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In vitro and *in vivo* assessment of Thidiazuron mediated micro-clones of *Dendrocalamus asper*, an ornamental bamboo species

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ABSTRACT

Dendrocalamus asper is an economically important ornamental as well as edible bamboo species. Long flowering cycle limits its propagation which has been surmounted a little by macropropagation. Tissue culture has already been proved as efficient method for large scale propagation. Despite the higher potentiality of thidiazuron as promising phytohoromone, no report is available in micropropagation of D. asper. A complete protocol of thidiazuron mediated micropropagation of D. asper is reported for the first time in the present report. Murashige and Skoog (MS) media with very low concentrations of thidiazurone (0.25 and 0.5 mg/l) were found equally effective with higher concentrations (3 and 4.5 mg/l) of 6-Benzyladenine (BA) for bud breaking and shoot length. Significant multiplication was observed when the single shoot from thidiazuron containing solid MS, was transferred to liquid MS medium supplemented with 3 mg l^{-1} BA, which is rarely possible in case of BA. Both root length and root numbers/plant was found maximum at ½MS with 1 mg l^{-1} IBA (Indole-3-butyric acid) during root induction. Simple cost effective hardening procedure using cocopeat (100%) followed by soil (100%) promoted high survivability during acclimatization. Healthy plantlets with higher survival rate were ascertained from a field trial of 18 months.

Keywords: *Dendrocalamus asper*, micropropagation, node, phytohormone, thidiazuron

Dendrocalamus asper, an exotic ornamental bamboo species, is native to China (Singh *et al*., 2012b) and was introduced in India by Indian Council of Forestry Research and Education (ICFRE) in 1994 (Singh *et al*., 2004). This economically important bamboo species is worldwide famous for its edible value as well as its higher preference in the paper and pulp industry (Arya *et al*., 2001). Besides, matured culm is also utilized for construction and handicraft (Kumar and Banerjee, 2004). In the year of 2000, USA spent US $16.8 million for the import of the edible bamboo shoots of this species from South Asian countries (Singh *et al.,* 2004). In India, *D. asper* is now gaining importance and recognized as one of the target species and considered for bamboo propagation through National Bamboo Mission (Nadha *et al*., 2013).

Despite of its economic potentiality, the farmers are not getting full benefits of the species as both seed and cutting- based vegetative propagation of *D. asper* is very troublesome. Seed based propagation is limited due to irregular long flowering cycle (Nadgir *et al*., 1984; Banerjee *et al*., 2011) where as the vegetative propagation is restricted because of 'bulkiness' of cutting materials (Arya *et al*., 1999), seasonal dependency (Saxena and Bhojwani, 1993) and low multiplication rate (Banerjee *et al.,* 2011). The micropropagation technique is already proven as very reliable and robust technique in multiplication/propagation of several woody plants including bamboo (Aitken-Christie *et al*., 1995; Bakshi *et al*., 2015). The micropropagation technique may be exercised to overcome not only the shortfall of saplings but also to conserve this exotic bamboo species in its natural habitat. Micropropagation of tree species having monopodial growth pattern with thidiazuron (TDZ) was found superior over common cytokinins because of its rapid shoot multiplication capacity (Huetteman and Preece, 1993; Faisal *et al*., 2005). Application of TDZ on bamboo micropropagation are reported in different species namely in *D. strictus* (Singh *et al*., 2001; Chowdhury *et al*., 2005; Kapruwan *et al*., 2014), *D. giganteus* (Ramanayake *et al*., 2001), *B. vulguris* (Ramanayake *et al*., 2006), *B. edulis* (Lin and Chang, 1998; Lin *et al*., 2004), *B. oldhamii* Munnro (Lin *et al*., 2007) and *D. hamiltonii* (Singh *et al*., 2012a). But in case of *D. asper,* reports are available on the use of 6-Benzyladenine (BA) (Arya *et al*., 2001; Kumar and Banerjee, 2014; Arya *et al*., 1999), and both of Kinetin (Kin) and BA (Banerjee *et al*., 2011; Singh *et al*., 2012b; Nadha *et al*., 2013). But there is no report available till now on this promising cytokinin TDZ in micropropagation of *D. asper*. This study was undertaken to develop TDZ mediated micropropagation protocol of *D. asper* emphasising the potentiality of use of TDZ in comparison to BA. The hardened saplings were also evaluated through field experimentation.

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MATERIALS AND METHOD

Collection of plant materials

Nodal explants of *D. asper* were collected from the green house of Department of Science and Technology (Govt. of West Bengal), Salt Lake, Kolkata, West Bengal, India (N 22°35´09.8´´/ E 88°24´53.2´´).

