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DEVELOPMENTAL AND HORMONAL REGULATION OF DIRECT SHOOTS AND ROOTS REGENERATION IN *COCCINIA INDICA* L

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ABSTRACT

Multiple shoots were induced from the nodal meristms in Coccinia indica L. in present study. Maximum number of shoots (4.7) per explant were observed on Murashige and Skoog (MS) medium supplemented with 2.5 mgl^{-1} 6-Benzylaminopurine (BAP). The response of explants towards the use of kinetin (Kn) was not impressive (3.6 shoots per explant) as compared to BAP. Application of auxin (Indole-3 acetic acid, IAA) in the medium caused cullogenesis from the basal part of the explants. Shoots were multiplied (47.3 shoots per culture flask) on MS medium supplemented with 1.0 mgl^{-1} each of BAP and Kn. Long and healthy shoots (4-5 cm long) were rooted (8-9 roots per shoot) on agar-gelled half-strength MS medium supplemented with 2.0 mgl^{-1} Indole-3 butyric acid (IBA). None of the shoot produced roots in ex vitro experiments even after five weeks in green house. In vitro generated plantlets were hardened in the green house and transferred to the pots and finally to the field with 100% success.

Keywords: *Coccinia indica* L., *In vitro*, Micropropagation, Rooting, Hardening.

Contribution/ Originality

This study documents the development of *in vitro* regeneration protocol for the *Coccinia indica* L. Micropropagation study is largely deficit in the literature on this plant species. The tissue culture method defined in present report is highly efficient and reproducible and 100% success has been achieved in the hardening and field transfer of the plantlets.

1. INTRODUCTION

Coccinia indica L., also known as ivy gourd, little gourd, baby watermelon, tindora or gherkin belongs to family Cucurbitaceae. *C. cordifolia* and *C. grandis* are the synonyms of *Coccinia indica*. It is a perennial herb which is growing in semi-arid regions [1]. It is widely distributed in the tropical regions of the world and has immense usages in everyday life from food to medicines, as it is rich in nutritious elements. The fruit is more interesting, which is slimy in touch, pulpy,

barrel shaped, green in raw form, and it becomes red when it ripens. It also possesses about ten white strips on its posterior portion.

In rural India, *C. indica* has been harvested and sold in urban and local markets as green vegetable providing a source of nutrition rich food supplement. It contains saponins, flavonoids, sterols and alkaloids. Saponin and flavonoid are found to be responsible for antidiabetic activity [2]. The fruit possesses mast cell stabilizing, anti-anaphylactic and antihistaminic potential [3].

This plant has been wildly used in Indian traditional medicinal systems. The whole plant extract of *C. indica*, showed the strong free radical scavenging activity almost same as that of Ginseng [4]. Leaf extract of Ivy Gourd is effective against malarial parasites. The crude methanolic extract of Ivy Gourd showed the highest larvicidal activity among the cucurbitaceous plants tested [5, 6]. In the global market, Ivy vine is a principal ingredient in several products. It is one of the important ingredient in medicinal supplements like, "Sugar balance, Fat free" etc. as a natural relief for diabetes, chronic sinuses, psoriasis itch, sores, pityriasis, gonorrhea, glycosuria, urinary tract infection and respiratory diseases [7-9].

Currently there has been an increased interest globally to identify antioxidant compounds in *C. indica* that are pharmacologically potent and have low or no side effects for use in preventive medicine and the food industry. Micropropagation technique is of special use for the conservation of this valuable genotype. The present study deals with the development of an efficient micropropagation protocol for *C. indica*, using somatic tissues as explants.

2. MATERIALS AND METHODS

2.1. Collection of Plant Material

Field surveys were conducted for identification and selection of mature and superior plants of *Coccinia indica* L. Fresh sprouts were collected from the east-coast region of India during the months of January, 2012 to March, 2013. Some plants were also maintained in the green house to get healthy sprouts which could be used for the initiation of cultures. Different types of explanting materials namely, axillary/terminal shoots, shoot apices/nodal shoot segments were harvested from selected plants. The fresh sprouts produced from pruned plants were brought to the Plant Biotechnology Laboratory.

2.2. Inoculation of Shoots from Nodal Segments

The explants were cleansed, dressed and treated with 0.1% solution of Bavistin, (a systemic fungicide, BASF, India Ltd.). These were surface sterilized with 0.1% $HgCl_2$ for 4-5 minutes and washed 6-8 times with autoclaved distilled water under laminar air flow cabinet (Technico Systems, Chennai, India). The surface sterilized explants were dipped in 90% ethanol for a short period (40 seconds) of time.

