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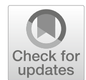

SHORT COMMUNICATION

Effects of Thidiazuron (TDZ) on Direct Shoot Organogenesis of *Gymnocladus assamicus*: A Threatened and Critically Endangered Species from Northeast India

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Abstract An efficient morphogenic potential was developed for direct shoot organogenesis of *Gymnocladus assamicus*, as an IUCN Red List of threatened and critically endangered species from Northeast India. This species is used as leech repellent of domestic animal, seedpod as detergent and roasted seed as a substitute for coffee and groundnut. The wild population is rapidly shrinking due to various anthropogenic pressure and poor regeneration. Therefore, the present study has been taken up for morphogenic potential through direct shoot organogenesis which is not reported. Nevertheless, cotyledonary nodal explants showed 100% responses in Murashige and Skoog (MS) medium fortified with 0.75 mg L^{-1} thidiazuron (TDZ) alone or in combination with 1 mg L^{-1} IBA, in comparison with other combinations tested. Cotyledonary node was found to be the best source of explant which produced 10.80 $\pm$ 0.39 shoots per explant. Further, shoots were transferred to proliferation and elongation medium fortified with 0.25 mg L^{-1} TDZ in MS medium which produced 12.06 $\pm$ 0.31 shoots per explant. MS medium fortified with 1.5 mg L^{-1} IAA showed highest root induction frequency (76%) with mean root number 2.03 $\pm$ 0.19 and root length 3.26 $\pm$ 0.27 cm. The micropropagated plantlets were transferred to soil after acclimatization with a 68% success rate.

Keywords Cotyledonary node · Thidiazuron · Direct shoot organogenesis · *Gymnocladus assamicus* · Fasciation · Micropropagation

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Gymnocladus assamicus is a critically endangered species as included in the IUCN Red List of threatened species (A2cd ver 3.1) owing to its dwindling population size [1], and also it is one of the prioritized plants listed for national recovery program under endangered and threatened species in India [2]. This species has been reported with 24 discrete subpopulations according to ecological niche modeling of western Arunachal Pradesh, India [3]. Thus, bottleneck population size of this species is due to various intrinsic and extrinsic factors responsible for poor regeneration, seed dormancy, hermaphrodite trees and also tremendous pressure from anthropogenic activity as road construction, agriculture and human colonization which may cause its extinction in near future [1, 3, 4]. Moreover, climate change as well as over-harvesting of any species leading to the extinction of species, especially those having sparse population density in their natural habitat, has a great chance of extinction through minor fluctuating event taking place in the ecosystem.

Mature seedpods are used as a substitute for detergent, socio-ritual activity, soap and anthelmintic of domestic livestock, and also roasted seeds are consumed as a substitute for coffee or groundnut [5–7]. The extract of

different parts of this species has been reported to have the antioxidant potential [8]. Biosynthesis of nanoparticle was reported as a potential catalytic activity of *Gymnocladus assamicus* [9], while other *Gymnocladus* species has been reported to have potent antifungal peptide, and it also inhibits immunodeficiency virus-1 reverse transcriptase [10]. In view of the above, and realizing the importance of this species, there is an urgent need for in vitro propagation of this species for its sustainable use and ex situ conservation.

The present study has improved regeneration capacity as well as the highest shoot number through direct shoot organogenesis which was not achieved in our previous studies [6]. Further, it has minimum chances of somaclonal variation in comparison with callus-mediated shoot organogenesis, and also it is the privilege for *agrobacterium* gene transfer technique. Our present study was developed for an efficient reproducible protocol which has not been reported so far.

Seedpod of *G. assamicus* was collected during 2008–2009 from the Dirang valley in Arunachal Pradesh, India, at an altitude of 1700–2000 masl. The petiole, cotyledonary node, cotyledonary leaf (in vitro germinated) and nodal (in vivo germinated) section (1–0.5 cm) excised from 20-day-old seedlings were used as a source of explants for the study on direct shoot organogenesis. All the explants were subjected to surface sterilization for in vitro seedlings; the nodal explants were disinfected with 10% (v/v) sodium hypochlorite solution with 2–3 drops of Tween 20 per 100 ml for 40 min followed by washing with sterile distilled water and finally treated with 0.1% (w/v) $HgCl_2$ for 1 min, followed by washing thoroughly with sterile distilled water under aseptic condition.

