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In Vitro Organogenesis of *Quisqualis indica* Linn. —An Ornamental Creeper

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ABSTRACT

Shoot organogenesis and plant regeneration were achieved on callus derived from leaf section and stem base explants of *Quisqualis indica* (Combretaceae). *In vitro* cultures were established using nodal segments obtained from mature field-grown shrubby plants. For the development of optimized protocol, different types and concentrations of plant growth regulators were used to induce adventitious shoot regeneration via callus from leaf section and one-node stem base explants obtained from *in vitro* regenerated micro shoots and direct field-grown newly flush-off shoots. The TDZ was considered to be the best among the cytokinins (6-benzyladenine (BA), 6-(γ-γ, dimethylallyamino purine) (2-iP) and thidiazuron (TDZ) added to the Murashige and Skoog's medium (MS) for adventitious shoot productions. A combination of 1.0 mg/L TDZ and 0.5 mg/L GA_3 was most effective in stimulating callus induction and adventitious shoot regeneration from the leaf section derived calli with an average of 6 shoots per callus explant and an average of 8 shoots per callus explant originated from one-node stem base explants. *In vitro* raised shoots were sub-cultured on MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L GA_3 for further shoot growth. Maximum rooting of *in vitro* regenerated shoots was obtained on MS medium supplemented with either 0.5 mg/L indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) individually or a combination of 0.5 mg/L IAA and 0.5 mg/L IBA. Plantlets raised *in vitro* were acclimatized and subsequently transferred to experimental field.

Keywords: Organogenesis; *Quisqualis indica*; Shoot Regeneration; Tissue Culture

1. Introduction

Rangoon Creeper scientifically known as *Quisqualis indica* originated from South East Asia and occurs all over Africa, Philippines, Vietnam, Malaysia, India, Bangladesh and Thailand. It has bright colored fragrant flowers and is one of the most stunning ornamental of the family Combretaceae. *Quisqualis indica* is grown as an ornamental garden plant for it's horizontally orientation to pendulous white, pink and red flowers that give out distinct perfume. The flowers contain high quantity of polyphenol that are believed to be strong antioxidants beneficial for human health [1-3]. This species is known to have free radical scavenging activity and alleviating flatulent distension of abdomen like that of the medicinal properties of *Terminalia chebula*, *T. belerica and Emblica officinalis* [4-6]. The plant parts such as roots, flowers and seeds of the plant are used for curing diarrhea, fever, rickets, rheumatism and nephritis [2,7]. The leaves, fruits and seeds of the plant have been used as anthelmintic for expelling round worms and thread worms [8-10].

This ornamental shrub is conventionally propagated through seeds and cuttings. However, according to Lambardi and Rugini [11] propagation through seeds renders undesirable variation whereas shoot cuttings of many genotypes do not respond to root inducing medium.

These difficulties may be overcome using in vitro tissue culture techniques. Plant tissue culture techniques are considered as easy and reliable for rapid up-scaling of shoot regeneration of elite genotypes independent of seasonal and environmental influences. In addition, production of transgenic plants relies on successful establishment of *in vitro* regeneration methods based on organogenesis and thus can complement conventional breeding technique.

Regeneration protocols have been reported for micropropagation of many species such as *Cinnamomum camphora* ([12], *Terminalia chebula* [13], *Saussurea obvallata* [14], *Terminalia bellirica* [15], *Terminalia arjuna* [16], *Aloe polyphyla* [17], *Vaccinium* species [18], *Pha-*

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seolus vulgaris [19], *Punica granatum* [20], *Trifolium alexandrinum* [21] from axillary meristems and shoot tips as well as shoot organogenesis from excised leaf explants, cotyledon and embryo axis with different plant growth regulators.

In vitro systems may offer the tools for rapid multiplication and conservation of genetic stocks and availability of wild *Quisqualis* germplasm for its medicinal and ornamental use besides its potential use in genetic engineering and plant breeding. However, there has been no report on the shoot organogenesis of *Quisqualis indica*. The aim of the present study was to establish an efficient regeneration protocol through callus mediated organogenesis of leaf and stem base explants of *Quisqualis indica*.

2. Materials and Methods

2.1. Culture Initiation

Nodal segments from newly flush off shoots of adult climber plants of *Quisqualis indica* were collected in December 2009, February 2010 and March 2011 and thoroughly washed under running tap water for 10 min and surface sterilized with 0.1% $HgCl_2$ for 5 min under aseptic condition in Laminar air flow. After five washings in sterile double distilled water for 10 min, the cut ends of the nodal segments were trimmed and cultured on MS [22] Murashige and Skoog, 1962) medium supplemented with the 1.0 mg/L benzyladenine (BA), 0.5 mg/L gibberellic acid (GA_3), 3% sucrose and gelled with 0.8% agar in 20 × 150 mm glass tubes and 100 ml conical flasks. The pH was adjusted to 5.8 with 1 M HCl or 1 M NaOH and autoclaved at 121˚C for 20 min. Cultures were incubated in a culture room maintained at 29˚C ± 2˚C under a 16/8 h photosynthetic photon density of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool white fluorescent tubes (40 W; Philips, India). Shoots of *in vitro* plants were cut into nodal segments and subcultured on MS medium supplemented with 1.0 mg/L 6-benzyladenine (BA) and 0.5 mg/L gibberellic acid (GA_3). Every three weeks, newly formed shoots were subjected to the same procedure of cutting nodal segments and subculture on the fresh medium.

