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HIGH FREQUENCY SHOOT TIP MULTIPLICATION AND *EXVITRO* ROOTING OF *CENTELLA ASIATICA* (L.) URB. - A INDUSTRIAL MEDICINAL HERB

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ABSTRACT

An efficient protocol was achieved for rapid clonal propagation of the medicinally important plant species, *Centella asiatica* (L.) Urban through shoot tip proliferation and *ex vitro* rooting. High frequency bud break (88%) and multiple shoot formation were induced from shoot tip segments are cultured on MS medium supplemented with BAP (17.76 µM) plus GA_3 (1.44 µM). Although callus free multiple shoot formation was a function of cytokinin activity alone it fastest bud break coupled with enhanced frequency of shoot development (88%) and internode elongation were dependent on the synergistic effect of GA_3 (1.44 µM). By repeated subculturing of nodal segments harvested from the newly formed axenic shoots, prolific shoot cultures, free of proximal callusing and showing a high frequency multiplication rate were established within three months. The percentage of shoot multiplication as well as the number of shoots per shoot tip attained the highest values (88%, 16.8 shoots/shoot tip) during the first two culture passages; beyond this there was a gradual decline in shoot bud differentiation. Half-strength MS medium fortified with NAA (10.74 µM) induced the highest number of roots. All *in vitro* rooted shoots survived in field. Dipping of the basal end of shoots collected from multiplication medium in IBA (10.74 µM) solution for 10 days induced roots and its transfer to small pots facilitated the survival of all rooted shoots (100%).

Rooting of *ex vitro* by direct transfer of shoots from multiplication medium exhibited 90% survival. Use of commercial sugar and tap water and also the omission of *in vitro* rooting reduce the propagation cost 50 – 70 %. The plantlets were hardened off and successfully established in the natural soil, where they grew and matured normally. The protocol enables to harvest more than 25,000 plantlets within 160 days starting from a single shoot tip explant.

KEYWORDS: *Centella asiatica,* Medicinal plant, Micropropagation, Explant source, Multiple shoot-bud induction, *Ex vitro* rooting.

Abbreviations BAP 6-Benzylaminopurine, KN Kinetin, GA_3 gibberillic acid, IBA-Indole-3-butyric acid, IAA- Indole-3-acetic acid, 2,4-D - 2,4-Dichlorophenoxy acetic acid, NAA - 1-Naphthalen acetic acid, MS - Murashige and Skoog.

INTRODUCTION

Centella asiatica (L.) Urban. Indian Pennywort (Umbelliferae), popularly known as `Brahmi` in India, is one of the sources of the medhya rasayan drugs (that counteract stress and improve intelligence and memory) of ayurveda. It is prescribed for a variety of therapeutic indications including various skin diseases, malaria, fever, epilepsy, ulceration, chronic rheumatism, leprosy and enlargement of glands.[1] It also acts as an alternative tonic when given internally and stimulant when applied externally.[2] The drug showed significant improvement in cases of anxiety neurosis[3] and peptic ulcer.[4] It has also been found to cure general debility[5] and improve the faculty of memory in mentally retarded children.[6] *Pharmaceutical* companies largely depend upon material procured from naturally occurring stands which are being depleted rapidly raising concern about possible extinction of the species and providing justification for the development of *in vitro* propagation techniques for this crop.[7] *C. asiatica* was placed third in a priority list of the most important Indian medicinal plants evaluated on the basis of their medicinal importance, commercial value and potential for further research and development.[8] *C.asiatica*, a weakly aromatic Smelling, stoliniferous, creeping herb that can be found in all plain districts in wet places, ponds, rice fields and tropical and subtropical regions of India and throughout the world. The triterpenoid compounds apparently represent the chief pharmacologically active martial in *C. asiatica.*[9] Asiaticoside, a trisaccharide triterpene, has been identified as the most active compound in the plant. Many commercial drug preparations of *C.asiatica* are available in West Germany and France.[10] The other chemical constituents of the plant include bramic acid, thankunic

acid, isothankuni-side, thankunidide, polyactylenes, asiatic acid, madecassic acid and two saponin glycosides.[8]

With an increasing world-wide demand for plant derived medicines and formulations[11], there has been a concomitant increase in the demand for raw material. Hence, there is a need to develop approaches for ensuring the availability of raw material of a consistent quality from regular and viable sources.