The petiole, cotyledonary node and cotyledonary leaf were obtained from in vitro germinated seedlings, whereas nodal explants were obtained from in vivo germinated seedlings which have been mentioned in our previous studies [11]. The collected fresh seeds were treated with sulfuric acid (36 N H_2SO_4) for 24 h, followed by surface sterilization method mentioned in our previous studies [6, 11]. All explants of 0.5 to 1 cm size excised were inoculated in MS [12] medium comprising Bacto agar 0.8% (w/v) (S D Fine-Chem, Mumbai, India) and sucrose (w/v) 3% supplemented with TDZ (0.25, 0.75, 1.25 mg L^{-1}) and BA (0.50, 1.50, 2.5 mg L^{-1}) alone or in combination with IBA or NAA (0.50, 1.0, 2.0 mg L^{-1}) (Table 1). MS basal medium served as control. The pH of the medium was adjusted to 5.8 before autoclaving for 20 min at 1.2 kg cm^{-2} and 121 °C. All the cultures were maintained at 25 °C ± 2 °C under 16-h photoperiod using warm white fluorescent lamps (40 W, Phillips, India) at an irradiance of 60 µmol m^{-2} s^{-1} and 65% humidity. The response of explants was recorded every week. After 7 weeks of initiation culture, shoot stubs were transferred to semisolid MS medium supplemented with TDZ (0.10, 0.25, 0.50 mg L^{-1}) or BA (0.50, 1.25, 1.5 mg L^{-1}) individually (Fig. 2a) against control medium for shoot proliferation and elongation. The number of shoots and length per explant were recorded after 7 weeks of culture.

The healthy microshoots of 1 to 1.5 cm length excised from the cluster of shoots were transferred to rooting induction media. Rooting induction media were composed of half-strength MS medium supplemented with 2% sucrose and either of IBA or IAA (0, 0.5, 1.0, 1.5, 2 mg L^{-1}) (Fig. 2b). All rooting data were recorded after 6 weeks of culture. The regenerated healthy in vitro rooted plantlets were washed carefully under running tap water to remove media and treated with a broad-spectrum fungicide 1% Bavistin (w/v) (BASF India Ltd., India) for 2 min. The plantlets were transferred to an earthen pot containing a mixture of 1:1 ratio of leaf mold and sand under greenhouse condition (24 °C and RH 90%) for acclimatization. All plantlets were irrigated with one-fourth strength of MS salt solution once in a week for 2 months. The successfully established plants were transferred to the botanic garden of BSI, Shillong.

For shoot bud and rooting induction were performed 30 replicates for each treatment, and all the experiments repeated three times. All data were subjected to analysis of variance (ANOVA, SPSS version 10). All the means were compared by the least significant difference (LSD) at the 5% level of significance.

Except for cotyledonary node (in vitro raised) and nodal explants (in vivo raised seedling), the petiole, root section and cotyledonary leaf (in vitro raised seedling) explants were failed to respond on different plant growth regulators tested for direct shoot organogenesis (Table 1). The shoot bud initiation was observed within 19 days on the epicotyl region. TDZ (0.75 mg L^{-1}) gave 100% response with maximum mean shoot bud number of 5.31 per explant. Direct shoot organogenesis in rooted hypocotyls, hypocotyl segments and cotyledonary leaf explants was reported in *Bixa orellana* (pink flowers variety) [13], and other eight cultivars of *Brassica oleracea* were tested the morphogenic potential of hypocotyl and cotyledon explants [14]. The effect of TDZ alone at low concentration (0.25 mg L^{-1}) was observed that shoot rose from the nodal portion of cotyledonary nodal explants, while on increasing concentration of TDZ, shoot bud originated from the epicotyl axis end of cotyledonary nodal explants. These shoot buds orientation might be the cause of interaction between exogenous and endogenous hormonal interference which could nurture in regeneration pattern of shoot buds. Thus, our studies also need further investigation on endogenous hormone profile for better understanding the regeneration pattern and orientation of shoot bud in *G. assamicus*.