2.2. Callus Induction

Field-grown matured plants as well as three-week old in vitro regenerated shoots were the sources of leaf section and one-node stem base explants. Explants from field grown plants were surface sterilized following the procedures used above for culture initiation. The petiole was removed and two cuts were made on each leaf. Stem base with one-node was used as another explant. The surface sterilized leaf section explants were inoculated with adaxial or abaxial side in contact with the medium. The stem base with one-node was inoculated with lower base inserted into the medium. The MS medium was gelled with 0.8% agar and different types and concentrations of plant growth regulators such as cytokinins, 6-benzyladenine (BA) or 6-(γ-γ, dimethylallylamino purine) (2-iP) or thidiazuron (TDZ) at the concentrations of 0.5 mg/L or 1.0 mg/L either individually or in combination with either 0.25 mg/L naphthaleneacetic acid (NAA) or 0.5 mg/L gibberellic acid (GA_3). Preliminary trials suggested that there were no marked differences in the calli induction potentials between the explants of field-grown and *in vitro* shoots, subsequent experiments based on explants of field-grown plants were considered. Every three weeks the developed calli were subdivided and subcultured on the same medium.

2.3. Shoot Regeneration and Proliferation

Isolated calli (0.5 - 1.0 cm^2) from leaf section and basal portion of one-node stem base explants were placed on MS medium supplemented with 0.5 mg/L or 1.0 mg/L BA or TDZ either individually or in combination with 0.25 mg/L α-naphthaleneacetic acid (NAA) or 0.5 mg/L gibberellic acid (GA_3) for regeneration of shoots for six weeks. Shoots of 5 - 8 mm in length were separated from the calli explants and subcultured on MS supplemented with 1.0 mg/L BA and 0.5 mg/L gibberellic acid (GA_3) for elongation and multiple shoot regeneration for four weeks. A total of five explants per conical flasks were used and each treatment was replicated four times. The experiment was repeated three times. The explants were incubated for six weeks to the same photoperiod, light intensity and temperature used for culture initiation. Data on percentage callus induction, percentage of organogenic calli, and number of shoots per callus explant and shoot length was recorded after four weeks of incubation and presented in **Figures 1**-**4**.

2.4. *In Vitro* Rooting of Shoots and Hardening

Six-week old in vitro shoots (2 - 3 cm) developed from calli were cultured on MS medium supplemented with 0.5 mg/L or 1.0 mg/L IAA or IBA either individually or in combination for rooting for a period of four weeks following a procedure for rooting of micropropagated shoots of *Quisqualis indica* (unpublished, data not shown). After this incubation period, *in vitro* raised plantlets with healthy root systems and without roots were thoroughly washed under running tap water and planted in plastic cups containing a 1:1 (v/v) mixture of sterilized vermiculite and garden soil moistened with 1/2 MS strength. These cups were wrapped with polyethylene bags ensuring high humidity and kept under the same growth conditions as for culture initiation for further development. Thereafter, the plants were nurtured

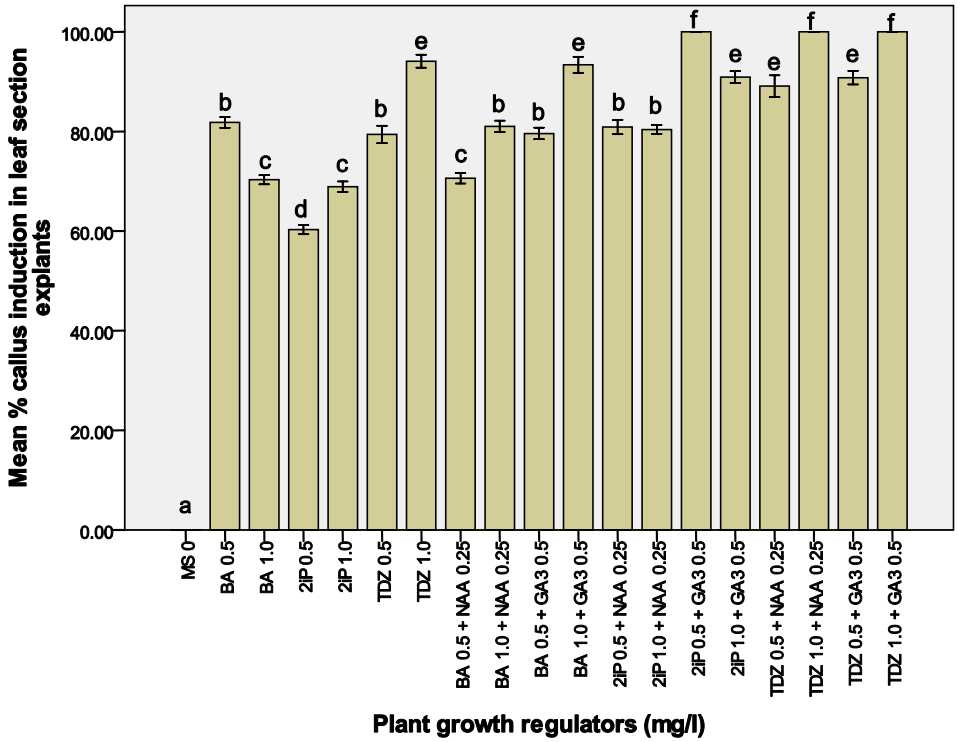


Figure 1. Mean % of callus induction of leaf section explants of *Quisqualis indica* on MS medium supplemented with different combination and concentrations of plant growth regulators. Different letter(s) indicate a significant difference between treatments at $P \leq 0.05$ according to Tukey test.