During the investigation presented here, we developed a tissue culture system for adventitious shoot-bud induction in *C.asiatica*. We also established suitable conditions for multiple shoot bud regeneration and shoot culture, which have the potential of providing a source for continuous supply of shoots at periodic intervals.

MATERIALS AND METHODS

Explant source: For experiments explant was obtained from *C. asiatica* plants growing in the medicinal plant garden of National college campus. New healthy young twigs (15 -20 cm) were collected during different months from the field grown plants in the morning. Individual shoot tips (3- 5 mm) were excised, washed under running tap water for 15 min, and then surface sterilized with a solution of teepol (2% v/v) for 5 min followed by 30 seconds in a solution of ethanol (70% v/v) and mercuric chloride (0.1% w/v) for 7 min. The shoot tips were finally washed for 5-6 times with double sterile distilled water.

Culture medium

Murashige and Skoog[12] culture medium (MS) supplemented with sucrose (3%, w/v), myo-inositol (100mg/l, w/v) and BAP (4.44 – 22.20 µM) and KN (4.64 – 27.84 µM) either individually or in combination with GA_3 (1.44 µM) was used. The pH of the medium was adjusted to 5.7 - 5.8 before gelling with agar (0.8%, w/v; Bacteriological grade, Hi-media, India). All chemicals were analytical grades (Sigma, S.d.fine and Hi-media). 10 ml of molten medium were dispensed into 50 ml boiling test tubes (Borosil, India). Test tubes were capped with cotton plug. The test tubes were autoclaved at 104 kPa and 121°C for 20 min. The surface sterilized shoot tips were explanted vertically into culture medium (2 buds/tube).

Culture conditions

Cultures were maintained in a culture room at 25 ± 2°C, under 16 h photoperiod provided by cool white fluorescent light (60 $\mu Em^{-2} S^{-1}$) with 55-60% relative humidity. The cultures were

grown for 35 days before data were collected. Each treatment consisted of 15 replicates and each experiment was repeated five times.

Multiplication of shoots

Primary shoots formed *in vitro* were isolated and sectioned into one shoot tip after removing the leaves. These shoot tip segments each containing the shoot tip, were cultured on MS medium fortified with BAP (17.76 µM) plus GA_3 (1.44 µM) for further multiplication. Subsequent subcultures were done in the same medium at a periodic interval of 4 weeks.

Rooting of shoots

For rooting, shoots (3-4 cm in length) formed *in vitro* were excised and transferred to half-strength MS medium supplemented with 0.54, 2.69, 5.37, 10.74 and 16.11 µM of NAA, 0.49, 2.46, 4.92 and 9.84 of IBA and 0.57, 2.85, 5.71 and 11.42 µM of IAA individually. The cultures were grown for 25 days after data were collected.

For *ex vitro* rooting, the well grown shoots (more than 4 cm long) from the shoot multiplication medium were directly transferred to small pots containing vermiculite and red soil (3:1) and also by transferring to pots after dipping the basal ends of the shoots in different concentrations of IBA solutions for 7 days.

Acclimatization and transfer of plantlets to soil

Well-rooted plantlets were removed from the culture medium and the roots were washed under running tap water to remove agar. The plantlets were transferred to plastic cups (5 cm diam.) containing red soil and vermiculite and maintained inside a plant growth chamber set at 25 ± 2°C, 16 h day length (60 µE m^{-2} S^{-1}) and 70-80% relative humidity. The potted plantlets were irrigated with 1/8 MS basal salt solution devoid of sucrose and myo-inositol, every 2-day for period of 2 weeks. The plants were then transplanted to earthenware pots (10 cm diam.) containing natural soil and manure and kept under shade in the glasshouse for another 2 weeks. The potted plants were then placed outdoors under full sun, with 95% survival. No morphological abnormalities were noticed in the potted plants.