The explants were inoculated horizontally and vertically on MS medium [10] for culture initiation. Sucrose and sugar cubes were added as a source of carbohydrate in the medium. Different concentration and combination of cytokinins (BAP and Kn ranging from 1.0 to 4.0 mgl^{-1}) and auxins (IAA and IBA ranging from 0.1 to 1.0 mgl^{-1}) were incorporated in the

medium to induce bud breaking. These cultures were incubated at 25±2°C in the dark for 2-3 days. Subsequently these were kept under diffused light (22 µmol m^{-2} s^{-1} Spectral Flux Photon, SFP) for 8-10 days.

2.3. Multiplication of Shoots

The *in vitro* regenerated shoots were multiplied by repeated transfer of mother explants and subculturing of *in vitro* generated shoots on fresh medium. The shoot-clumps were subcultured in the culture flasks. MS medium supplemented with various concentration and combination of cytokinins (BAP and Kin ranging from 0.1 to 2.0 mgl^{-1} and IAA; 0.5 to 1.0 mgl^{-1}) were used for multiplication of cultures. The cultures were incubated at 25±2°C temperature, 60-70% RH and 30-35 µmol $m^{-2}s^{-1}$ SFP for 12 h/d.

2.4. Induction of Roots from the *In vitro* Regenerated Shoots

Experiments were conducted to induce roots from the *in vitro* raised shoots. The long (4-5 cm) and healthy shoots were separated from the bunch of multiplied shoots. These were inoculated vertically on full and half strength MS medium. The medium was incorporated with auxins (IAA and IBA) with different concentrations (ranging from 0.5 mgl^{-1} to 3.0 mgl^{-1}). Activated charcoal (200 mgl^{-1}) was also added to the medium for better root initiation and multiplication. The cultures for rooting experiments were kept in dark for one week. Experiments were also conducted to induce *ex vitro* rooting from the cut ends of *in vitro* raised shoots. 100 to 500 mgl^{-1} IBA was used for pulsing to induce roots from the shoots. The pulse treated shoots were put in the bottles containing autoclaved soilrite for rooting as well as hardening in the green house.

2.5. Hardening of Plantlets

The *in vitro* rooted plantlets were taken from the medium and washed with distilled water to remove adhered nutrient agar. These were then transferred to the bottles containing autoclaved soilrite and moistened with autoclaved half strength MS salts. The *in vitro* rooted plantlets and *ex vitro* rooting experiments (shoots) were hardened in green house. The caps of bottles were gradually loosened after fifteen days. The hardened plantlets were transferred to the pots after five weeks.

2.6. Observation and Data Analysis

The cultures were regularly subcultured on fresh medium after 4-5 weeks interval. The observations were taken after every five days of inoculation. The experiments were repeated thrice with ten replicates per treatment. The data were statistically analyzed using SPSS ver. 17 (SPSS Inc., Chicago, USA). The significance of differences among mean values was carried out using Duncan's multiple range tests at $P < 0.05$.

3. RESULTS AND DISCUSSIONS

3.1. Collection of Explants and Initiation of Cultures

Various parts of the *Coccinia indica* have been used as traditional medicine in India from time immemorial. The medicinal utilities have been described, especially for leaf, fruit, root and stem. The leaves contain some pharmaceutically important compounds like alkaloids, proteins and amino acids, tannins, saponins, flavonoids, phytosterol, triterpenes [2]. Studies were conducted to establish cultures from selected plants of *C. indica* during present investigation. Plants were first established in the green house for better management of the source plants, so as to facilitate harvesting desired type(s) of explanting materials at appropriate time.

The stem of *C. indica* being soft and delicate is very sensitive to physical handling and surface sterilization. In case of *C. indica* extra care must be taken while selecting the explant and surface sterilizing it. The explant age (physiological status), the season of collection, size of explants and plant quality are some of the factors which play important role in the initiation and multiplication of culture [11-13]. The explants harvested during the months of January-March found to be the most suitable for the initiation *in vitro* cultures of *C. indica*. The nodal meristem was induced within seven days after inoculation.

