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# HIGH FREQUENCY OF PLANTLET REGENERATION AND MULTIPLE SHOOT INDUCTION FROM LEAF AND STEM EXPLANT OF *CITRULLUS COLOSYNTHIS* (L.) SCHRAD, AN ENDANGERED MEDICINAL CUCURBIT.

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## ABSTRACT

Efficient plant regeneration via organogenesis was established using leaf and stem explant. Callus cultures from the leaf and stem explants were tested for growth and organogenic capacity on MS medium fortified with different concentrations and combination of 2,4-D with BAP and 2,4-D with TDZ. The maximum morphogenic callus induction rate (65%) was observed from leaf explant by culturing in MS medium supplemented with 1.5 mg/l 2,4-D + 1.0 mg/l BAP when compared to 2.0 mg/l 2,4-D + 1.0 mg/l TDZ (50%). High frequency shoot regeneration (75%) from leaf derived callus was observed on MS medium supplemented with 2.5 mg/l 2,4-D and 0.5 mg/l TDZ. At the end of 3 weeks the regenerated shoots were transferred on the same medium (MS + 2.5 mg/l 2,4-D + 0.5 mg/l TDZ) for further proliferation and elongation. The regenerated shoots were rooted with high frequency (60%) in MS medium supplemented with 1.5 mg/l IBA when compared to other auxin NAA. The in vitro raised plantlets were successfully established in green house and transplanted to natural conditions with 70% survival.

## KEY WORD

*In vitro*, organogenesis, thiadiazuron, rhizogenesis, *Citrullus colocynthis*.

## INTRODUCTION

*Citrullus colocynthis* (L.) Schrad grows widely in Asia, especially East Asian Countries. It grows wild in Israel/Palestine. The colocynth, is also known as bitter apple or bitter cucumber. The white flesh character is associated with bitterness[1]. The bitter taste is caused by high concentration of a substance called Cucurbitacin E. glycoside or colocynthine[2]. The fruit is used in herbal medicine by traditional herbalist for treatment of diabetes, jaundice and urinary diseases. Dried unripe fruit pulp consist the drug 'colocynth' which is very strong laxative. It also stimulates liver and regularizes bile secretions. Also used as antirheumatic, antihelminthic, as a remedy for skin infection and controlling menstrual disorders [3,4]. It is more pronouncedly used in antitumerous drugs as it contained antitumerous agent like Cucurbitacin B and Cucurbitacin E.[5]. Colocynthis an important

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ingredient in Narayanchurn. Colocynth entered into the composition of numerous valued medicines such as Stahls's pills, Morison's pills, Barclays's antibilios pills etc.

## MATERIAL AND METHODS

*Citrullus colocynthis* (L.) Schard plants were collected from river valleys of koonoor Warangal, A.P. The collected plants were maintained at the campus department garden. Leaf and stem explants were excised from the garden-grown plants and washed thoroughly in running tap water for 5-10 minutes under aseptic condition. Explants were surface sterilized by dipping in 0.1% fresh aqueous mercuric chloride ($HgCl_2$) solution for 1-2 minutes, subsequently washed thoroughly with double distilled water to remove traces of $HgCl_2$. The pH of the medium was adjusted to 5.7 either 0.1 NaOH (or)1 N HCl before autoclaving. About 10 ml of the medium were dispensed in each culture tube and sealed with non-absorbent cotton plugs prior to autoclaving at 121°C for 15 min under 15 psi. The leaf and stem explants were cultured on MS medium fortified with different strengths of plant growth hormones 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 2000 lux. Results were observed at regular intervals and tabulated.

Sterlized stem and leaf explants were cultured on MS sterilized medium supplemented with various concentrations of 2,4-D with BAP and 2,4-D with TDZ (Table 1). After 3 weeks, efficient callus was induced and were subcultured into fresh media with various concentrations and combinations of 2,4-D with BAP and TDZ for developing potentially organogenic nature (Table 1). Nodular and friable calli are potentially organogenic and were subcultured for adventitious shoot bud induction and plantlet regeneration. The regenerated shoots were subcultured onto the same shoot induction medium after 21-28 days for shoot proliferation and elongation. *In vitro* raised microshoots after attaining a height of 1-1.5 cm were transferred to MS medium fortified with different concentrations of IBA and NAA for root induction (Table 2).