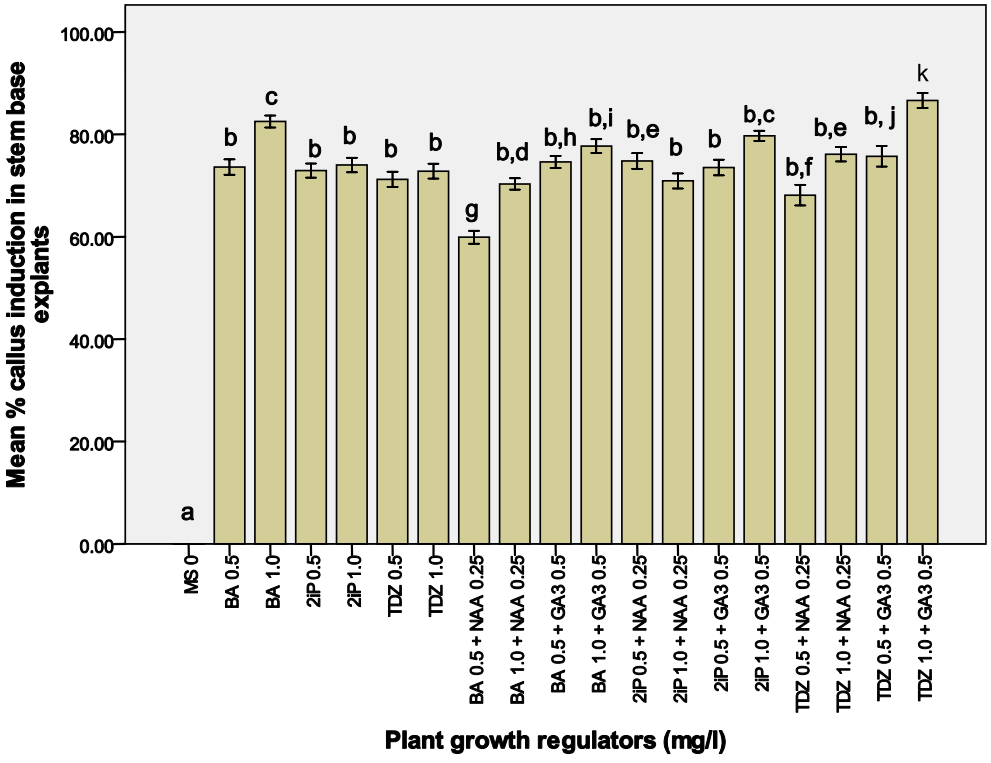
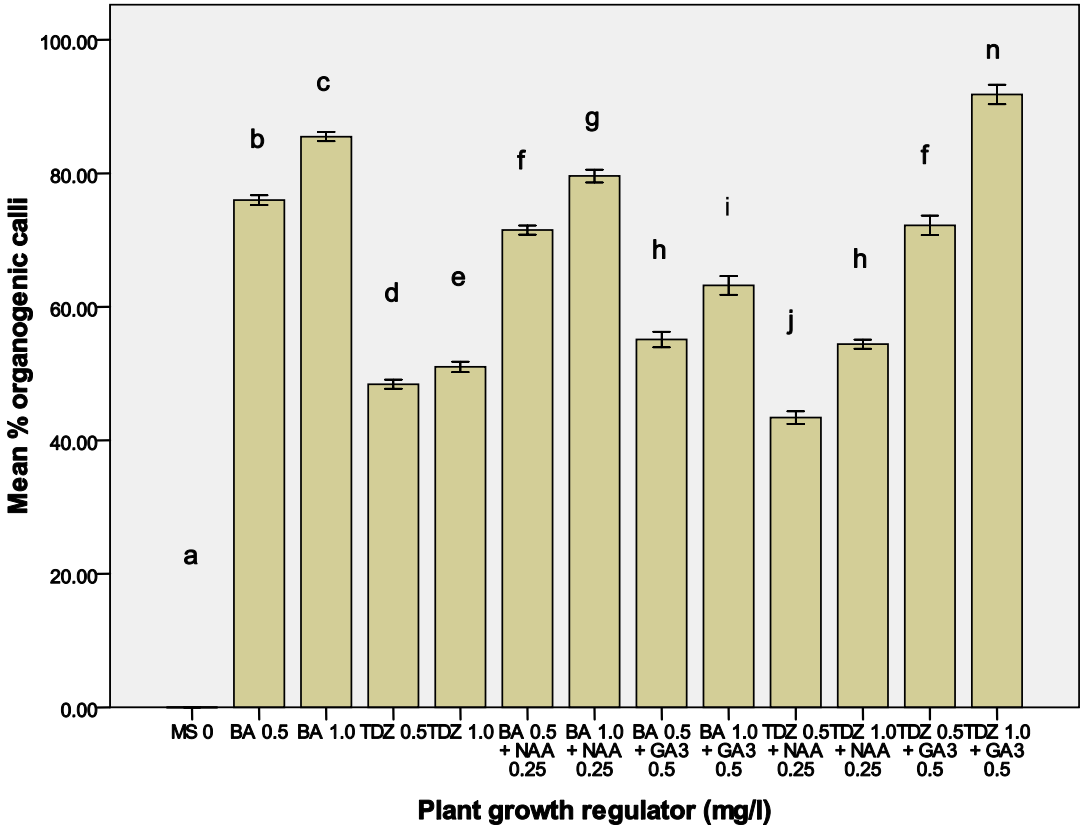