Shoot induction was not found on basal medium even after four weeks of culture inoculation. The explants were found to be swollen and they produced two to three shoots within three - four weeks after inoculation on MS containing BAP and Kn in combination. The number of shoots was increased up to four when the explants cultured on MS medium augmented with 2.5 mgl^{-1} BAP alone (Figs. 1A, 4A). Both shoot tips and nodal explants responded identically in the same medium. Kn alone and Kn with IAA were not found suitable for shoot induction as shown in Fig. 1B. Protocol for indirect organogenesis and plantlets regeneration in *Coccinia grandis* from nodal explants was reported by Thiripurasundari and Rao [14]. The maximum frequency of organogenic callus induction was observed with MS medium supplemented with 0.1 mg^{-l} naphthalene acetic acid (NAA) and 1.0 mg^{-l}benzyladenine (BA) by them. But in case of *C. indica* all the shoots produced buds while subjected to either BAP or Kn in present study.

The effect of auxins on multiple shoot induction was also investigated. The addition of IAA in the medium along with BAP and Kn caused callus formation from the base of the explants, especially the part of explants which is submersed in the medium. There was no effect of IAA on the induction of number of shoots from the nodal meristem. Repeated subcultures of explants on fresh medium helped to achieve continuous production of healthy shoot buds and shoots at least through five to 6 subculture cycles (Fig. 4B).

3.2. Multiplication of Shoots in Cultures

Among the many newly differentiated shoots from the nodal meristem, only one to two shoots were elongated as a result of apical dominance. To achieve uniform growth of differentiated shoots and to eliminate apical dominance, apices of leader shoots were excised. The amendment promoted differentiation of 10-15 shoots. Such amendment was also found necessary for uniform growth of shoots during subculturing and propagation.The shoots that proliferated

from the original explants were harvested and the initial explants along with the base of the regenerated shoots were repeatedly transferred onto the fresh medium. Fresh crop of shoots (12-13 shoots per cycle per node; average shoot length 4-5 cm) could be harvested from the original explants up to four cycles at intervals of four weeks.

Maximum numbers of shoots (47.3) per culture bottle were regenerated when the shoot clumps were inoculated on MS medium supplemented with 1.0 mgl^{-1} each of BAP and Kn in the present investigation (Figs. 2, 4C-4E). Thiripurasundari and Rao [14] have achieved maximum multiple shoots on MS medium with the increased numbers of shoots (8.7) and the shoot mean length (8.8cm) on 0.1mg^{-l} NAA, 1.0 mg^{-l} BA and 0.5 mg^{-l} Kin in *C. grandis*. *In vitro* regenerated shoots exhibited varied responses when subcultured on various other media combinations. These were generally exhibiting severe defoliation after two weeks of subcultures under the same conditions. Less numbers of shoots (24.2) were produced on the higher concentrations of BAP; however, these shoots were stunted in growth response and observed vitrified. Similar types of results were reported in case of *Arnebia hispidissima* and *Salvadora persica* on MS medium supplemented with BAP [15, 16]. Cullogenesis was observed when auxins (IAA) were added in the medium even at low concentrations. This affects the rate of multiplication *in vitro* and the shoots were not healthy for root induction in the next stage.

3.3. Rooting and Hardening

In vitro rooting of individual shoots was achieved on ½ strength agar-gelled MS medium containing 2.0 mgl^{-1} of IBA without activated charcoal. Cent-percent response was recorded in terms of *in vitro* rooting with IBA. About 8-9 roots of length 3 to 4 cm were obtained after four weeks of inoculation (Figs. 3A, 4F, 4G). The *in vitro* root induction was low (4-5 roots per shoot) on medium supplemented with IAA (Fig. 3B). Diffused light (10-20 $\mu molm^{-2}s^{-1}$ SFP) also favored *in vitro* root induction. Delayed rooting was observed under higher light intensity (30 to 40 $\mu mol\ m^{-2}s^{-1}$ SFP). Induction of rooting is affected by several intrinsic and extrinsic factors [17, 18]. *In vitro* produced shoots could not be rooted under *ex vitro* treatment conditions. Thick shoots of length 4 to 5 cm were harvested and then pulse treated with IBA. None of the shoots rooted after four weeks of this treatment.

The concentration of IBA and way of its treatment influenced the root induction [19]. Higher concentrations (more than 2.0 mgl^{-1}) of IBA decrease in root number and reduce the root length but it favors callus formation at the cut ends of the shoots. Similar results were also reported in case of *Momordica dioica* and *Arnebia hispidissima* [15, 20]. These rooted plantlets were then subjected to harden in the green house (Fig. 4H, 4I). About 95% plantlets were successfully hardened and transferred to the field. The results of the present investigation demonstrated the establishment of a reliable *in vitro* plant regeneration protocol for *Coccinia indica*.

