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Full Length Research Paper

High frequency plant regeneration from shoot tip explants of *Citrullus colocynthis* (Linn.) Schrad. – An important medicinal herb

Mahesh Chand Meena, Ramraj Meena* and Vidya Patni

Plant Pathology, Tissue culture and Biotechnology Laboratory, Department of Botany, University of Rajasthan, Jaipur-302004, India.

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A high frequency and rapid regeneration protocol was developed from shoot tip explants of *Citrullus colocynthis* on Murashige and Skoog (MS) medium supplemented with N^6-benzylamino-purine (BAP, 0.5 mg/l) and α-naphthalene acetic acid (NAA, 0.5 mg/l). Highest number of shoots (23.0 ± 0.567) was obtained on MS medium containing BAP (0.5 mg/l) and NAA (0.5 mg/l). The regenerated shoots were further elongated on same medium. *In vitro* shoots were excised from shoot clumps and transferred to rooting medium containing indole-3-butyric acid (IBA, 4.0 mg/l) with 0.2% activated charcoal. The rooted plants were hardened in polycups containing sterile soil and vermiculite and finally well established in the field; survival rate was 60%. This is the first report of direct *in vitro* plantlet regeneration in *C. colocynthis* from shoot tip explant.

Key words: *Citrullus colocynthis*, regeneration, shoot tip.

INTRODUCTION

Plant based remedies have always been an integral part of traditional medicine throughout the world. The demand for herbal remedies has been increasing significantly (Suchitra et al., 1999). Constant demands for some of the medicinal plants have affected their availability to a large extent. Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened with extinction. Greater demand for these plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats. Micropropagation offers a great potential for large scale multiplication of such useful species and subsequent exploitation (Boro et al., 1998).

Among the important medicinal plants, *Citrullus colocynthis* (Linn.) Schrad (Cucurbitaceae) commonly known as, Tumba or Indrayan or bitter apple, is a perennial herb and a common desert plant having a wide range growing in most barren and arid situations. *C. colocynthis* has been utilized as a general promoter of health in medicine.

C. colocynthis is used in folk medicine by people in rural areas as purgative, antirheumatic, anthelmintic, anti-cancerous and as remedy for skin infection. The pulp of the fruit has antibacterial activity. The dried and powdered pulp is taken orally to cause abortion. Seeds are purgative and leaves are used against migraine and neuralgia. Root has a beneficial action in opthalia, uterine pains, jaundice and is also useful against boils, pimples and enlarged abdomen. The root base mixed with cow milk is applied on hypogastrium for easy delivery (Yadav et al., 2006). The glycosidic extract of the whole plant is also used for lowering glucose level (Bharate and Gnana, 2006). An attempt on the determination of extractive value has been done (Meena and Patni, 2007b) and it was found to be maximum with ethyl alcohol among various solvents used in soxlet extraction.

Due to unsuitable exploitation of eco-resources, several plant and tree species of great medicinal importance have become threatened and may become extinct due to

*Corresponding author. E-mail: ramra1986@gmail.com.

Abbreviations: MS, Murashige and Skoog; **BAP,** N^6-benzylamino-purine; **Kn,** kinetin; **NAA,** α-naphthalene acetic acid; **IBA,** indole-3-butyric acid.

lack of proper planning for their conservation and multiplication. Due to excessive and destructive exploitation of *C. colocynthis,* it is getting fast depleted (Bhandari, 2004). Therefore, it is time to conserve this plant species by means of micropropagation through tissue culture technique. Tissue culture is an age-old practice for *in vitro* regeneration of plants, especially the economically valuable plant or plants that are difficult to propagate in natural environment. This method is also used for studying the plant development at the molecular level thereby artificially increasing the plant output molecules. So we describe here an efficient procedure for the rapid clonal propagation of *C. colocynthis* through shoot tip culture, which is often used to produce disease free stock of plants. Shoot tip culture is widely used for rapid propagation of many species due to its advantages over traditional methods (Hu and Wang, 1983). The main objective of the present investigation is to select the best *in vitro* responding media combinations for inducing morphogenesis in higher frequencies and optimization of conditions for complete plantlet development.