Experimental deign and analysis of data

All experiments of the completely randomized design and repeated five. Each treatment consists of 15 replicates and each replicates had 2 explants. Means were compared using Duncan's multiplication range test.

RESULTS

Bud breaks and shoot proliferation

Bud break and development of shoots from shoot tip explants was a function of cytokinin activity. The morphogenic responses of shoot tip explants collected during September through December to various concentrations of cytokinins are documented in Table-1. There was no sign of bud break even after 30 days on MS basal medium without any growth regulator supplement. Of the two cytokinins tested, BAP was more effective than KN in inducing bud break as well as multiple shoot formation. There was a linear correlation between the increase in concentration of BAP upto the optimal level (17.76 µM) and percentage shoot development. The number of shoots per explant also increased with increments in BAP concentrations upto the optimal level (17.76µM). MS medium containing 17.76µM BAP induced bud breaks in 80% of the shoot tip explants. The explants cultured on this medium showed their first response by an initial enlargement of the existing shoot bud following bud break within 8-10 days (Fig.1a). From each developing bud a single shoot elongated within 12-15 days. New shoots (9-10 shoots/bud) developed adjacent to this primary shoot tip within 20-25 days (Fig. 1b). These shoots attained a highest averaging 5.64-cm in 35 days bearing 3 or 4 and 6 to 9 leaves (Table-1). All regenerated shoots were free from callus at their proximal ends. The percentage of bud break and induction declined with the increase in BAP concentration beyond the optimal level (17.76 µM Table-1). Above 17.76µM BAP caused a suppression of sprouting. Multiple shoots were induced from each sprouted bud at higher concentrations of BAP (22.20 µM), but these failed to elongate there by resulting in rosette shoot clumps (Table-1).

Table 1. Morphogenic response of shoot tip explants of *Centella asiatica* L. to different concentrations of cytokinins (BAP, KN) and GA_3. Data were collected after 45 days.

Growth Regulator (µM/l)	Percentage of response (%) mean	Number of shoots mean	Shoots length(c.m) mean
0.00	--	--	--
BAP			
4.44	59.00^{f}	2.60^{g}	0.64^{gh}
8.88	63.00^{ef}	3.20^{fg}	1.34^{g}
13.32	66.00^{e}	4.80^{f}	2.72^{f}
17.76	80.00^{bc}	11.6^{cd}	6.60^{cd}
22.20	73.00^{cd}	8.00^{de}	6.4^{ef}
KN			
4.64	38.00^{h}	2.20^{g}	0.48^{h}
9.28	49.00^{g}	3.00^{fg}	0.84^{gh}
13.92	55.00^{fg}	4.40^{f}	1.22^{g}
18.56	65.00^{e}	8.20^{de}	4.46^{e}
23.20	71.00^{d}	9.60^{d}	5.74^{de}
27.84	66.00^{e}	6.20^{e}	4.40^{e}
BAP + GA_3			
17.76 + 0.01	63.00^{ef}	8.60^{de}	5.93^{d}
17.76+ 0.14	70.00^{d}	9.20^{d}	6.54^{cd}
17.76+ 0.29	80.00^{bc}	12.8^{c}	8.64^{bc}
17.76+ 1.44	88.00^{a}	16.8^{a}	11.0^{a}
17.76+ 2.89	81.00^{b}	15.4^{ab}	7.76^{c}
KN + GA_3			
23.20 + 0.01	55.00^{fg}	6.40^{e}	3.32^{ef}
23.20 + 0.14	61.00^{ef}	8.00^{de}	5.38^{de}
23.20 + 0.29	69.00^{de}	11.2^{cd}	7.72^{c}
23.20 + 1.44	76.00^{c}	14.6^{d}	9.26^{b}
23.20 + 2.89	65.00^{e}	11.6^{cd}	8.36^{bc}

Each value was the average 15 replicates and repeated 5 times.