4. ACKNOWLEDGEMENTS

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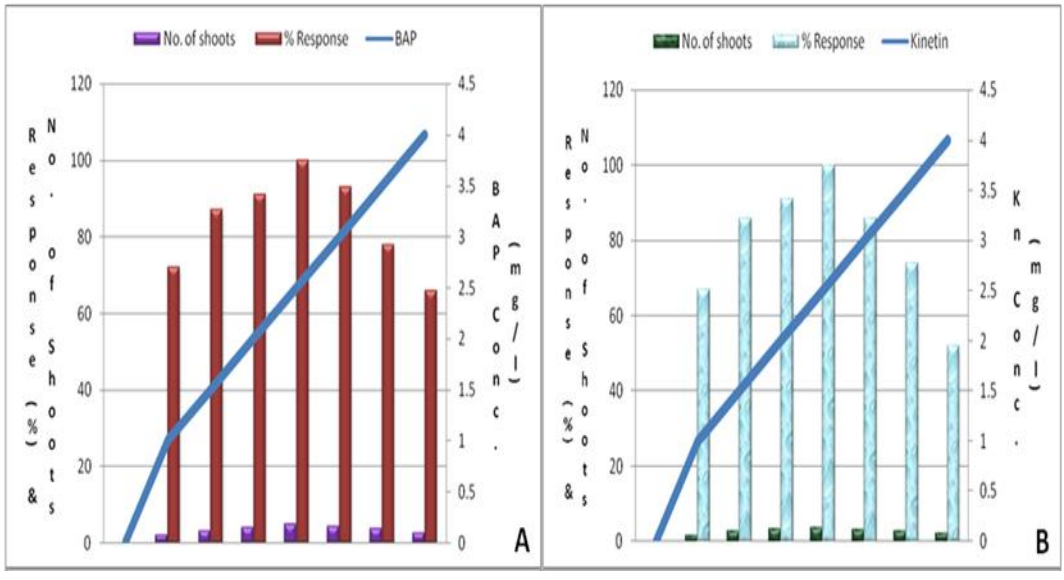
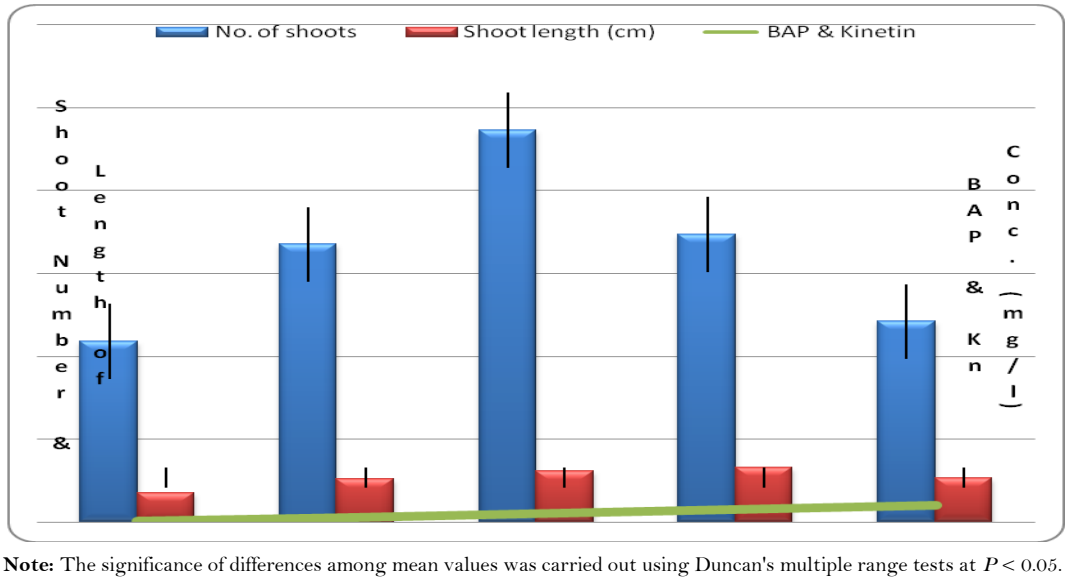
FIGURES

Fig-1. (A) The effect of BAP on induction of shoots from the explants of *C. indica*on MS medium, (B) Effect of Kn on induction of shoots.