MATERIALS AND METHODS

Plant material and surface sterilization

Shoot tip bearing shoot segments were collected from mature plants growing in the campus of the University of Rajasthan, Jaipur, India. Explants were initially washed under running water with Labolene solution (0.1% v/v) for 5 min and sterilized with 0.05% (w/v) mercuric chloride for 2 - 3 min. Shoot tips were rinsed with sterile distilled water 4 - 5 times to remove traces of mercuric chloride. These shoot tips were inoculated on MS-medium (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of phytohormones.

Culture condition

All the media contained 3% (w/v) sucrose. The pH of the media was adjusted to 5.8 and solidified with 0.8% agar before autoclaving at a pressure of 15 psi and 121°C temperature for 20 min. All the cultures were maintained at a temperature of 25 ± 2 °C under a 16/8-h (light/dark) photoperiod provided by cool white fluorescent light. Multiple shoots obtained were later transferred to elongation medium supplemented with different growth regulators. The cultures were regularly subcultured on fresh medium at four week intervals and observations were recorded. Elongated and healthy plantlets were transferred to rooting medium containing 4.0 mg/l concentration of indole-3-butyric acid (IBA) with activated charcoal (0.2%). Regenerated plantlets were then transferred to pots containing sterilized soil and vermiculite (3:1). The plantlets were kept in a green house for one week under normal light and temperature. New leaves emerged from the plantlets after 10 - 15 days in the green house; they were then placed in the normal environment for 1 h and assessed for signs of wilting. The exposure time was increased daily until the plants were established fully under normal environmental conditions; they were then transferred into the field for normal growth. For each treatment, 8 replicates were used and each experiment was repeated at least five times.

The cultures were examined periodically. The means and standard errors of the results were also calculated.

RESULTS AND DISCUSSION

Shoot tip of Indrayan (*C. colocynthis*) were isolated aseptically and cultured on MS-medium supplemented with cytokinins and auxins for initiating vegetative growth and inducing maximum number of plantlets. MS medium was found to be most suitable compared to other media tried viz. B5, WB, Schenk and Hildebrandt, etc.

The shoot tip explants were inoculated on MS medium containing different concentrations of N^6-benzylaminopurine (BAP) (0.5 - 5.0 mg/l) and kinetin (Kn) (0.5 - 5.0 mg/l) alone and in combination (0.5 - 3.0 mg/l BAP and 0.5 - 3.0 mg/l Kn and 0.5 - 3.0 mg/l BAP and 0.5 - 3.0 mg/l α-naphthalene acetic acid (NAA)) (Figure 1A). Among different concentrations used, best response towards shoot proliferation from shoot tip explant was obtained on MS medium with BAP (0.5 mg/l) and NAA (0.5 mg/l). Initiation of axillary bud started after one week of inoculation (Figure 1B). Maximum multiple shoots (23.0 ± 0.567) were obtained on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) (Figure 1C). Shoots elongated on the same medium (Figure 1D). From the results (Table 1), it is clear that a combination of BAP (0.5 mg/l) and NAA (0.5 mg/l) at lower concentration was suitable for shoot multiplication as well as shoot elongation.

The effective role of NAA in combination with BAP for the induction of multiple shoots has been reported in *Basilicum polystachyon* (Chakraborty et al., 2006), *Musa sapientum* L. (Kalimuthu et al., 2007), *Rauwolfia serpentina* (Baksha et al., 2007), *C. colocynthis* (Meena and Patni, 2007a) and *Bupleurum distichophyllum* (Karuppusamy and Pullaiah, 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).

Rooting of the developed shoots was usually achieved in auxin containing medium (Gaspar and Coumans, 1987). Root formation is an energy demanding process and thus, exogenous supply of carbohydrates is required. However, this being the last stage of *in vitro* culture, it is important to transform the plant from heterotrophic to autotrophic mode of nutrition. Thus, the supply of exogenous sugars should be reduced at this time. The rooting response differed according to different concentrations and combinations of auxins used (Table 2). In the present study, rooting was achieved on full strength of