Table 1 Direct shoot organogenesis from cotyledon node and nodal explants of *G. assamicus*, on MS medium supplemented with TDZ individually or in combination with either BA or IBA, after 8 weeks of culture

Treatments (mg $L^{-1)}$				Cotyledon nodal explants			Nodal explants		
TDZ	BA	IBA	NAA	% of response	No. of shoot buds	Shoot length (cm)	% of response	No. of shoot buds	Shoot length (cm)
0	0	0	0	14	1.16 ± 0.04 o p	3.18 ± 0.05 h i j k	0	1 ± 00 o p	2.62 ± 0.06 l m n
0.25	0	0	0	77	3.81 ± 0.20 h i	**4.84 ± 0.07 a**	71	2.52 ± 0.12 j k	2.90 ± 0.08 h i j k l
0.75	0	0	0	100	**5.31 ± 0.14 f**	3.82 ± 0.06 d e	100	**6.93 ± 0.14 a**	**3.92 ± 0.06 a**
1.25	0	0	0	65.55	3.10 ± 0.19 k	3.60 ± 0.08 e f	81	4.14 ± 0.20 d	3.40 ± 0.08 c d e f
0.75	0.5	0	0	100	6.31 ± 0.15 e	3.97 ± 0.08 b c d	100	4.85 ± 0.18 c	3.80 ± 0.08 a b
0.75	1.5	0	0	100	9.24 ± 0.26 b	4.10 ± 0.06 b c	100	4.10 ± 0.21 d e	3.56 ± 0.10 b c
0.75	2.5	0	0	78	4.13 ± 0.24 h	3.25 ± 0.11 g h i j	82	3.71 ± 0.22 d e f g	3.13 ± 0.09 f g h i j
0	0.5	0	0	44	1.45 ± 0.05 o	2.71 ± 0.10 l m	30	1.31 ± 0.05 o	2.50 ± 0.10 n o
0	1.5	0	0	100	4.80 ± 0.26 f g	2.90 ± 0.08 l	100	3.32 ± 0.18 g h i	3.48 ± 0.12 c d e
0	2.5	0	0	91	2.35 ± 0.11 l m n	2.62 ± 0.11 m n	90	2.25 ± 0.11 k l m	3.15 ± 0.13 f g h i
0.75	0	0.5	0	100	7.43 ± 0.23 c	3.40 ± 0.13 f g h	100	5.64 ± 0.29 b	3.50 ± 0.12 c d
0.75	0	1.0	0	100	**10.80 ± 0.39 a**	4.23 ± 0.10 b	72	3.90 ± 0.27 d e f	3.22 ± 0.11 d e f g
0.75	0	2.0	0	93	7.12 ± 0.26 c d	3.51 ± 0.14 f g	68	3.55 ± 0.25 f g h	3.18 ± 0.10 f g h
0.75	0	0	0.5	100	3.72 ± 0.18 h i j	3.30 ± 0.12 g h i	90	2.90 ± 0.22 i j	3.02 ± 0.14 g h i j k
0.75	0	0	1.0	80	2.92 ± 0.17 k l	2.60 ± 0.09 m n o	48	2.40 ± 0.22 j k l	2.90 ± 0.13 h i j k l
0.75	0	0	2.0	75	2.70 ± 0.19 k l m	2.27 ± 0.10 p	40	1.92 ± 0.18 l m n	2.85 ± 0.15 j k l m
LSD				–	0.58	0.27	–	0.54	0.30

Means followed by the same letters within a column are not significantly different (LSD; $P < 0.05$)