Mean separation using Duncan's Multiple Range Test (DMRT).

Means within column with different letters are significant at 5% level.

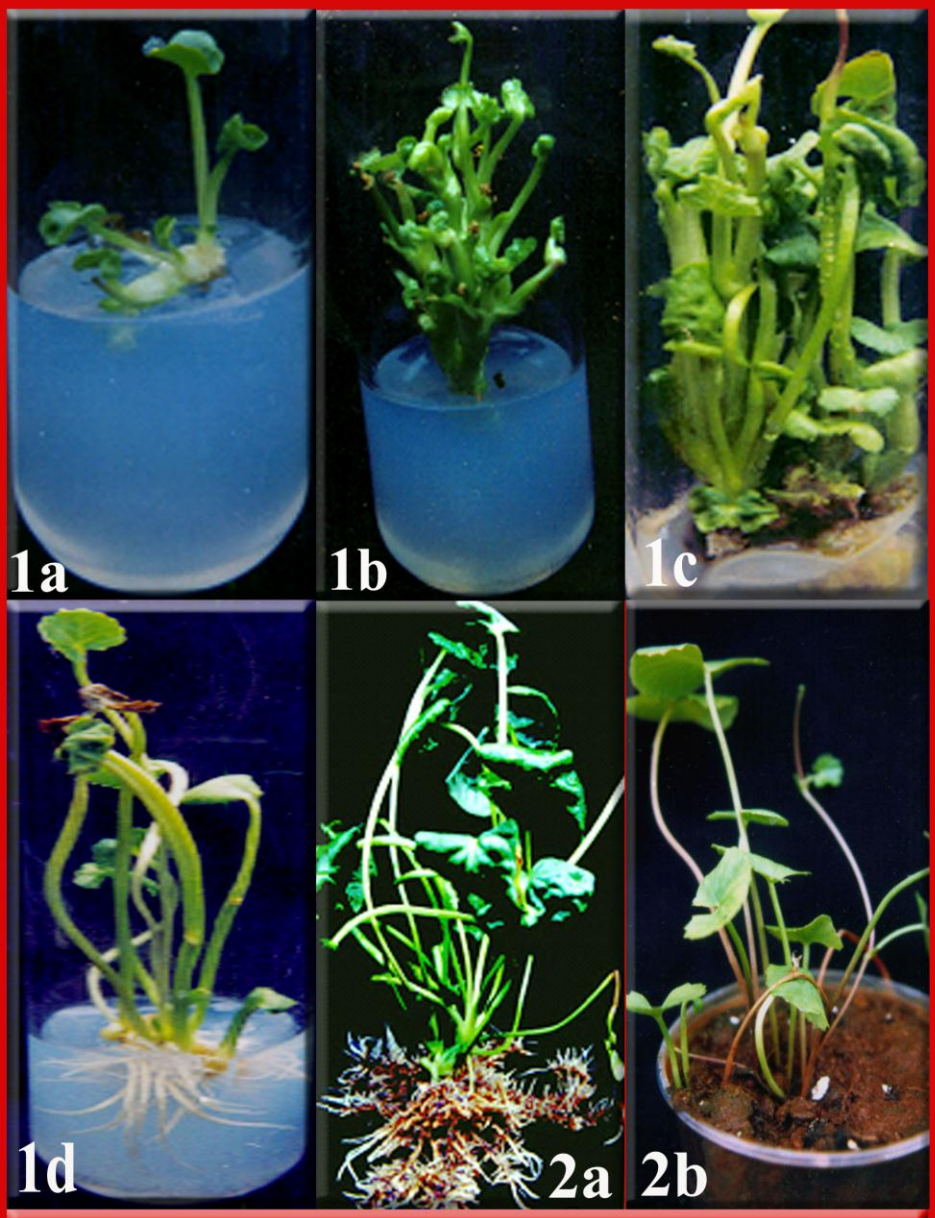

Figure 1: In vitro multiplication and Ex vitro rooting of *Centella asiatica* (L.) Urban. 1a. Initiation of bud ;1b. Enhanced shoot development ; 1c. Enhanced number of shoots during 3rd subculture; 1d. Healthy shoots with root ; 2a. Ex vitro rooting; 2b. Hardened plantlets in small plastic cup.

