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Direct regeneration of plantlets from shoot tip explants of a vulnerable medicinal plant – *Celastrus paniculatus* Willd.

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Abstract

A study was undertaken to develop a rapid efficient direct propagation protocol of *Celastrus paniculatus* Willd, a medicinal vulnerable plant. Half strength Murashige and Skoog's (MS) medium supplemented with GA_3 showed maximum percentage (82.4 ± 0.50) embryo response through embryo rescue method. Shoot tip explants were transferred from cotyledonary node and inoculated to shoot induction medium supplemented with cytokinins (BAP, TDZ and Kin) and highest response (87 ± 0.70) with 3.8 shoot number was achieved in BAP 1.0 mg L^{-1}. Shoot multiplication was achieved with combination of BAP (1 mg L^{-1}) with meta-Topolin (1 mg L^{-1}) which showed highest response (91.0 ± 1.10) with 10.2 shoots within 10 days after inoculation. The *in vitro* regenerated shoots were transferred carefully to the half strength and full-strength MS medium supplemented with GA_3 (0.1 to 0.5 mg L^{-1}) for elongation. The *in vitro* elongated shoots were treated with different auxins (IAA, IBA and NAA) individually for early rooting and treated shoots were transferred to the half strength MS medium. At 0.3 mg L^{-1} IBA concentration, 91 % rooting was observed. The regenerated plantlets were acclimatized in pots containing sterilized soil and sand with 3:1 ratio and plantlets were then transferred to the field conditions. Ninty percent of the regenerants survived well. The result of this study revealed the pioneer report on *in vitro* plant regeneration of *C. paniculatus*. by using shoot tip explants.

Key words: *Celastrus paniculatus* Willd., embryo rescue method, shoot tip explant, *in vitro* micropropagation.

Introduction

Celastrus paniculatus Willd., commonly known as Prog or Malkangi, Jyotsmati or Bitter Sweet, is an important medicinal plant belonging to the family Celastraceae and known for centuries as the 'Elixir of Life'. It is a woody, climbing shrub with a height upto 10-18 m and common to all over the hilly parts of the India. In Indian traditional system of medicine, it is believed to sharpen the memory and also used to cure depression, paralysis, leprosy, fever and antiemetic (Handa, 1998; Warrier and Nambiar, 1993; Kumar and Gupta, 2002) and its roots are also used in the treatment of cancerous tumors (Parrotta, 2001). India is the largest producer of medicinal plants and is rightly called the Botanical garden of the world (Govind and Madhuri, 2006). Meditional plants are economically important in pharmaceutical industry as raw material and essential for human health, hence it is essential that one should focus on micropropagation of these plants so as to develop new or more safe drugs (Ikram, 1983) but unfortunately indiscriminate collection from natural sources of these plants poses a serious threat to existence in the wild. Rekha *et al.* (2005) reported, in conventional methods of propagation of *C. paniculatus,* poor seed viability and less percentage of germination (11.5 %) is a major issue of using the seed in multiplication.

It is well known that *C. paniculatus* seed oil contains sequiterpene alkaloids such as celapanin, celapanign and celapagin which are making this plant as highly potential meditional plant (Godkar *et al.*, 2004). Due to the unreliability on harvest of phytochemicals from natural sources and the complexity in producing natural products through chemical synthesis, one has to look for an environment friendly and sustainable production system of the plant to fulfill growing needs of pharmaceutical industry.

Natural regeneration has major limitations like long life cycle, seasonal response and loss of viability (Parimala *et al.*, 2009). *In vitro* propagation is in use to maintain the superior, genetically stable and virus free material (Winton, 1970). *In vitro* shoot regeneration has been achieved from mature explants of several woody climbers and trees (Mascarenhas and Muralidharan, 1989 and Anitha and Pullaiah, 2002) and shrubs (Cheepala *et al.*, 2006; Nagori and Purohit, 2006; Javadian *et al.*, 2017). *In vitro* studies such as induction of multiple shoots and complete plantlets from plant tissue culture is very less reported in *C. paniculatus*. Regeneration in *C. paniculatus* via callus cultures, shoot bud differentiation and nodal segments obtained from 12 years old plant but there is no report on its regeneration via shoot tip explants of cotyledonary derived seedlings.