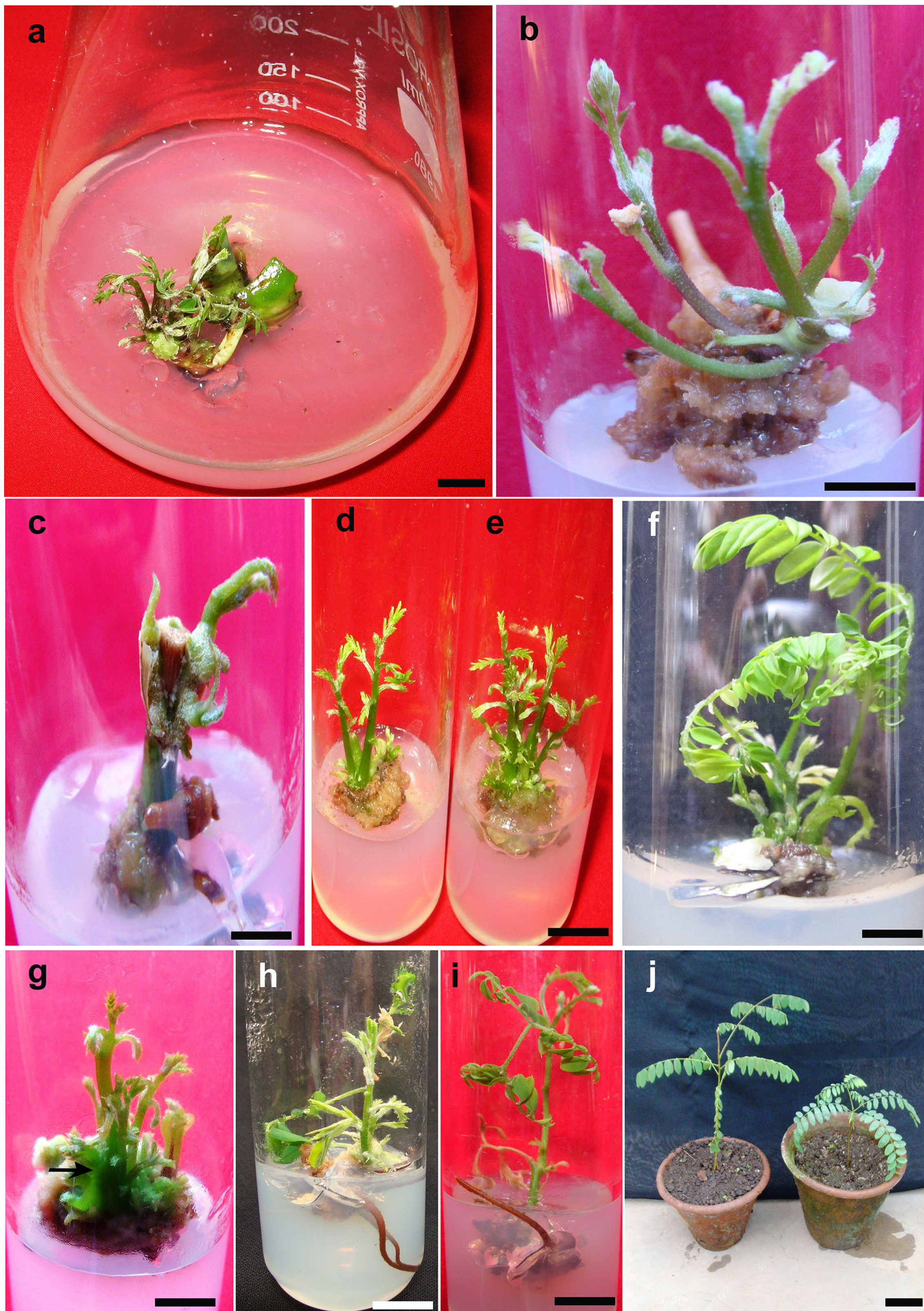
Bold shown as comparatively most significant result among treatments

The present study was found to be synergetic effects in the combinations of TDZ (0.75 mg L^{-1}) and IBA (1 mg L^{-1}). It showed higher shoot organogenesis in cotyledonary node explants response with 100% efficiency and mean shoot bud number of 10.80 ± 0.39 (Fig. 1a). TDZ has been reported to promote direct plant regeneration from cotyledonary nodes in several plant species [15–17] which could be attributed to the enhanced rate of accumulation and translocation of auxins within the tissues. Direct shoot organogenesis without intervening callus phase was well recognized by several authors as higher genetic solidity than those produce via callus [18–20]. Thus, in the present study, it was observed that explants of *G. assamicus* responded well in combination with TDZ and IBA, without callogenesis which is a preferred path for plant regeneration. Thidiazuron is considered for gratifying both the cytokinin and auxin requirements for diverse regeneration responses [17]. It can influence meristem formation, promote shoot development from preexisting meristems and induce adventitious shoots bud regeneration in a number of species including recalcitrant woody plant species [21–23].