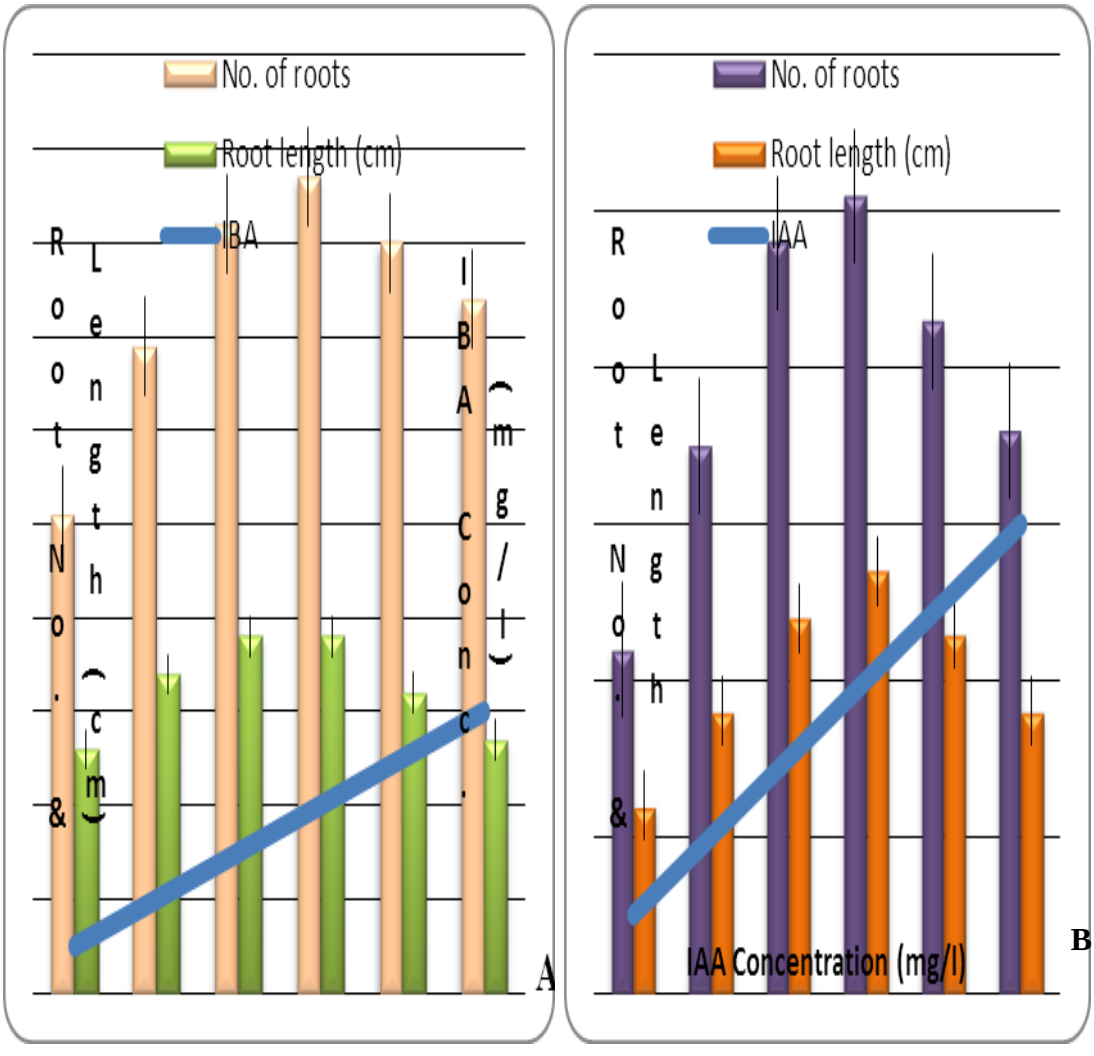
Note: The significance of differences among mean values was carried out using Duncan's multiple range tests at $P < 0.05$.

Fig-2. The effects of Cytokinin (BAP and Kn) and their concentrations on multiplication of shoots from sub-cultured shoots on MS Medium.