Fig. 1: *In vitro* multiplication and *Ex vitro* rooting of *Centella asiatica* (L.) Urban

On the other hand, when MS medium containing a singular supplement of GA_3 was used the sprouting period was lengthened, but percentage bud break and shoots per explant were substantially reduced. A maximum of 15-20% of the shoot buds sprouted within 25-30 days of culture on MS with an optimal concentration of GA_3 (1.44 µM) alone. Only one or two shoots were formed which attained a height averaging 4.8 cm within 35 days of culture. Concentrations of GA_3 higher than 1.44 µM had no promotive influence on percentage shoot development and on shoot number per explant. However, there was a progressive increase in shoot length at increasing GA_3 concentrations. A combination of optimal concentration of BAP (17.76 µM) and GA_3 (1.44 µM) in the culture medium not only induced a faster bud break (within 7 day) but also enhanced the frequency of bud break (88%). In addition a BAP-GA_3 coupling had a synergistic effect on multiple shoot formation (16.8 shoots / shoot tip explant (Fig. 1b) as well as on internode elongation (11 cm high shoots after 35 d; Table-1). The synergistic effect was also noticed in a combination of KN (23.20 µM) and GA_3 (1.44 µM), but with respect to percentage (76%) shoot development, shoot number (14.6 shoots/explant) and shoot length (9.26 cm) it was inferior to BAP plus GA_3 coupling. Besides, when the multiple shoot clumps were maintained in culture on a KN plus GA_3 medium for a very long period (50-55d), they occasionally developed few roots. These roots were slender, thin and hairy and often developed directly from the nodal region of the shoots immediately above the culture medium (Fig. 1c).

Influence of Explanting Season on Culture Establishment

The shoot proliferation was greatly influenced by the month of the year during which the explants had been collected. Highest frequency bud break (88%) coupled with maximum number of shoot formation (16.8 shoots/explant) occurred with shoot tip explants excised between September through December representing autumn season in India. Other explanting periods comparatively less suitable (Table-2), were the between January to April (winter season).

Shoot Multiplication of Axenic Shoots

Shoots excised from the primary, *in vitro* raised shoots cultured on MS medium containing BAP (17.76 µM) plus GA_3 (1.44 µM) produced 10-12 shoots per shoot tip within 4 wk. By repeated subculturing of shoots from the newly formed axenic shoots harvested from each culture passage at every 4 wk interval prolific shoot cultures free from basal callusing was established within three months. The percentage shoot multiplication as well as the number of

shoots per shoot tip attained the highest values (88%, 16 shoots/shoot tip) during the first two culture passages, beyond which there was a gradual decline in shoot bud differentiation (Fig. 1c).

Rooting of shoots

Excised shoots failed to root on half- strength MS medium without any growth regulators supplement. Addition of auxin to the culture medium induced rooting (Table-3). Of the three auxins tested, NAA (10.74 µM) was found to be most effective in inducing roots. About 90% of the excised shoots developed roots (18-19 roots per shoot) within 12-15 d of culture on half-strength MS fortified with NAA at an optimal concentration of 10.74 µM (Fig. 1d). In 30 d 25-28 roots developed from each shoot with root length averaging 4.9 cm (Table-3). On the medium supplemented with IBA (9.84 µM) the excised shoot failed to elicit rooting response even after 30 d of culture. Upto a maximum of 69% rooting could be induced on the medium fortified with 4.92 µM IBA. IAA at 0.57 – 11.42 µM induced light-brown callus formation at the proximal end of the excised shoots and two to three roots were occasionally developed after 30 days.

Table 2. Influence of different auxins on rhizogenesis of *in vitro* formed shoots of *C.asiatica.* L. Data were collected after 20 days.