On the other hand, with treatment at low concentration of 0.25 mg L^{-1} TDZ alone, some of the nodal explants responded by initiation of shoot bud above the nodal junction, i.e., apical end of nodal explant within 3 weeks (Fig. 1c), while on increasing concentration of TDZ (0.75 mg L^{-1}), all shoot buds originated from preexisting adventitious bud of nodal explants. TDZ (0.75 mg L^{-1}) alone gave 100% regeneration frequency with highest mean shoot number and length, respectively, 6.93 ± 0.14 and 3.92 ± 0.06 cm (Table 1; Fig. 1b). The synergetic effects of TDZ (0.75 mg L^{-1}) + IBA (1 mg L^{-1}) were optimum responses in cotyledonary nodal explants, whereas nodal explants reduced threefold in mean shoot number. Thus, cotyledonary nodal was the best source for explants.

Fig. 1 Morphogenic response of cotyledonary node and nodal explants. **a** Shoot bud proliferation after 8 weeks of culture on 0.75 mg L^{-1} TDZ and 1 mg L^{-1} IBA. **b** Shoot bud proliferation from nodal explant after 8 weeks of culture on 0.75 mg L^{-1} TDZ and 1 mg L^{-1} IBA. **c** Shoot bud initiation after 3 weeks from nodal apex of nodal explant. **d** Shoot stub transferred to control media. **e** and **f** the effects of shoots proliferation and elongation media at 0.25 mg L^{-1} TDZ after 5 and 7 weeks of culture. **g** The arrow mark shows fasciation and fragile shoots at the high concentration of TDZ (1.25 mg L^{-1}) after 9 weeks of culture. **h** Rooting in ½ MS +1.50 mg L^{-1} IAA after 6 weeks of culture. **i** Rooting in ½ MS +1.5 mg L^{-1} IBA after 6 weeks of culture. **j** 3-month-old acclimatized plant. At the right side of the bottom in all figures is represented bar of 1.5 cm ▶

The highest mean shoot number and length per explant were recorded at 0.25 mg L^{-1} TDZ (Fig. 2a). Of the two hormones, the optimal concentration of TDZ (0.25 mg L^{-1}) produced maximum shoot number of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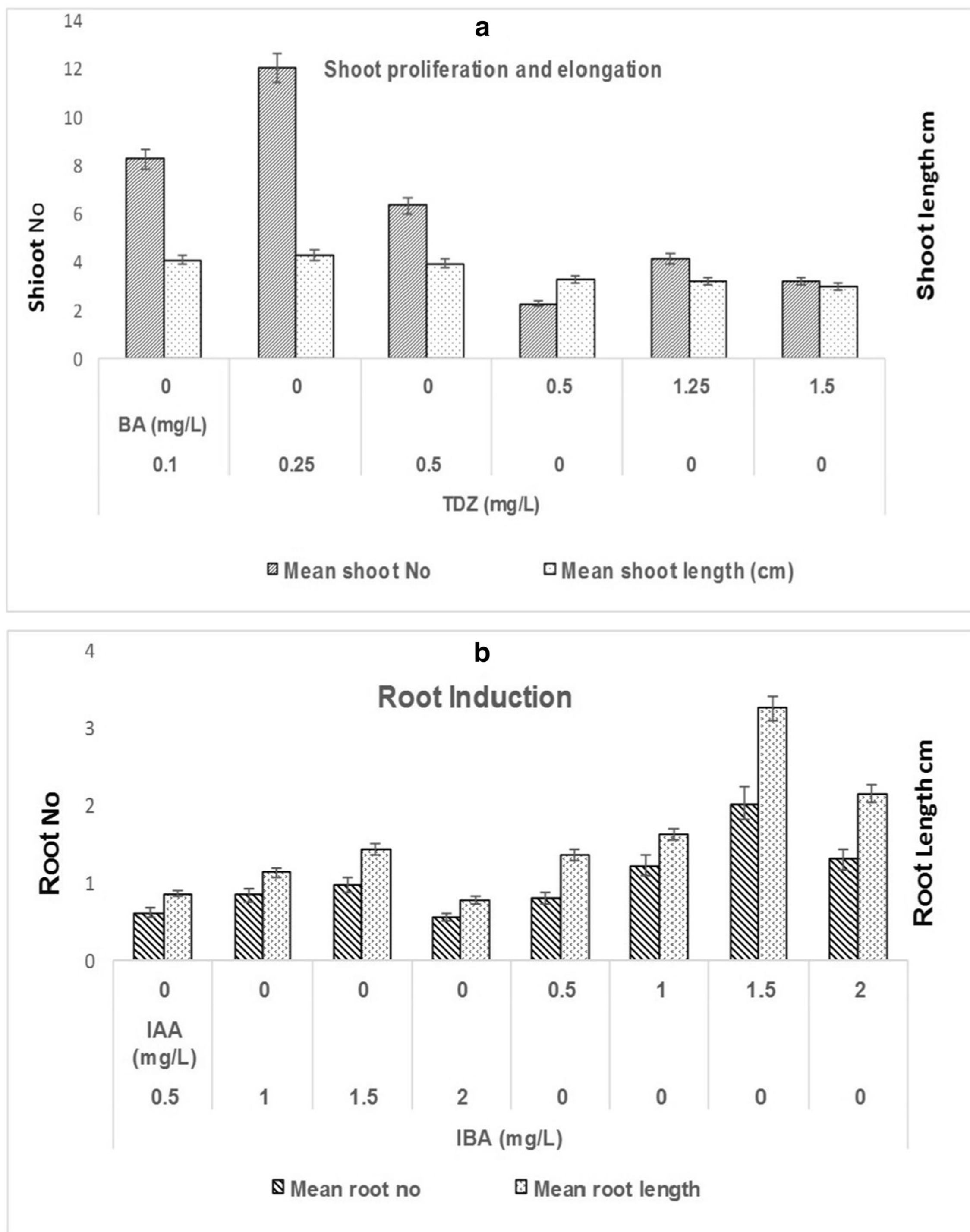