In view of its medicinal, industrial application, the species has been over exploited and now is considered a vulnerable species (Martin *et al.*, 2006; Lal and Singh, 2010; Singh *et al.*, 2017), mainly in Western ghats of South India (Rajesekharan, 2002). Moreover the poor seed germination is because of inhibitory compounds present in the seed (De Silva and Senarath, 2009). With this background the present investigation aimed to develop the germination percentage by embryo rescue method and develop regeneration protocol by using shoot tip explants derived from seedlings of *C. paniculatus* willd.

Materials and methods

Media and culture conditions: The nutrient medium used in all the experiments consisted of Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962), 3.0 % sucrose (Hi-Media, Mumbai, India) with various concentrations of different plant growth regulators (PGR's) and pH was adjusted to 5.6 to 5.8 by using 1N HCl or NaOH. The media was solidified with 0.7 % (w/v) agar and was sterilized for 15 min at 121 °C. The culture was maintained at temperature of 25±2 °C, 16 h photoperiod having a light intensity of 3000 lux supplied by cool white fluorescent lamps (Philips, India) and > 85 % relative humidity.

Embryo rescue method: The seeds were surface sterilized with 0.1 % mercuric chloride ($HgCl_2$) for 5 min followed by washing with distilled water four to five times. Seed material were then taken to the laminar airflow chamber and treated with 0.1 % $HgCl_2$ for 2 to 3 times and washed with autoclaved double distilled water to remove the remaining mercury traces. Total of 100 healthy seeds were selected, 50 seeds were selected for embryo rescue method and another 50 seeds used as such for germination. The explants were inoculated on full strength and half strength MS medium alone and alongwith gibberellic acid (GA_3) to determine the optimum concentration for embryos responding.

Shoot initiation, elongation and multiplication: For shoot initiation, the *in vitro* regenerated shoot tip explants were excised aseptically and entrenched on MS medium supplemented with different combinations of BAP, TDZ and Kin (0.0 to 2.0 mg L^{-1}) and incubated in 16.8 h light/dark conditions for 2 weeks. Even though multiple shoots were induced from shoot tip explants, for increasing the multiplication rate, regenerated aseptic cultures (shoots) were subsequently transferred on the fresh MS medium supplemented with BAP best concentration for shoot initiation in combination with meta-Topolin (1.0 mg L^{-1}) for shoot multiplication. Shoots with 7-8 cm length were excised on full and half strength MS medium supplemented with gibberellic acid ranged from 0.1 to 0.5 mg L^{-1} for shoot elongation. All cultures were maintained under the same incubation conditions as have been described above. The frequency of explants producing shoots per explant and shoot length was recorded after every 2 weeks of culture.

Rooting, hardening and acclimatization: The *in vitro* regenerated shoots were excised in aseptic condition and implanted on full strength and half strength MS medium with and without growth regulators (IAA, IBA and NAA) ranging from 0.1 to 1.0 mg L^{-1}. The rooted plantlets were taken out from rooting medium and washed with autoclaved water to remove the traces of agar-agar. The well rooted plantlets were covered with transparent polythene bags to ensure high humidity and maintained in growth chamber and watered every alternative day with half strength MS salts lacking organic supplements (Ahmed *et al.*, 2017). The acclimatized plants were transferred to pots containing normal garden soil and sand (autoclaved) in 2:1 ratio and maintained in green house conditions for further growth and for field experiments.

Statistical analysis: Every treatment conducted in a completely randomized design of ten replicates contained one explant and every experiment was repeated three times. The data were subjected to analysis of variance (ANOVA) using SPSS. The significance of differences among means was carried out using Duncan's multiple range test at P=0.05. The results were represented as mean ± standard error of three repeated experiments.