### *Acclimatization of the plantlets*

After *in vitro* rooting the regenerated plantlets were taken out and were washed carefully to remove agar and then transferred to pots containing sterile vermiculite. Each pot was enclosed in a polyethylene bag after watering and maintained in a plant growth chamber at 25±1°C under 16-h illumination with fluorescent lamps. Bags were progressively opened weekly. After 3 weeks of field. The percentage of survival was found to be 70% and the plants were morphologically identical to the acclimatization, plantlets were transferred to large pots filled with garden soil and farmyard manure (1:1) in the open parental plants.

In all experiments a minimum of three plates were cultured. Each single treatment consisted of five to ten explants per plate. Data recorded at 3 weeks included the number of shoots per explant, length of shoots and rooting were statically analysed using one-way analysis of variance.

## RESULTS

The leaf and stem induced efficient callus on MS medium containing 1.5 mg/l 2,4-D + 1.0 mg/l BAP and 2.0 mg/l 2,4-D + 1.0 mg/l BAP respectively (Plate I, Fig 1 & 2 and Table 1). Highest growth response was obtained with 2,4-D and BAP

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(65%) than with 2,4-D and TDZ (50%). Levels above or below this gradually decreased the frequency of callus induction. After 3 weeks, the actively growing callus were subcultured on fresh medium of culture on the same composition of medium enhanced peripheral greening of callus inducing shoot buds (Plate I, Fig 3).

**Table 1.**

***Frequency of callus induction and shoot regeneration from leaf and stem explants of citrullus colocythis(L.) schrad on MS medium containing auxin and cytokinin.***

| GROWTH REGULATORS | % OF CALLUS GROWTH RESPONSE | | % OF SHOOT REGENERATION | |
|---|---|---|---|---|
| MG/L | LEAF | STEM | LEAF | STEM |
| **MS + 2,4-D + BAP** | | | | |
| MS + 1.0 + 0.5 | 35.2 | 27.4 | 18.8 | 26.3 |
| MS + 1.5 + 1.0 | 65.1 | 45.3 | 21.4 | 30.1 |
| MS + 2.0 + 1.0 | 40.5 | 30.9 | 30.6 | 41.8 |
| MS + 2.5 + 1.5 | 28.3 | 27.8 | 45.8 | 50.6 |
| MS + 3.0 + 2.0 | 22.1 | 20.1 | 28.6 | 22.8 |
| **MS + 2,4-D + TDZ** | | | | |
| MS + 1.0 + 2.0 | 33.6 | 25.4 | 20.3 | 26.6 |
| MS + 1.5 + 1.5 | 40.2 | 33.3 | 25.2 | 31.4 |
| MS + 2.0 + 1.0 | 50.1 | 40.6 | 33.1 | 56.6 |
| MS + 2.5 + 0.5 | 26.6 | 28.1 | 50.8 | 75.1 |
| MS + 3.0 + 0.5 | 20.8 | 16.6 | 30.9 | 35.8 |

Combination of 2,4-D and TDZ showed low response to callus formation compare to 2,4-D and BAP showed best results from stem and leaf. The calli derived from leaf and stem explants were best for regeneration and were subcultured on MS medium with 2.5 mg/l 2,4-D + 1.5 mg/l BAP and 2.5 mg/l 2,4-D + 0.5 mg/l TDZ. After 2 weeks shoot buds from green callus were regenerated to plantlets (Plate I, Fig 4). The combination of 2.5 mg/l 2,4-D and 0.5 mg/l TDZ is most effective and induced maximum percentage of (75%) in plantlet formation when compared to 2.5 mg/l 2,4-D + 1.5 mg/l BAP (50%) (Table 1). The percentage of culture response in inducing callus and regeneration from callus derived from leaf is high when compared to stem. The regenerated microshoots (Plate I, Fig 5) were subcultured on the same composition of medium for further shoot proliferation and elongation. *In vitro* grown healthy microshoots (1-2 cm) were excised and cultured on MS supplemented with different concentrations of IBA at 2.5, 2.0,1.5, 1.0 and 0.5 mg/l and NAA at 3.0, 2.5, 2.0, 1.5 and 1.0 mg/l (Table 2). IBA (1.5% mg/l) alone is most potential

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in inducing high percentage (60%) of rooting with highest number of roots per shoot (14) when compared to NAA (1.5 mg/l) induced (50%) of rooting with highest number of roots (9) per shoot (Table 2). Most of the shoots had produced roots within 2 weeks after placing on rooting medium (Plate I, Fig 6). Low concentration of auxin facilitated better rooting.