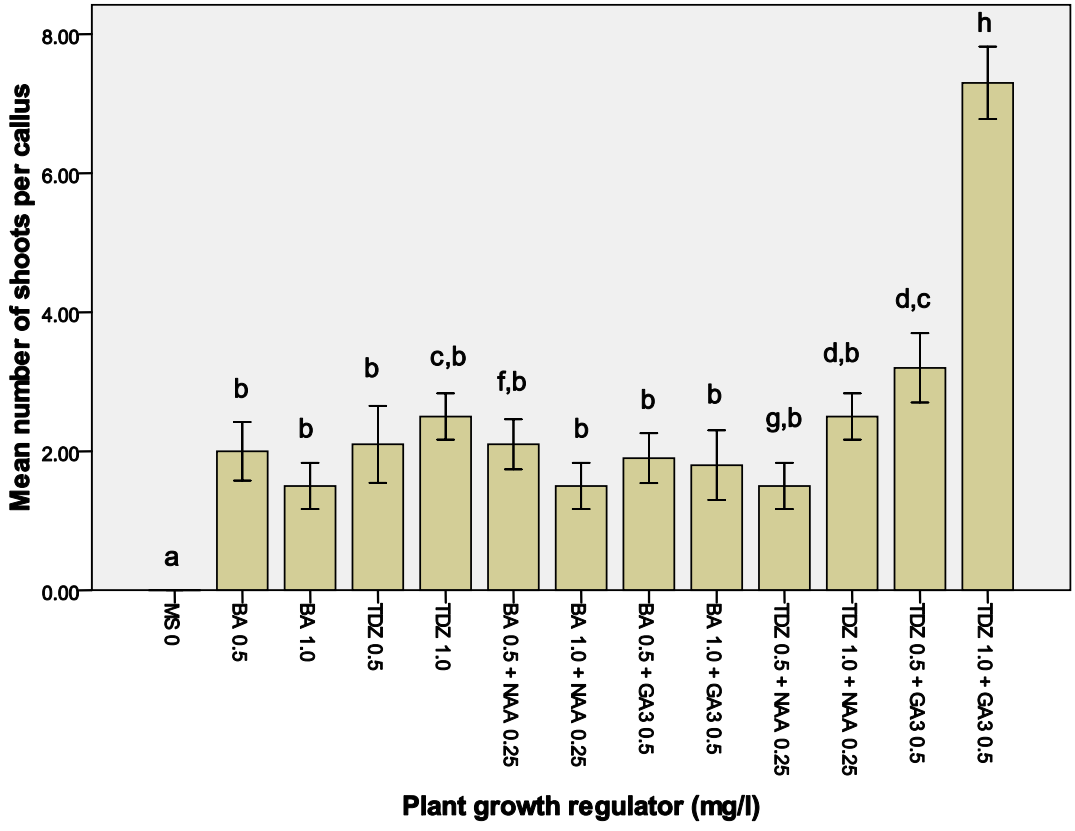