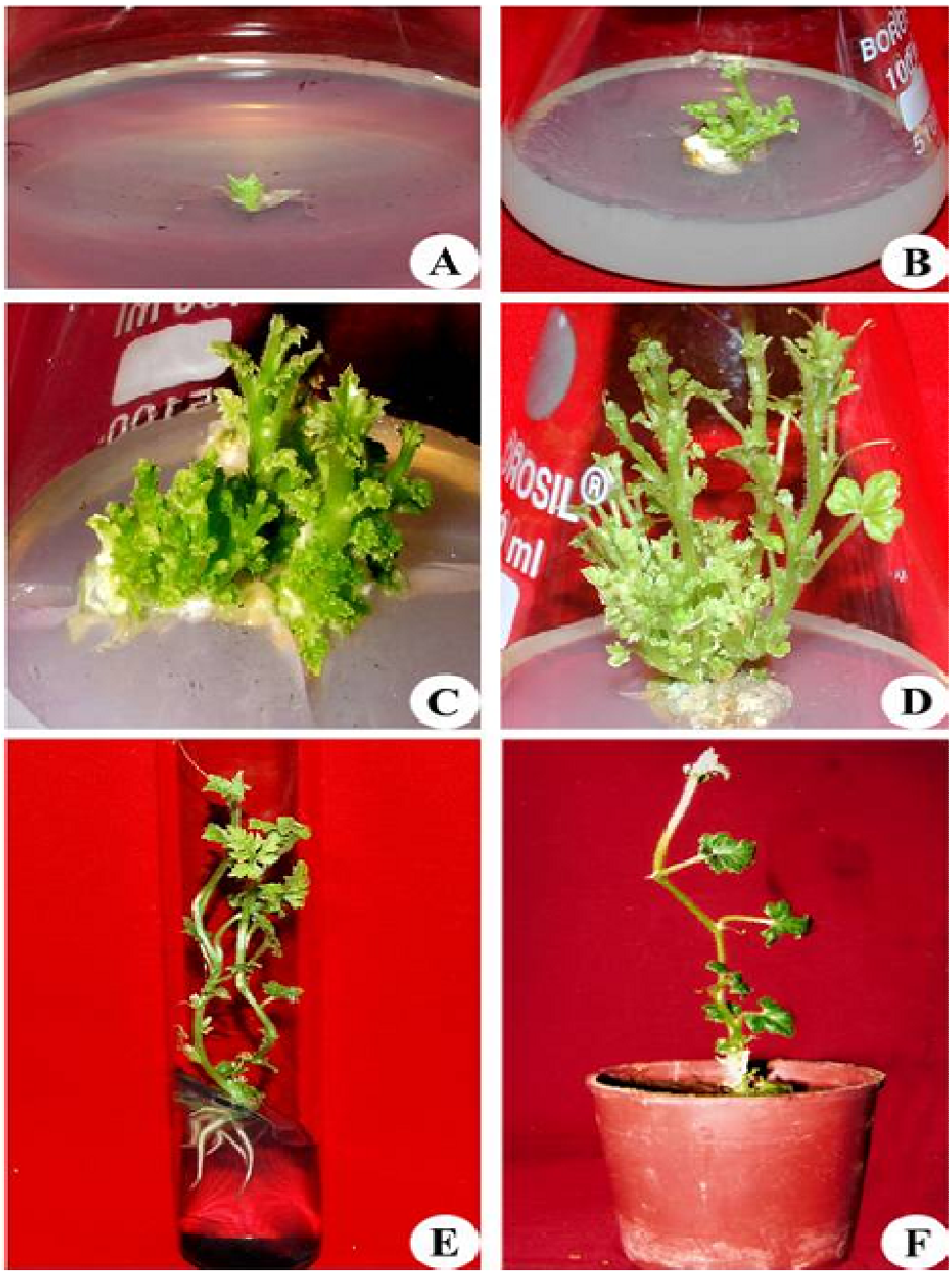

Figure 1. *In vitro* shoot multiplication and acclimatization of *C. colocynthis* through shoot tip culture. A. Shoot tip explant on MS medium; B and C. multiple shoot induction after two weeks and three weeks on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l); D. elongated shoots on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l); E. *In vitro* rooting of regenerated shoot on MS medium supplemented with IBA (4.0 mg/l) and 0.2% activated charcoal; F. hardening of *in vitro* raised plantlets.