Fig. 2 **a** Effect of lower concentrations of TDZ and BA hormone on shoots proliferation and elongation. **b** Effect of different auxins on rooting induction after 6 weeks of culture

12.06 ± 0.31 and length 4.25 ± 0.11 cm (Fig. 1e) within 5 weeks of culture. Controls were also recorded for mean shoot number of 4.22 ± 0.18 and length of 3.50 ± 0.14 cm (Fig. 1d). Moreover, fasciation and fragile shoots were observed during prolonged culture in high concentration of TDZ (1.25 mg L^{-1}) which has earlier been reported [24], shown as arrow in Fig. 1g. Thus, prolonged culture on TDZ is not advantageous; the use of pulse medium has been reported in mulberry [25]. Of these two hormones, TDZ alone at the concentration of 0.25 mg L^{-1} (Fig. 1e, f) enhanced maximum mean shoot number and length in *G. assamicus*. In addition, result also

observed that shoot stubs on prolonging culture in TDZ produced friable nonorganogenic callus at the base of shoot stubs (Fig. 1d, e).

Auxins-free medium failed to produce any root. Of the two auxins evaluated, IAA (1.50 mg L^{-1}) obtained a significantly good response with a maximum rooting frequency of 76% with a mean root number and length, respectively, of 2.03 ± 0.19 and 3.26 ± 0.27 cm (Fig. 1h) in comparison with IBA. A similar observation of IAA effect on rooting was reported in a legume species, *Acacia nilotica* [26]. Callus growth was also observed in all the treatments; a similar observation was also reported in *Acacia mangium* [27]. Thus, overall rooting of the present study showed a similar trend with our previous work [6]. Plantlets were transferred successfully with 68% survival (Fig. 1j).

In conclusion, the present investigation displays efficient reproducible method developed through direct shoot organogenesis using cotyledonary node explant of critically endangered species *G. assamicus*. In addition, the synergetic effect was observed in the combination of optimal concentrations of TDZ (0.75 mg L^{-1}) and IBA (1.0 mg L^{-1}) without intervening callus in cotyledonary nodes. Rooting was successfully induced. In vitro grown rooted plantlets were successfully acclimatized and transferred to natural habitat. The present study is an efficient protocol and best suited for in situ conservation of *G. assamicus* which is not reported elsewhere.

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Authors' Contribution SG implemented all the experiments, analyzed the data and written the manuscript. AAM and SS planned the work, edited the manuscript and monitored the entire research work.

Compliance with ethical standards

Conflict of interest All authors declare that they have no any conflict of interest in this publication.

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