Figure 2. Mean % of callus induction of stem base explants of *Quisqualis indica* on MS medium supplemented with different combination and concentrations of plant growth regulators. Different letter(s) indicate a significant difference between treatments at $P \leq 0.05$ according to Tukey test.



(a)



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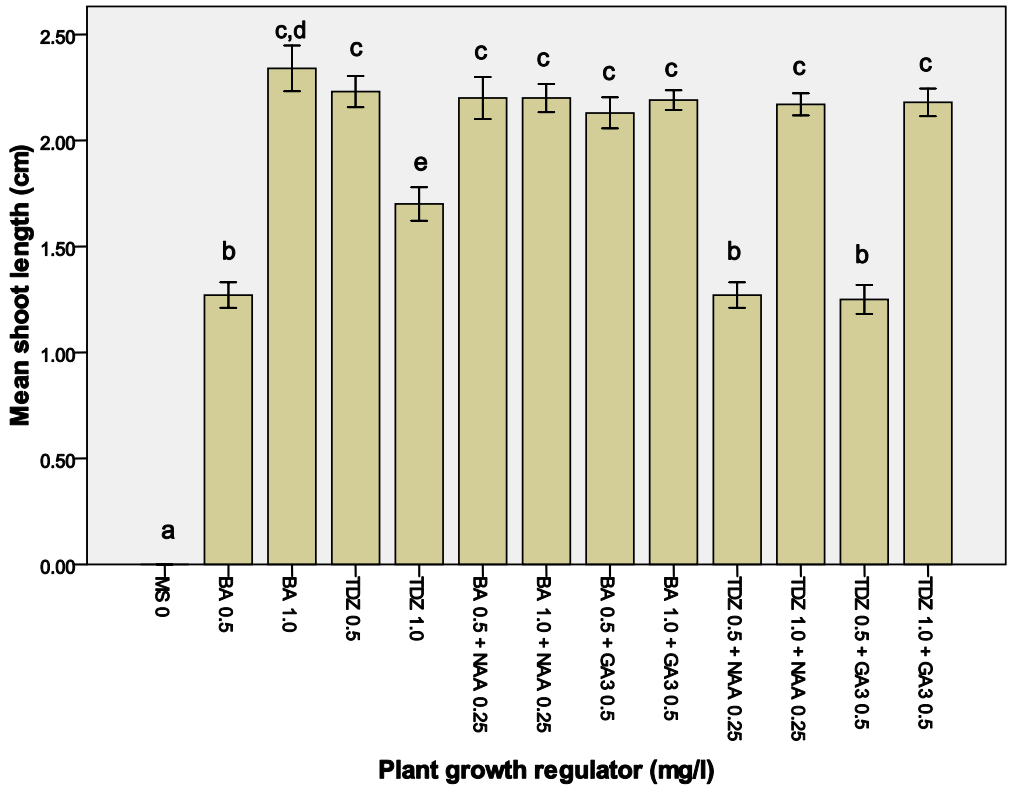
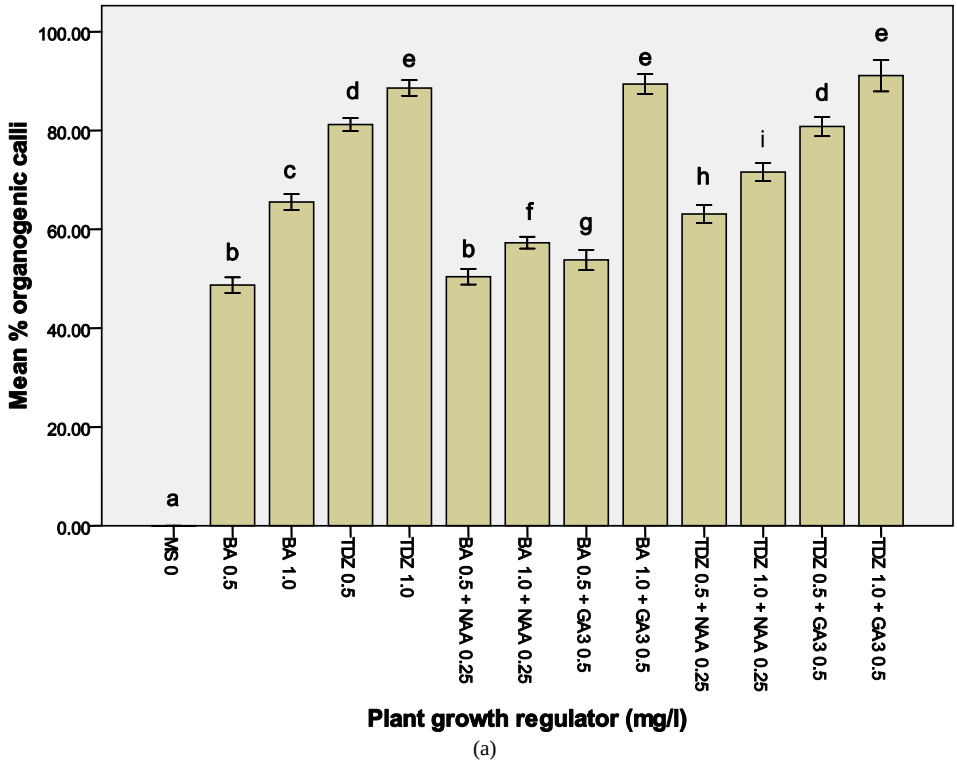
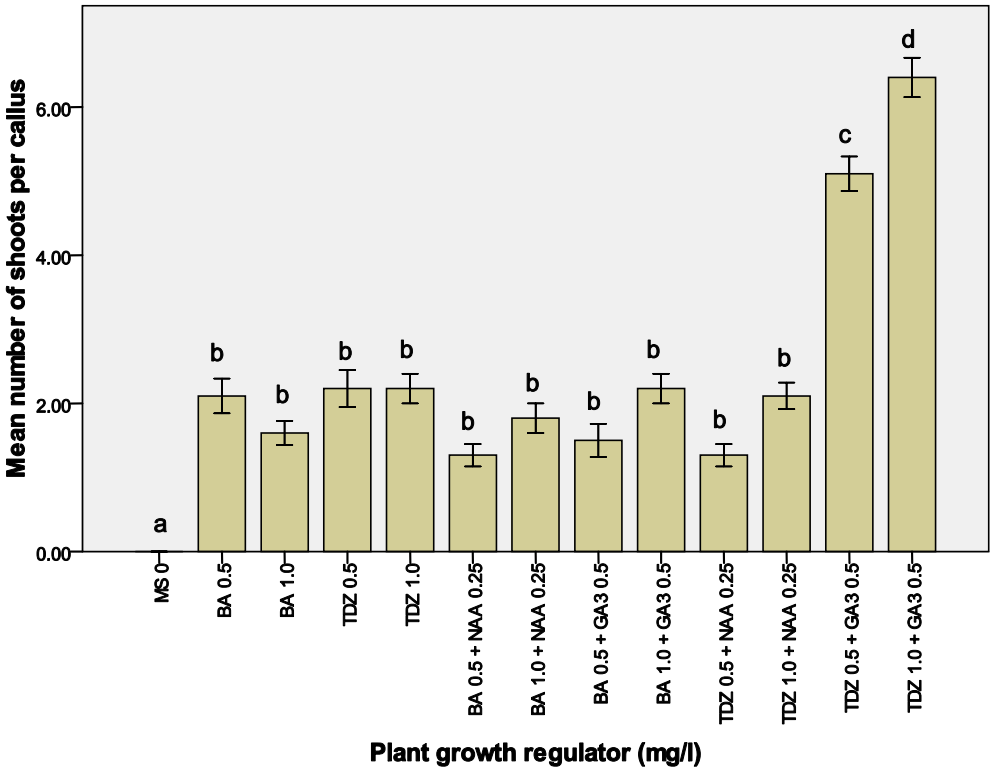


(c)

Figure 3. (a)-(c) Organogenic responses of stem base explants of *Quisqualis indica* on MS medium supplemented with different combination and concentrations of plant growth regulators after 30 d. (a) % *Organogenic calli* regenerating shoots; (b) Mean number of shoots per callus; (c) Mean shoot length (cm). Different letter(s) indicate a significant difference between treatments at $P \leq 0.05$ according to Tukey test.