**Table 2.**

***Effect of IBA, NAA and BAP in various concentrations on rhyzogenesis from stem derived regenerated plant of citrullus colocynthis(L.) schrad.***

| Growth regulators (mg/l) | | No. of inoculated calli | No. of calli forming roots | Differentiation frequency (%) |
|---|---|---|---|---|
| IBA | NAA | | | |
| 1.5 | 1.0 | 21 | 8 | 35.2 ± 0.2 |
| 2.0 | 1.5 | 22 | 16 | 44.4 ± 0.1 |
| 2.5 | 2.0 | 18 | 6 | 32.2 ± 0.3 |
| 1.5 | 1.0 | 12 | 4 | 31.1 ± 0.1 |
| 2.0 | 1.5 | 19 | 12 | 40.0 ± 0.4 |
| 2.5 | 2.0 | 14 | 6 | 37.2 ± 0.2 |

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PLATE - I

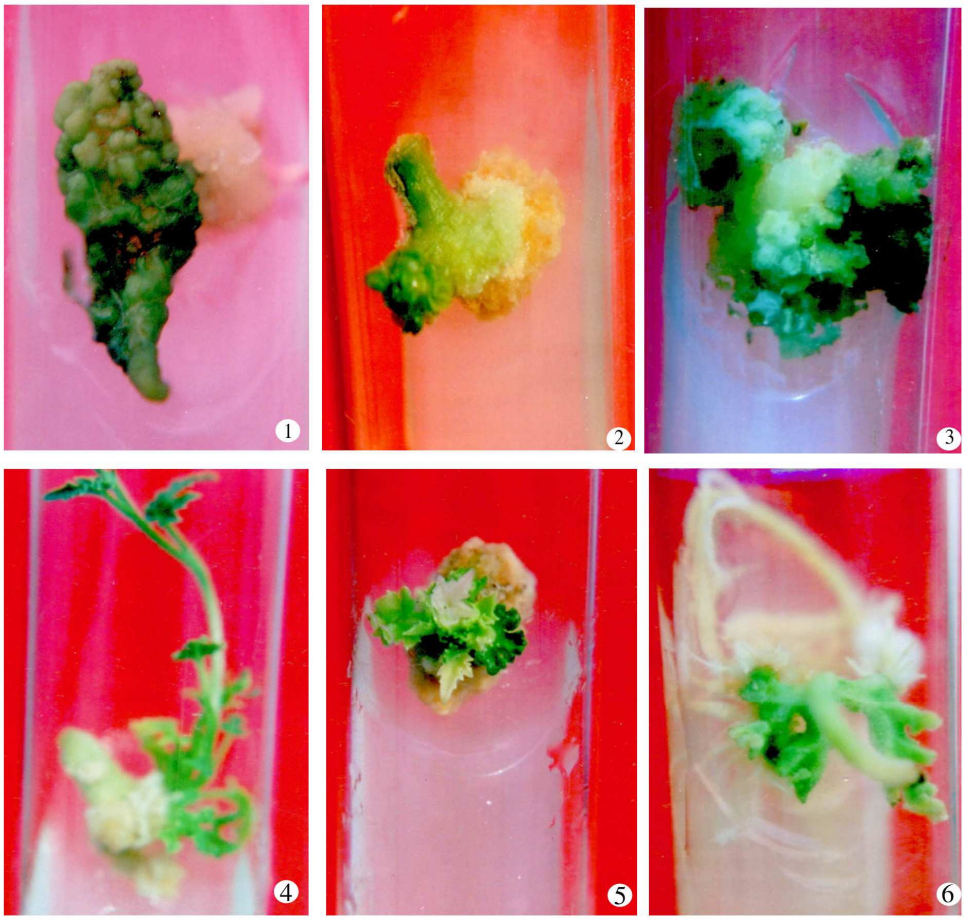


Fig.1 : Initiation of callus from leaf explant on MS+2.0 mg/l 2, 4-D+1.0 mg/l BAP

Fig.2 : Greening of callus derived from stem explant on MS +1.5 mg/l 2, 4-D+1.0 mg/l BAP

Fig.3 : Excessive greening of leaf callus on MS +2.0 mg/l 2, 4-D+1.0 mg/l TDZ

Fig.4 : Regeneration of plantlets from callus cultures on MS+2.0 mg/l 2, 4-D+1.0 mg/l TDZ after 4 weeks of culture.

