitious rooting in *Citrullus colocynthis* reported (Shrivastava and Roy, 2011).

In the present study, 60-80% rooting occurred on medium containing NAA at low levels. The highest number of roots/shoot formed at 2.0 mg L⁻¹ NAA whereas, the lowest-number of roots/shoot found at 1.0 mg L⁻¹ NAA. Shoot elongation was simultaneously observed along with root induction in 2.0 mg L⁻¹ NAA in *Zehneria scabra* and *Citrullus lanatus*. Anitha and Pullaiah (2002) in *Decalepis hamiltonii* and Latha *et al.* (1998) in *Porteresia coarctata* also demonstrated similar results.

In some cases, rooting in PGR free medium during organogenesis has been reported in *C. pepo* (Ananthakrisnan *et al.*, 2003) and *C. maxima* (Lee *et al.*, 2003). Rao *et al.* (1992) was able to regenerate adventitious shoots and roots from dissected seed cotyledons and the embryo axis of *Cucumis melo* in MS media with 5.0 mg L⁻¹ Kn+CM.

The present investigation, results reveals that combination of auxins, IAA+IBA and NAA+IBA are best terms of rooting ability. Explants leaf, cotyledon and stem responded well for rhizogenesis than hypocotyls and leaf. This might be due to the genotypic variation of the explants and along with the cultural and environmental conditions.

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