(a)



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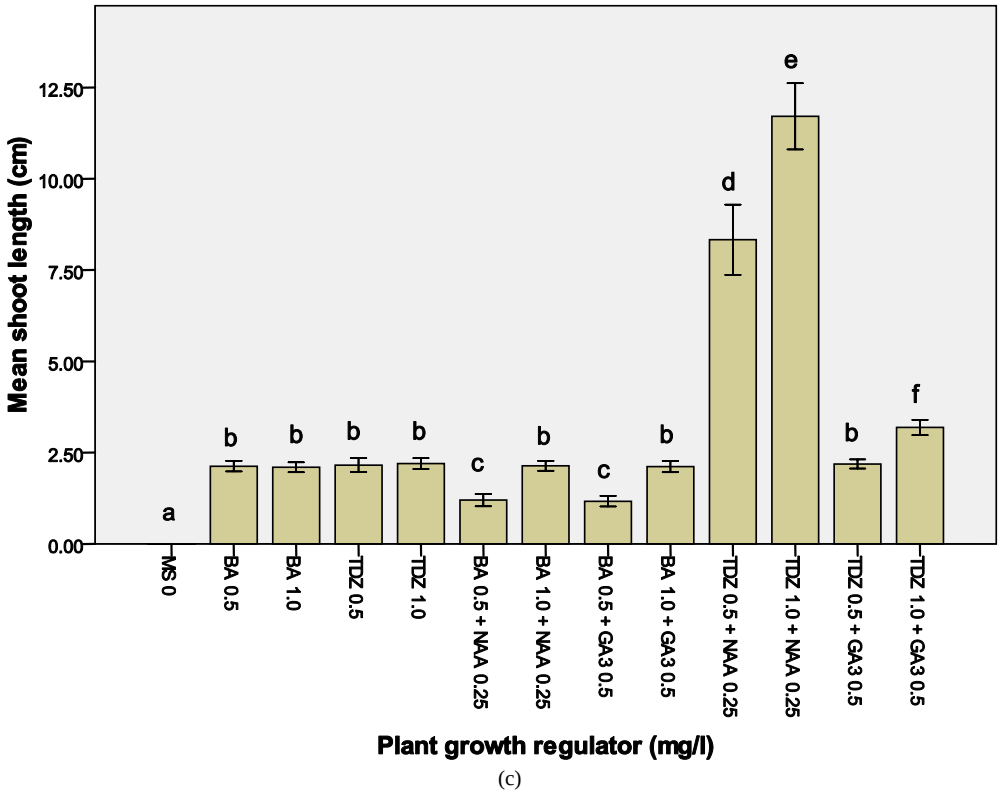

(c)

Figure 4. (a)-(c) Organogenic responses of leaf section explants of *Quisqualis indica* on MS medium supplemented with different combination and concentrations of plant growth regulators. After 30 d. (a) % *Organogenic calli* regenerating shoots; (b) Mean number of shoots per callus; (c) Mean shoot length (cm). Different letter(s) indicate a significant difference between treatments at $P \leq 0.05$ according to Tukey test.

under a shade (50% light cut off) for a period of two weeks before transfer to the experimental field

2.5. Data Analysis

All the experiments were carried out in a completely randomized design and repeated thrice. Means and standard error of means for all the dependent variables such as, callus induction shoot regeneration shoot number and shoot length under different plant growth regulator concentrations were computed and found out the significant differences between means using Tukey test.

3. Results

3.1. Culture Initiation

Shoot cultures of *Quisqualis indica* were initiated successfully from nodal segments with 100% bud break. Preliminary experiments suggested the inclusion of BA and GA_3 in the MS medium for bud break and shoot proliferation. The highest bud break (100%) with an average of 20 shoots per node explant was observed on the MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L GA_3 within two weeks of inoculation. In contrast, 75% of the explants developed multiple shoots with an average of 2 - 3 shoots per node explant on MS medium supplemented with 1.0 mg/L TDZ or 2-iP in combination with 0.5 mg/L GA_3 (data not shown). The MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L GA_3 was chosen for further up-scaling of *in vitro* shoot regeneration because of its increased shoot formation potential.

3.2. Callus Induction

Leaf section and one-node stem base explants were incubated on different media to promote the induction of calli (**Figures 1** and **2**). Callus was induced from both the explants: the leaf section explants produced the higher frequency of callusing than the one-node stem base explants. Initiation of callus was observed within seven days from cut ends of leaf section and basal cut end region of one-node stem base explants on MS medium supplemented with plant growth regulators. Leaf section explants showed the highest percentage of callus induction (100 ± 0) on MS medium supplemented with 0.5 mg/L 2-iP and 0.5 mg/L GA_3 or 1.0 mg/L TDZ in combination with 0.25 mg/L NAA or 0.5 mg/L GA_3 after four weeks (**Figure 1**). In contrast, the highest callus induction frequency (86.6 ± 2.3) (significantly different, $P < 0.05$) from basal cut end of one-node stem base explants was observed on MS medium supplemented with 1.0 mg/L TDZ and 0.5 mg/L GA_3 after four weeks (**Figure 2**). The calli were compact and green in color (**Figures 5(a)**, **(b)** and **(d)**). Thus the results of the investigation showed higher frequency of callus induction through leaf section explants than the one-node stem base explants (**Figures 1** and **2**).