Fig.5 : Formation of multiple shoots on MS +2.5 mg/l 2, 4-D+0.5 mg/l TDZ

Fig.6 : Induction of rhizogenesis from regeneration plant on MS+2.0 mg/l IBA+1.5 mg/l NAA

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## DISCUSSION

In our study, high rate of callus growth was induced on MS medium containing 2-4-D and BAP than 2,4-D and TDZ. 2,4-D is widely used for *in vitro* callus induction in a wide range of plant species. Combination of 1.5 mg/l 2,4-D + 1.0 mg/l BAP induced potentially organogenic callus from stem and leaf explant. Levels above or below this gradually decreased the frequency of callus induction. Similar results were reported previously in *Cucurbit pepo* L.[6] Moreover, there is a report of induction of organogenic calli using a combination of BAP and NAA in *Citrullus vulgaris* [7]. Friable and creamy calli derived from leaf and stem explant were subcultured on 2.0 mg/l

2,4-D + 1.0 mg/l TDZ are suitable for acquiring green granular organogenic callus with subsequent shoot bud induction after 2 weeks. In *Cucumis metuliferus* and *Cucumis figrei*, highest regeneration frequency (92.5%) was achieved under the combination of 1 mg/l BAP and 0.2 mg/l TDZ [8, 9]. Regeneration of plantlets were observed on the same medium after four weeks of subculture. Organogenic response in Cucurbitaceae is highly genotype dependent. An expressive organogenic response in cotyledon explant from *Citrullus colocynthis* using only BA as growth regulator was reported[10]. Also, organogenesis depends on the endogenous concentration of plant growth regulator. An adverse effect of prolonged *in vitro* cultures reduce shoot organogenesis was reported[11, 12]. Shoot bud proliferation is satisfactory by cytokinin BAP (1.0 mg/l) alone in *Citrullus lanatus*[13]. Among different concentration it was concluded that 2.5 mg/l 2,4-D with 0.5 mg/l TDZ was impressive and best suitable phytohormones for shoot regeneration from leaf explants of *Citrullus colocynthis.* Further an increase or decrease of this hormone level showed a negative trend in multiple shoot formation.

In our studies, TDZ at low concentration (0.5 mg/l) increased shoot differentiation and marked effect on the quality of regenerated plants when compared to BAP. At higher level of TDZ lead to undifferentiated hard green callus development. In the present investigation rooting occurred in all concentrations, but with different rooting percentages. Highest number of roots were produced at 2.0 mg/l IBA and 1.5 mg/l NAA. When exposed to high concentration above 3.0 mg/l IBA/ NAA shoots become necrotic, lost leaves and the shoot tips died gradually. While at lower below 1.0 mg/l concentration of IBA and NAA low frequency number of roots was induced.

The present study reveals that auxin, IBA is better than NAA in inducing rooting ability. Among all plants growth regulators, IBA is widely used for root induction in Cucurbits[14], while NAA is also used[15]. Efficient rooting was achieved in *Trichosanthes dioica* at different concentration if IBA (0.5 mg/l) and NAA (2.0 mg/l)[16]. Variation in rooting response may be a result of genotype or culture conditions. Subsequently, the rooted plantlets were removed from agar medium, washed thoroughly and placed in soil pots after 2 weeks for acclimatization and initial hardening under culture room conditions. Almost 70% of these regenerants

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survived and developed new branches and were ready for planting in the field for further growth.

The importance of *Citrullus colocynthis* as a medicinal cucurbit is growing up substantially with increasing and stronger reports in support of its multifarious therapeutic uses. Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Greater demand for these plants especially for the purpose of food and medicine is one of their rapid depletion from primary habits[17]. In the present study, shoot induction and plantlet regeneration from leaf explant is the best and first report to our knowledge on large scale multiplication in a short period of time for conservation of an endangered medicinally important species *Citrullus colocynthis.*

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