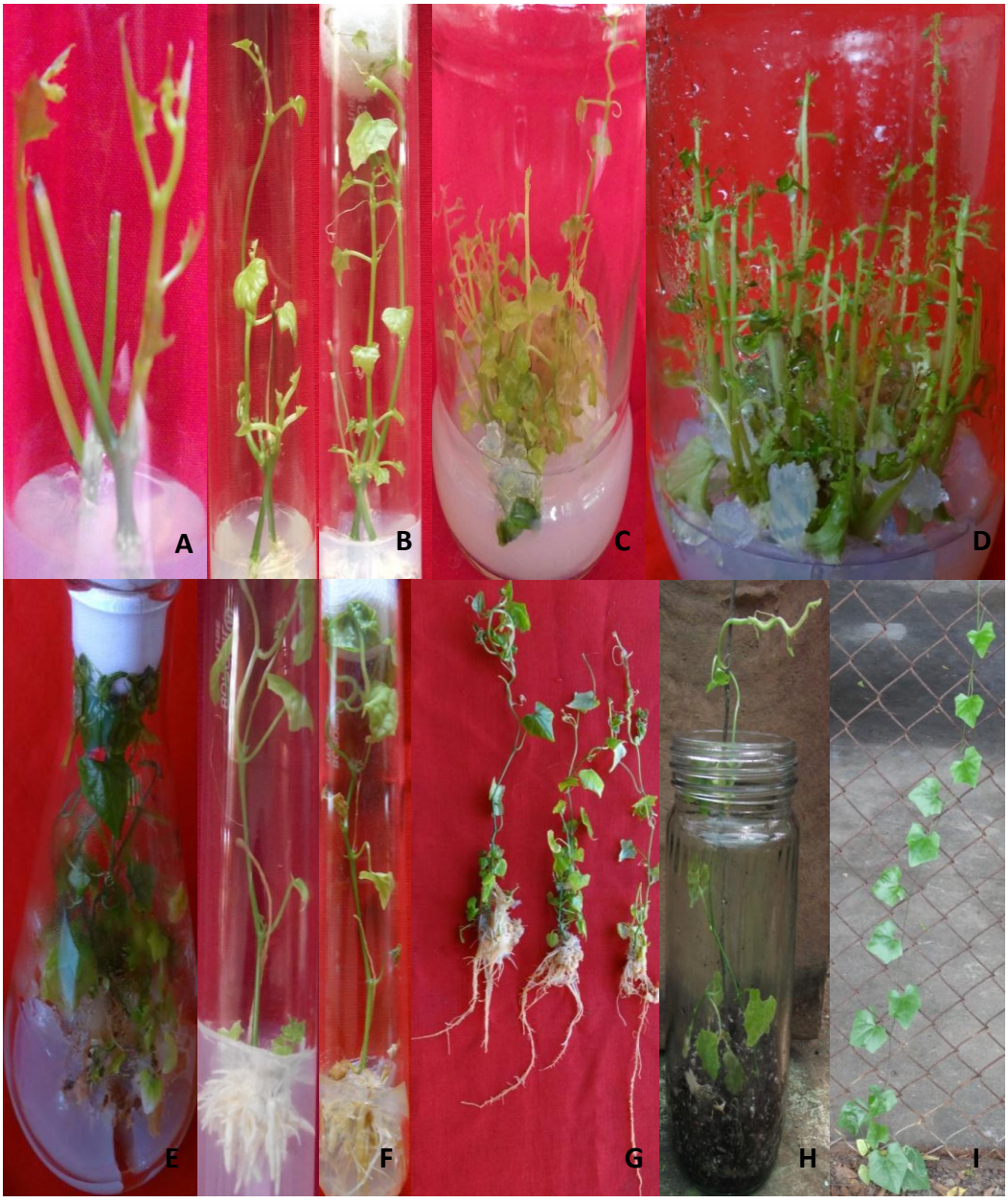
Note: The significance of differences among mean values was carried out using Duncan's multiple range tests at $P < 0.05$.

Fig-3.(A) The effect of IBA on *in vitro* induction of roots from *in vitro* generated shoots on half strength MS medium, (B) Effect of IAA on induction of roots from the shoots *in vitro*.



Note: The significance of differences among mean values was carried out using Duncan's multiple range tests at $P < 0.05$.

Fig-4. (A) Induction of shoots from the nodal meristems of explants, (B) Elongation of shoots, (C, D and E) Multiplication of shoots of *C. indica in vitro*, (F and G) Induction of roots from the base of shoots with IBA, (H) Hardening of *in vitro* raised plantlets in green house and (I) Hardened plant in the field.



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