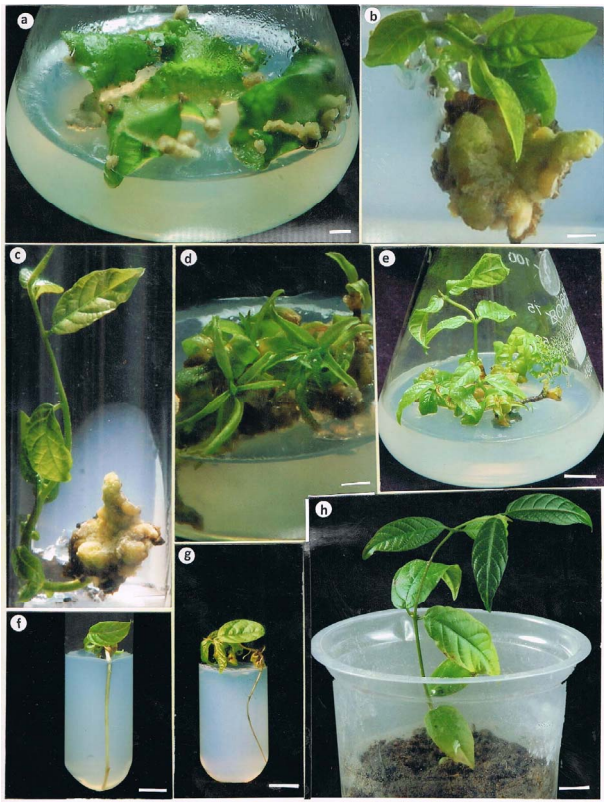

Figure 5. (a)-(h) *In vitro* plant regeneration of *Quisqualis indica*. (a) Callus formation from leaf section explants after 30 d of culture on MS medium supplemented with 1.0 mg·l^{-1} TDZ 2 iP and 0.5 mg·l^{-1} GA_3. (b) Shoot regeneration from leaf section callus after 21 d culture on MS medium supplemented with 1.0 mg·l^{-1} TDZ and 0.5 mg·l^{-1} GA_3; (c) Shoot elongation after 30 d following subculture on the same medium; (d) Induction and proliferation of multiple shoots from one-node stem base derived callus culture after 30 d culture on MS medium supplemented with 1.0 mg·l^{-1} TDZ and 0.5 mg·l^{-1} GA_3. (e) Elongation of regenerated shoots after 30 d of culture on MS medium supplemented with 1.0 mg·l^{-1} BA and 0.5 mg·l^{-1} GA_3; (f) *In vitro* shoot produced healthy root after 30 d of culture on MS medium supplemented with 0.5 mg·l^{-1} IAA and 0.5 mg·l^{-1} IBA; (g) Rooting of regenerated shoot after 45 d of culture on MS medium supplemented with 0.5 mg·l^{-1} IAA; (h) An established plant of *Quisqualis indica* after 30 d of transfer to garden soil. Bars = 0.7 cm (a); 2 cm (b); 2 cm (c); 1 cm (d, e, f, g), and 1 cm (h).

3.3. Shoot Regeneration and Proliferation

In the present investigation, the highest frequency of organogenic calli (91.1 ± 2.5) (significant differences, $P < 0.05$) was obtained from leaf section explants on MS medium supplemented with 1.0 mg/L TDZ and 0.5 mg/L GA_3 inducing an average of 5.4 shoots per callus explant with an average shoot length of 3.19 cm (**Figures 4(a)**,

(b), **(c)**, **5(b)** and **(c)**), whereas the organogenic calli (91.8 ± 2.3) (significant differences, $P < 0.05$) derived from one-node stem base explant induced an average number of 7.3 shoots per callus explant with an average shoot length of 2.18 cm on this medium (**Figures 3(a)**, **(b)**, **(c)** and **5(d)**).

The total number of regenerants increased as the concentrations of TDZ increased in combination with 0.5 mg/L GA_3 or 0.25 mg/L NAA (**Figures 3(b)** and **4(b)**). In the present investigation, 71.6% of the organogenic calli derived through leaf section explants on MS medium supplemented with 1.0 mg/L TDZ and 0.25 mg/L NAA produced an average of 2.1 shoots per callus explant averaging a length of 11.7 cm (**Figures 4(b)** and **(c)**). The regenerated shoots were excised as node explants and subcultured on the MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L GA_3 for further shoot proliferation and elongation growth (**Figure 5(e)**).

3.4. *In Vitro* Rooting of Shoots and Hardening

In vitro shoots of *Quisqualis indica* regenerated via shoot organogenesis rooted spontaneously on MS basal medium. Besides, the MS medium supplemented with different concentrations of IAA or IBA either individually or in combination induced root initiation (**Figures 5(f)** and **(g)**). IAA at a concentration of 1.0 mg/L showed decrease in root production while IBA at this level was inhibitory to root induction. The inhibitory action was possibly due to the less quick metabolism of IBA than that of IAA [23]. The action of IAA and IBA at a lower concentration of 0.5 mg/L was found to increase the number of roots per explant. *In vitro* raised plantlet was acclimatized and maintained in experimental field where 90.0 % of plantlets survived (**Figure 5(h)**).

4. Discussion

In the present investigation, the MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L GA_3 was chosen for further up-scaling of in vitro shoot regeneration because of its increased (20 shoots per node) shoot formation potential. This result was similar to the report of Tzitzikas *et al*. [24] who achieved regeneration of 22 shoots per node explant of *Pisum sativum* on MS3 medium supplemented with 1.0 mg/L GA_3 and 1.0 mg/L BAP. In contrast, in the present investigation, the less percentage and poor shoot regeneration (2 - 3 shoots) on MS medium containing 1.0 mg/L 2-iP and 0.5 mg/L GA_3 was possibly due to phytotoxicity of cytokinin (2-iP). The similar results of less percentage of shoot regeneration and stunted shoot growth through nodal segments of *Vaccinium species* [18] were obtained on woody and plant medium [25] supplemented with 25 µM 2-iP.