Results and discussion

Embryo rescue method: It is well known that *Celastrus paniculatus* seeds are deeply dormant because seed coat contains seed inhibitors (De Silva and Senarath, 2009). Because of this reason this study was aimed to increase the germination by using embryo rescue method. For the germination, seed coat was removed very precisely and embryo was placed half strength and full-strength MS medium with different concentrations of GA_3. Among the concentrations, the best response was achieved at half strength MS in combination with GA_3 (0.15 mg L^{-1}) (Table 1). GA_3 250 ppm produced a maximum of 74.7 % of germination in *Parthenium argentatum* gray seed (Dissanayake *et al.*, 2010). Our results supported the findings of Dayarani *et al.*, (2014) who found that embryo rescue method is suitable for increasing the germination percentage in *Musa ornata*.

Table 1. *In vitro* response of cultured embryos of *C. paniculatus* on Murashige and Skoog basal medium containing plant growth hormone GA_3

MS + GA_3 (mg L^{-1})	MS/2 + GA_3 (mg L^{-1})	Embryos responded (%)
Control	Control	10.2 ± 0.68^{i}
0.05	-	34.4 ± 2.30^{h}
0.10	-	41.8 ± 1.48^{g}
0.15	-	76.4 ± 1.51^{b}
0.20	-	66.8 ± 1.30^{c}
0.25	-	53.2 ± 0.83^{e}
-	0.05	42.0 ± 0.70^{g}
-	0.10	46.8 ± 1.30^{f}
-	0.15	82.4 ± 1.14^{a}
-	0.20	65.0 ± 1.58^{d}
-	0.25	54.6 ± 1.14^{e}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Shoot initiation, elongation and multiplication: The development of plant regeneration protocol from shoot tip segment is an effective way to multiply selected variety true to its type showing the same agronomic characteristics (Ahmed and Anis, 2014). In the present experiment, explants cultured on MS basal medium without any PGRs (Plant Growth Regulators) did not induce any morphogenetic response and failed to initiate the shoots. Shoot induction was observed when shoot tip explants were cultured on a medium supplemented with different concentrations of BAP, Kin and TDZ (0.5 to 2.0 mg L^{-1}) (Table 2). Shoots were initiated from shoot tip explant after 10 to 15 days of inoculation. Highest mean shoot length (2.26 ± 0.06 cm) with 87 % response was observed in MS medium supplemented with BAP 1.0 mg L^{-1}, whereas with increasing the concentration of BAP, shoot length was decreased (Table. 2). Kin and TDZ was found to be less effective in causing shoot initiation. The

Table 2. Effect of cytokinin's (BAP, Kin and TDZ) on shoot initiation from Shoot tip explants of *C. paniculatus*

BAP (mg L^{-1})	Kin (mg L^{-1})	TDZ (mg L^{-1})	Percentage of Response	Shoot Number (Mean ± SE)	Shoot Length (cm) (Mean ±SE)
0.0	-	-	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.0
0.5	-	-	41.33 ± 0.37^{d}	1.8 ± 0.37^{bcd}	0.86 ± 0.05^{d}
1.0	-	-	87.00 ± 0.70^{a}	3.8 ± 0.35^{a}	2.26 ± 0.06^{a}
1.5	-	-	54.66 ± 0.69^{c}	2.2 ± 0.31^{bc}	1.22 ± 0.03^{c}
2.0	-	-	39.33 ± 0.70^{d}	1.4 ± 0.24^{cd}	0.74 ± 0.04d^{ef}
-	0.0	-	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
-	0.5	-	29.66 ± 0.50^{f}	1.6 ± 0.40^{bcd}	0.66 ± 0.09^{e}
-	1.0	-	69.33 ± 0.80^{b}	2.8 ± 0.37^{ab}	1.40 ± 0.05^{b}
-	1.5	-	40.66 ± 1.20^{d}	2.4 ± 0.50^{bc}	1.12 ± 0.03^{c}
-	2.0	-	25.00 ± 0.70^{g}	1.4 ± 0.24^{cd}	0.78 ± 0.04^{de}
-	-	0.0	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
-	-	0.5	34.33 ± 0.65^{e}	1.4 ± 0.33^{cd}	0.72 ± 0.02^{def}
-	-	1.0	71.33 ± 0.31^{b}	2.3 ± 0.38^{bc}	0.59 ± 0.06^{fg}
-	-	1.5	54.66 ± 0.69^{c}	1.7 ± 0.42^{bcd}	0.43 ± 0.09^{gh}
-	-	2.0	35.00± 0.31^{e}	0.9 ± 0.24^{d}	0.31 ± 0.09^{h}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