Growth Regulator (µM/l)	Percentage response (%) rooting Mean	Number of roots Mean	Root length (cm) Mean
0.00	--	--	--
IAA			
2.85	--	--	--
5.71	--	--	--
11.42	23.0^{f}	2.6^{f}	0.48^{g}
17.13	Bc	Bc	Bc
IBA			
2.46	26.0^{f}	4.66^{e}	1.00^{f}
4.92	69.0^{c}	8.82^{d}	3.64^{bc}
7.38	64.0^{cd}	7.80^{de}	2.88^{d}
9.84	57.0^{d}	4.50^{e}	2.60^{de}
14.76	50.0^{de} (Bc)	3.8^{ef}(Bc)	2.0^{e} (Bc)
NAA			
2.69	44.0^{e}	12.68^{cd}	2.94^{d}
5.37	65.0^{cd}	14.70^{c}	3.36^{c}
8.06	81.0^{ab}	18.10^{ab}	4.02^{b}
10.74	90.0^{a}	27.66^{a}	4.90^{a}
13.43	80.0^{b}	20.06^{b}	4.64^{ab}
16.11	72.0^{bc}	18.20^{ab}	4.06^{b}

Bc: basal callus, -- no response.

Each value was the average 15 replicates and repeated 5 times.
Mean separation using Duncan's Multiple Range Test (DMRT).
Means within column with different letters are significant at 5% level.

Reduction in cost of *in vitro* propagation is much fascinated in recent time. Reduction in the cost of micro propagation and also the time from lab to field by *ex vitro* rooting was tried. In first method, the basal portion of the rootless shoots was kept in different concentration of IBA solutions for 10 days (Table 4). Of the different concentrations of IBA solution, 9.84 µM of IBA was most effective. The shoots started to induce roots within 10 days. All shoots developed roots. Shoots developed mean of 30.32 roots/shoot (Fig. 2a; Table. 2). Shoots rooted by this method transplanted to small pots exhibited 100% survival. In the second method of *ex vitro* rooting, the healthy shoots excised from the shoot multiplication medium after 30 days of culture were directly transferred to small pots containing red soil and vermiculite (1:3). Among 100 plants transferred, 88 plants (88%) survived.

Transfer of plantlets to soil

Plantlets (Fig. 2b) (6-8 cm high) with 3-4 fully expanded leaves and well-developed roots were successfully transferred to soil. 75-80% of the plants survived transfer to growth chamber for 2 weeks in vermiculite and red soil (3:1). The plantlets were then transferred into the glasshouse, potted in natural soil for another 2 weeks, and the potted plants were then placed outdoors under full sun. 80-85% of the plants survived transfer from the growth chamber to the glasshouse. All the potted plants placed outdoors under full sun survived. Within 8 weeks of transfer to the outdoors, it grew upto 30-40 cm with a few axillary branches developed flowered umbels and produced fertile seeds. The regenerated plants did not show any detectable variation in morphological or growth characteristics when compared with the donor plants.

DISCUSSION

The stimulatory effect of a singular supplement of BAP on bud break and multiple shoot formation in *Centella* was similar to that reported earlier in other medicinal species including *Curcuma spp. and Zingiber officinale*[13], *Hybanthus enneaspermus*[14], *Piper spp.*[15], *Ocimum spp.*[16,17], *Aegle marmelos*[18], *Tridax procumbens*[19], *Azadirachta indica*[20], *Wedelia chinensis*[21], *Ophiorrhiza mungo*[22] and *Solanum trilobatum.*[23,49,50] Our observations on the suppression of sprouting at higher KN concentrations were in consonance with those of Ahuja *et al*[24] in *Ocimum gratissimum* and *O. viride* and more recently of