MS-medium fortified with IBA (4.0 mg/l) and activated charcoal (0.2%). On this medium, rooting percentage (95%), number of roots (24.0 ± 1.678) and root length (3.0 ± 0.16) were maximum. Roots were thick and with white root hairs. Similar effect of IBA on rooting in several medicinal plant species were also observed by several workers in different plant species viz. *Mentha piperita* (Sunandakumari et al., 2004), *Azadirachta indica* (Shahin-uz-zaman et al., 2008), *Cyphomandra betacea* (Chakraborty and Roy, 2006) and *Pluchea lanceolata* (Arya et al., 2008).

Plantlets with 6-7 leaves and well developed root system were removed and transferred to pot containing soilrite (Figure 1F). These pots were kept in growth chamber

Table 1. Effect of plant growth regulators (PGR) on shoot formation from shoot tip explant of *C. colocynthis* on MS-medium.

S. No.	PGR's mg/l			No. of shoots buds per explant
	BAP	Kn	NAA	Mean ± SE
1.	0.5	-	-	5.6 ± 0.220
2.	1.0	-	-	7.0 ± 0.756
3.	2.0	-	-	11.2 ± 0.180
4.	3.0	-	-	10.2 ± 0.336
5.	4.0	-	-	8.6 ± 0.220
6.	5.0	-	-	6.5 ± 0.220
7.	-	0.5	-	2.4 ± 0.220
8.	-	1.0	-	3.6 ± 0.359
9.	-	2.0	-	5.8 ± 0.595
10.	-	3.0	-	8.2 ± 0.336
11.	-	4.0	-	6.6 ± 0.608
12.	-	5.0	-	4.2 ± 0.336
13.	0.5	0.5	-	7.0 ± 0.401
14.	1.0	1.0	-	9.4 ± 0.220
15.	1.5	1.5	-	13.4 ± 0.463
16.	2.0	2.0	-	11.0 ± 0.284
17.	2.5	2.5	-	7.4 ± 0.588
18.	3.0	3.0	-	7.2 ± 0.336
19.	0.5	-	0.5	23.0 ± 0.567
20.	1.0	-	1.0	16.8 ± 0.180
21.	1.5	-	1.5	14.6 ± 0.220
22.	2.0	-	2.0	12.8 ± 0.336
23.	2.5	-	2.5	10.0 ± 0.634
24.	3.0	-	3.0	7.8 ± 0.595

Table 2. Effect of auxin (IBA) along with 0.2% activated charcoal on root induction from shoots of *C. colocynthis* after 4 weeks of culture.

IBA (mg/l)	% of cutting rooted	No. of roots per shoots	Average length of the root (cm)
1.0	80	1.8 ± 0.456	2.0 ± 0.16
2.0	70	8.0 ± 0.283	2.2 ± 0.18
3.0	75	13.8 ± 0.335	2.5 ± 0.40
4.0	95	24.0 ± 1.678	3.0 ± 0.16
5.0	85	16.0 ± 0.671	2.6 ± 0.63

Values are mean ± SE from 5 replicates in each treatment.

for 15 days at 26 ± 2°C and 2000 lux intensity for acclimatization. In order to maintain high humidity, the pots were covered with inverted glass beaker. After six months when new leaves emerged from these plantlets, they were taken outside the growth chambers and kept in shady place under normal temperature and light. A 60% survival rate was obtained when acclimatized plantlets were transferred to green house.

The present paper describes a prime and easy-to-use protocol for large scale production of plantlets through shoot tip culture of *C. colocynthis* and the method is useful for the *ex-situ* conservation of other threatened medicinally important species. This is the first report of *in vitro* culture of this wild endangered medicinal herb through shoot tip explant. So, the result of the present investigation forms the basis of further research. Since this plant has soil binding property when multiplied in bulk, it can be used to control soil erosion as well as spreading of Thar desert in Rajasthan. Many wild cultivated species are the major sources of genes for making hybrid

varieties (Turtumba with *Citrullus vulgaris* and *C. colocynthis*). *C. colocynthis* being an endangered wild medicinal herb has to be propagated and conserved. This work is part of the efforts taken towards that direction.

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