capability of BAP in shoot initiation was observed in nodal explants of *C. paniculatus* (Phulwaria *et al.*, 2013) and other species (Kumar *et al.*, 2010; Singh and Tiwari, 2012; Pathak *et al.*, 2017) as well. The increased shoot length with BAP than Kin and TDZ may be because of its effectiveness to stimulate plant tissues to metabolize the natural endogenous hormone to induce the shoot organogenesis (Ahmed and Anis, 2014). It is well known that meta-Topolin plays positive influence than other plant growth regulators in shoot multiplication (Wojtania and Agnieszka, 2010). In this study to increase the shoot multiplication in the subsequent cultures, shoots were sub cultured on MS medium containing BAP (1.0 mg L^{-1}) in combinations with meta-Topolin (0.5-2.0 mg L^{-1}). Among the combination's BAP (1.0 mg L^{-1}) with meta-Topolin (1.0 mg L^{-1}) was found to be most suitable for shoot multiplication and growth (Table 3).

Earlier reports stated that meta-Topolin plays a significant role in controlling the hyper hydricity, necrosis, and also delay senescence (Dobranszki *et al.*, 2002; Aremu *et al.*, 2012; Malá *et al.*, 2013). The advantage of meta-Topolin over BAP was reported in some crops such as *Beta vulgaris* (Kubalakova and Strnad, 1992), *Malus domestica* (Dobranszki *et al.*, 2002) and banana (Bairu *et al.*, 2008). This is the first report to regenerate *C. paniculatus* by using shoot

Table 3. Effect of BAP optimum concentration with meta-Topolin on multiple shoot production from BA derived shoots of *C. paniculatus* Willd

BAP (mg L^{-1})	Meta-Topolin (mg L^{-1})	Percentage of Shooting	Shoot Number (Mean ± SE)	Shoot Length (cm) (Mean ±SE)
1.0	0.5	55.33 ± 1.02^{c}	6.2 ± 0.37^{c}	3.2 ± 0.37^{b}
	1.0	91.00 ± 1.1^{0}a	10.2 ± 0.35^{a}	6.2 ± 0.36^{a}
	1.5	61.66 ± 0.80^{b}	7.2 ± 0.37^{b}	3.6 ± 0.24^{b}
	2.0	37.66 ± 0.70^{d}	3.6 ± 0.24^{c}	1.6 ± 0.24^{c}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Table 4. Effect of GA_3 on shoot elongation of *C. paniculatus* Willd

MS + GA_3 (mg L^{-1})	MS/2 + GA_3 (mg L^{-1})	Percentage of Shoots Respond	Length of Shoot (mean ± SE)
0.1	-	61.66 ± 0.92^{c}	4.02 ± 0.37^{c}
0.2	-	73.00 ± 0.50^{b}	5.60 ± 0.08^{b}
0.3	-	90.33 ± 1.06^{a}	8.10 ± 0.22^{a}
0.4	-	78.33 ± 1.22^{b}	5.98 ± 0.20^{b}
0.5	-	63.33 ± 0.70^{c}	4.80 ± 0.28^{c}
-	0.1	40.66 ± 0.81^{e}	2.90 ± 0.05^{d}
-	0.2	52.6 ± 0.58^{d}	4.24 ± 0.13^{c}
-	0.3	67.00 ± 0.70^{c}	6.04 ± 0.08^{b}
-	0.4	53.33 ± 0.86^{d}	4.66 ± 0.09^{c}
-	0.5	42.66 ± 1.12^{e}	3.80 ± 0.15^{cd}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Table 5. Effect of auxins on root induction of *C. paniculatus*