Pattnaik & Chand[16] in *O. americanum* and *O. sanctum*, Sahoo and Chand[19] in *Tidax procumbens* and Malathy suryanarayanan and Jagadish[25] in *Coleus forskhlii*. In the present investigation, a BAP - GA_3 coupling had a noticeable synergistic influence on multiple shoot formation. The promotive effect of GA_3 in combination with BAP on shoot bud induction as well as internode elongation in culture was reported earlier in *Centella asiatica* [7,25,26, 48] and other perennial medicinal herbs including *Ocimum americanum*[16], *O. sanctum* and *O. basilicum*[17], *Tridax procumbens.*[19] On the contrary, GA_3 has been shown to suppress shoot bud differentiation in *Plambago indica.*[27] Thus, the role of gibberellic acid with respect to shoot bud induction in medicinal plant species remains controversial. Synergistic effect of a range of growth regulators in combination with BAP on promotion of shoot multiplication of *C. asiatica* was previously documented.[28] Various successful combinations have been reported such as BAP+IAA for *Curculico orchioides*[29], *Alpinia galangal*[30], BAP plus NAA for *Gomphrena officinalis*[31] and *Rauwolfia serpentine*[32], BAP plus GA3 for *Tridax procumbens*[19], BAP plus 2,4-D for *Withania sominifera*[33], BAP plus IBA for *Rheum emodi*[34] and *Gardenia jasminoides*[35], and BAP plus KN for *Feronia limonia*[36], *Kaempferia galangal.*[37]

Fluctuations in environmental factors in different seasons had a definite effect on shoot bud differentiation from explanted shoot tip segments in *Centella* as has been reported in other medicinal herbs including *Tridax* species[19] and *Ocimum* species.[16,24]

The multiplication rate of the shoot cultures derived from the shoot tip explants of the primary shoots was dependent on the number of the subsequent culture passages. The gradual decline in the frequency of shoot development and the number of the shoots per shoot tip as also reported earlier for *Tridax procumbens*[19] and *Ocimum basilicum*[17] is indicative of a gradual loss of the morphogenic potential concomitant with advance in culture.

Successful induction of rooting by basal dipping of *in vitro* developed shoots in IBA has also been reported in *Panax ginseng*[38] and *Ceratonia siliqua.*[39]. Rooting *ex vitro* has also been reported in *Veronica*[40], *Gardenia jasminoides*[41] and *Actinidia deliciosa.*[42] The shoots revived growth within 15 days after transfer. According to Debergh and Maene[43] cost reduction by *ex vitro* rooting accounts 40 – 75 % of the total cost of plants propagated through tissue culture, depending on the species. *Ex vitro* rooting saves the expensive growth room space also. The growth rate of the plantlets was similar to that transferred after *in vitro* rooting. In all cases the plantlets established in the field exhibited morphological characters

(stem colour, growth pattern and leaf morphology etc,) similar to that on mother plant. Even though direct *ex vitro* rooting was with less per cent of survival compared to *in vitro* rooting and IBA dipped methods, considering the reduction in cost by avoiding the *in vitro* rooting and use of IBA and the reduction in labor and time of establishment from lab to land, *ex vitro* rooting advantageous.

According to Mitra *et al.*[44] and Martin *et al.*[21] sucrose and gelling agent constitute about 80% of the media cost. Use of tap water and commercial sugar (cost Indian Rupees. Rs. 15/Kg) respectively in place of double distilled water and tissue culture grade sucrose (tissue culture grade sucrose usually costs more than $25 / Kg) bring down the cost at least by 50 – 70 percent. Cost reduction by using tap water and commercial grade sugar has been documented in banana.[45] Kodym and Zapata-Arias[46] has also reported cost reduction by use of commercial sugar in micropropagation of banana. Martin *et al.*[21] reported that use of commercial sugar and tap water along with *ex vitro* rooting makes the protocol more economic. Moreover, acquaintance of small scale farmers with low cost tissue culture technologies make possible to fulfill the pilot project " biocentres in biovillages" emphasized in the dialogue` Biotechnology in agriculture: Reaching the unreached'.[47]

The present protocol facilitates cost effective production of more than 25,000 plantlets within 160 days starting from a single shoot tip explant using single medium and enables to meet the need in time without exploiting the natural resources of this valuable medicinal plant.

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