The source of explants is an important factor in determining the ability to induce callus. This suggests that the endogenous hormone levels as well as hormone responsivity vary among the different organs. Thus the results of the investigation showed higher frequency of callus induction through leaf section explants than the one-node stem base explants. Similar to these results, the addition of TDZ to MS medium resulted to successful calli induction from leaf explants of *Echinaceae purpurea* [26]. Stimulated callus growth was observed from epicotyl explants of *Phaseolus lunatus* [27] on MS containing 0.5 mg/L TDZ and 0.05 mg/L IAA. The best calli induction response from root segments of *Dorem ammoniacum* was reported by Irvani *et al*. [28] on MS medium supplemented with 2.0 mg/L BA and 1.0 mg/L NAA whereas cotyledon explants of *Punica granatum* [20] showed the highest frequency of callus induction on MS medium supplemented with 21 µM NAA and 9 µM BA.

On the other hand, similar to present investigation, calli formation from cut ends of slender stem of *Oldenlandia umbellata* has been reported by Siva *et al*. [29]. The successful induction (85%) of callus like tissue from base of node stem explants of *Pisum sativum* was reported by Tzitzikas *et al*. [23] on MS3 medium supplemented with 2.2 mg/L TDZ. Physiological gradient in explants holds key to the frequency of callus induction as observed in different species including *Fagus sylvatica* [30].

The balance between cytokinins and auxins holds key to the differentiation of organogenic calli into shoot bud induction and plantlet development during shoot organogenesis [31,32]. In the present investigation, it was observed that the total number of regenerants increased as the concentrations of TDZ increased in combination with 0.5 mg/L GA_3 or 0.25 mg/L NAA. This was in line with the observations on differentiation of organogenic callus into plantlets in *Hovenia dulcis* [33] on MS medium supplemented with 0.23 µM gibberellic acid and 0.46µM kinetin. Similarly maximum number of shoots was achieved from leaf section callus of *Hyptis suaveolens* [34] on MS medium containing 0.5 mg/L BA and 0.5 mg/L GA_3. In the present investigation, 71.6% of the organogenic calli derived through leaf section explants on MS medium supplemented with 1.0 mg/L TDZ and 0.25 mg/L NAA produced an average of 2.1 shoots per callus explant which was consistent with the previous reports such as, *Saussurea obvallata* [14] inducing 12 shoots per callus explant on MS medium supplemented with 5.0 µM BA and 1.0 µM NAA, *Astragalus cariensis* [35] producing 23 shoots per callus explant on MS supplemented with 4.0 mg/L BA and 0.5 mg/L NAA, *Kosteletzkya pentacarpos* [36] showing high shoot induction on medium containing 1.0 mg/L kinetin and 2.0 mg/L

IAA. Similarly Tan *et al*. [37] could induce plantlet regeneration in *Vanilla planifolia* from leaf and node derived callus on MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L NAA. There were reports of the best shoot regeneration response from leaf explants of *Brunfelsia calycina* [38] on MS medium supplemented with 2.85 µM IAA and 4.44 µM BA or 4.54 µM TDZ and hypocotyl derived organogenic calli of *Dorem ammoniacum* [28] on MS medium with 2.0 mg/L BA and 0.2 mg/L IBA. Likewise, the MS medium supplemented with cytokinins and auxins (TDZ and NAA) induced adventitious shoot regeneration in many species such as, *Prunus serotina* [39], *Aechmea fasciata* [40] and *Echinacea purpurea* [26]. Ghimire *et al*. [41] obtained highest mean number (10.65) of shoots per explant from *in vitro* leaves of *Drymaria cordata* on medium containing 1.0 mg/L BA and 0.1 mg/L NAA. Similar to the present results of indirect shoot organogenesis, successful callus initiation and proliferation and adventitious shoot production through nodal explants of silver maple and *Acer* species were achieved by Preece *et al*. [42] and Marks and Simpson [43] respectively. This was ascribed to the auxin accumulation at the excision sites by downward movement which in turn causes cell proliferation especially in the presence of cytokinins [43].

The Rooting efficiency of IAA or IBA has been documented for many medicinal and ornamental plants [16, 20]. The inhibitory action of IBA at 1.0 mg/L compared to IAA was possibly due to the less quick metabolism of IBA than that of IAA [23]. The action of IAA and IBA at a lower concentration was found to increase the number of roots per explant. This result was consistent with the previous report on rooting of *Ailanthus triphysa* [44].

5. Conclusion

In the present investigation, the TDZ treatment was superior to that of 2-iP and BA in combination with either GA_3 or NAA in terms of inducing the formation of multiple shoots from organogenic callus derived from leaf section and one-node stem base explants of this ornamental shrubby climber *Quisqualis indica*. This was attributed to the special property of TDZ and NAA to fulfill the capability of the ratios of cytokinin and auxin responses in plant species for achieving adventitious shoot production from callus tissues. The development of an efficient plant tissue culture system for the micropropagation of *Quisqualis indica* through organogenic callus derived from leaf section and one-node stem base explants holds promise to facilitate conservation and the commercial production of this plant for its medicinal and ornamental use, besides its potential use in plant breeding and the production of transgenic plants through genetic engineering.

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