Plant Growth Hormone	Conc-entration (mg/L)	Percentage of rooting	Root number (mean ± SE)	Root length (mean ±SE)
IBA	0.1	55.33 ± 0.02^{f}	4.2 ± 0.37^{de}	3.2 ± 0.37^{f}
	0.3	69.33 ± 0.88^{cde}	6.2 ± 0.33^{c}	4.5 ± 0.42 ^{cde}
	0.5	91.66 ± 0.10^{a}	10.1 ± 0.35^{a}	6.2 ± 0.36^{a}
	1.0	72.00 ± 0.15^{cd}	7.0 ± 0.65^{bc}	5.0 ± 0.75^{bcd}
IAA	0.1	56.00 ± 0.72^{f}	3.8 ± 0.53^{def}	3.6 ± 0.24^{ef}
	0.3	63.33 ± 0.55^{def}	4.7 ± 0.22^{d}	4.2 ± 0.22^{def}
	0.5	82.66 ± 0.70^{b}	7.3 ± 0.37^{b}	5.6 ± 0.24^{ab}
	1.0	65.33 ± 0.23^{def}	3.1 ± 0.24^{fg}	4.2 ± 0.53 ^{de}
NAA	0.1	49.00 ± 0.55	3.6 ± 0.26^{ef}	3.9 ± 0.53^{ef}
	0.3	61.33 ± 0.78^{ef}	4.5 ±0.33^{de}	4.1 ± 0.42^{def}
	0.5	75.00 ± 0.68^{bc}	6.8 ±0.42^{bc}	5.2 ± 0.33^{bc}
	1.0	60.00 ± 0.52$_{ef}$	2.5 ± 0.83^{g}	3.9 ± 0.37^{ef}

Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

tip explants generated via cotyledonary node. Soon after multiplication, shoots were transferred to the half strength and full-strength MS medium supplemented with gibberellic acid and best response, 90.33 % with 8.10 cm shoot length of shoot elongation in *C. paniculatus,* was noticed at 0.3 mg L^{-1} GA_3 (Table 4). The elongated shoots were transferred to half strength MS medium with and without growth regulators (0.1-1.0 mg L^{-1} IAA, IBA and NAA). The highest percentage was achieved at IBA (0.5 mg L^{-1}) after 15 days of culture (Fig.1). Pulwaria *et al.* (2013) also reported *ex vitro* rooting of micropropagated shoots in *C. paniculatus* and found IBA as a better auxin for shoot induction. Although the roots are initiating from shoots, time taken for initiating roots is more. To reduce the number of days for initiating the roots, shoots were pretreated with liquid MS medium (without agar) supplemented with different auxins (IAA, IBA and NAA) for

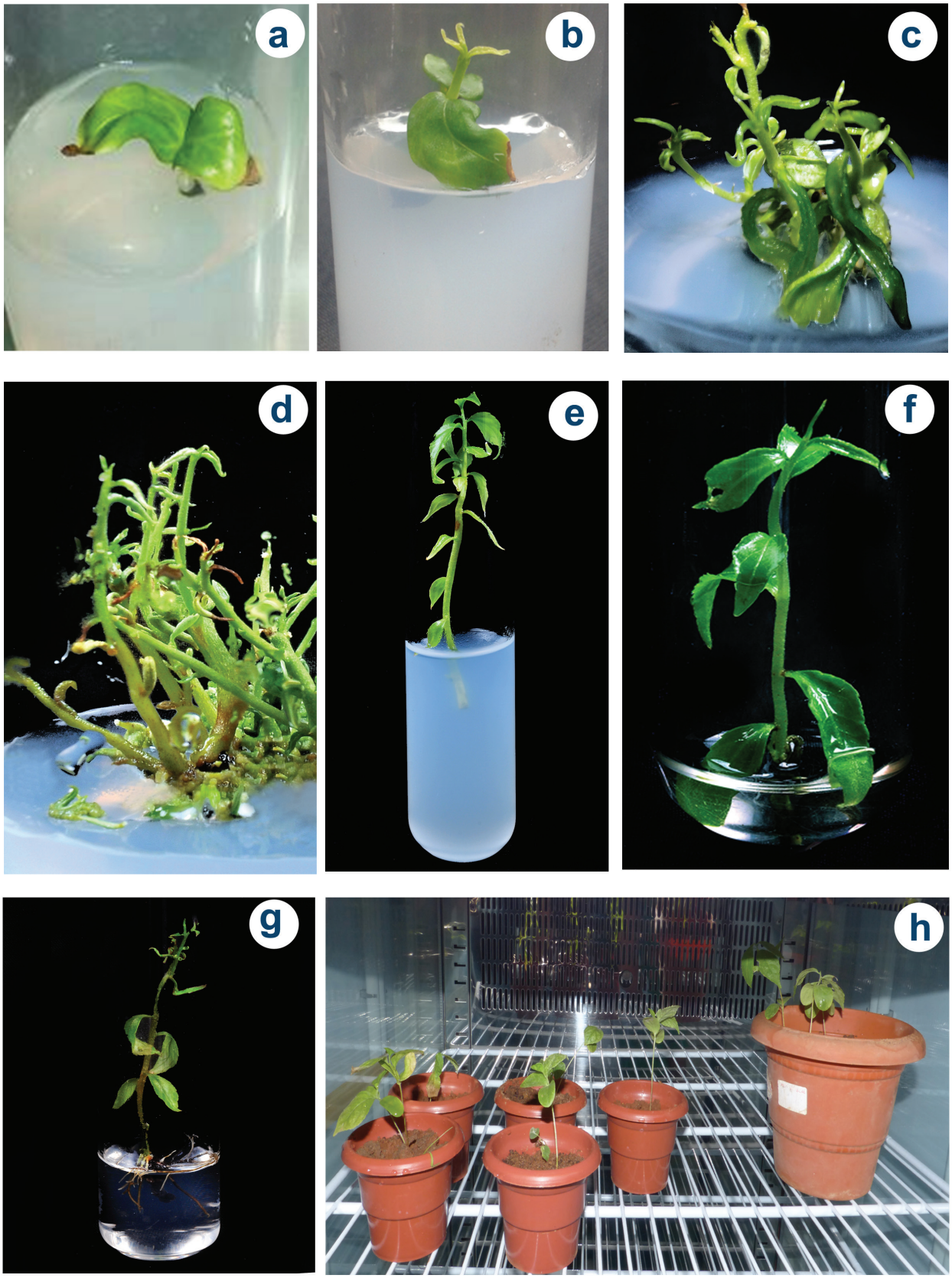

Fig. 1. Effect of plant growth hormones on direct regeneration of *C. paniculatus* Willd. a. Germination of embryos b. Initiation of shoot tip c. Shoot multiplication from shoot tip region d. Multiple shoots e. Shoot elongation, f. Shoot treatment with IBA solution for root initiation, g. Rooting, h. Hardening of plants.

3 h and transferred to the MS medium supplemented with agar. Interestingly, number of days for root forming was reduced from 15 to 10 by pretreating the shoots with above said auxins.

The highest rooting efficiency (91 %) was obtained in the half strength MS media supplemented with 0.5mg/L IBA (Fig.1) with an average of 10 roots/shoot (Table 5). In contrary, Senapati *et al.* (2013) reported highest rooting percentage (73.3) with IAA by using nodal explants in *C. paniculatus* Willd. This could be due to the ability and type of explants used for the study. Similar result was reported by Sharada *et al.* (2003) who obtained 85 percentage of rooting in woody plant medium supplemented with IBA in *C. paniculatus*. Similar effect was reported by investigators in other plant species such as chickpea, *Mentha × piperital* L, *Zhumeria Majdae* Rech, and *Wendelbo* (Ahmed *et al.*, 2019; Fallah *et al.*, 2019; Vaidya *et al.*, 2019.) The rooted plantlets were transferred into plastic pots for acclimatization and about 90 % of plantlets survived.

From the present study, it was observed that seed germination was very low in *C. paniculatus* Willd. due to the presence of seed germination inhibitors and endophytic microflora interference. Hence a reproducible and efficient protocol has been standardized for direct regeneration of plantlets through embryo derived shoot tip explants.

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