Experimental method

The explants were rinsed in running tap water to clean and remove the dirt and debrises. Before surface sterilization, the leaf sheath tissues covering the axillary bud were removed carefully by sharp scalpel without damaging the bud. Nodal segments were surface sterilized with 1% (savlon and tween 20) and subsequently were treated with bavistin (1%) and 0.1% mercuric chloride ($HgCl_2$) for 10 minutes each. After washing with $HgCl_2$, explants were washed four times with sterile double distilled water and the cut ends of the either sides of the nodal segments were trimmed to avoid any residual $HgCl_2$ in the cut ends. Then explants were aseptically transferred into MS media (Murashige and Skoog, 1962) supplemented with 4 different concentrations of BA (Merck, India) *i.e.* 1.5, 3, 4.5, 6 mg/l and TDZ (Duchefa Biochemie, Netherlands) *i.e.* 0.25, 0.50, 0.75, 1 mg l^{-1} and 3% (w/v) sucrose (Titan Biotech Pvt. Ltd., India), gelled with 0.8 % (w/v) agar (Hi-media Lab. Pvt. Ltd., India). The whole inoculation process was carried out under aseptic condition using laminer air flow (Klenzaids Bioclean Device Pvt. Ltd., India). The pH of the medium was modified to 5.70 with 1 (N) sodium hydroxide and 1 (N) hydrochloric acid prior to autoclaving at 121°C for 15 minutes. All cultures were maintained at 23±2°C, under a 16 hours photoperiod. After 3 weeks, shoots (single or two shoots bearing explants) regenerated from node were transferred to the liquid medium having 3 mg l^{-1} BA for multiplication. At an interval of three weeks (considered as one cycle for multiplication), subculture was done. After 10-12th subculture the plant propagules having more than 2-3 shoots culture materials were transferred to rooting media. For rooting, ½MS media containing different concentrations *i.e.* 1, 3 and 5 mg/l of indole-3-butyric acid (IBA) were used. After rooting, the plantlets were transferred to green house for hardening. Prior to primary hardening, plantlets were treated with bavistin (1%) for 15 minutes and after that primary hardening was done using cocopeat (100%) in the green house of Department of Science and Technology, Salt Lake, while after two weeks the plantlets were transferred to soil (100%) for secondary hardening. Data were recorded with respect to response variables (Fig. 1 a-d ; 2 and 3.a-c) at each stage (shooting, rooting and multiplication) with three replications and ten explants per replication for statistical analysis.

Field trial

Field trial was done in Gossairhat beat, Morgahat Beat range, Jalpaiguri range, West Bengal. Sixty saplings were planted and data on four morphological characters were taken at 6,12 and 18 month of planting (Fig. 4 a-c).

Statistical analysis

Statistical analyses of recorded data were performed using SPSS v16.0 for Windows (SPSS Inc., USA) with 5% probability level of statistical signiûcance.

RESULTS AND DISCUSSION

Effect of TDZ on shoot induction

Nodal explants after surface sterilization were found responsive in both cytokinins (BA and TDZ) as responses in both phytohormones were found better than control. As far as our knowledge goes, this is the first report on effect of TDZ on nodal explants of *D. asper* and assessment of this phytohormone was done with most widely used cytokinin BA. Among the various concentrations of phytohormones, TDZ was found to be more effective for early bud breaking than BA even at very low concentration (data regarding time is not given). High bud breaking at 5 mg l^{-1} BAP for the same species were recorded whereas similar bud breaking and shoot length were recorded in our experiment at lower concentration of TDZ (0.5 mg l^{-1}) (Bakshi *et al*., 2015). Rapid shoot proliferations (within a month of incubation) at comparatively low BA (4.5 mg l^{-1}) were observed. Multiple branching (more than 2 shoots) after 8 weeks of incubation under high BA (5 mg l^{-1}) found for *D. asper* (Arya *et al*., 2001). After three weeks of different treatments, shoot length was found maximum (6.57 cm) in TDZ (1 mg l^{-1}) coupled with highest bud breaking (95.83%) at 0.5 mg l^{-1} TDZ. The bud breaking at lower concentrations of TDZ (0.25-0.5%) were statistically as par to 4.5 mg l^{-1} BA (Fig. 1.a) that indicates its superiority over BA. Comparatively lower numbers of shoots (1.33-1.96) or leaves (1.67-2.42) in each plant were found in TDZ treated plants. The highest number of shoot (3.12) and leaves (3.29) per plant were observed at 4.5 mg/l BA (Fig. 1. d and c). The low concentration of TDZ is reported for shoot proliferation in *B. edulis* (Ramanayake *et al*., 2006) and *D. strictus* (Singh *et al*., 2001) that was again confirmed by the present study in *D. asper* also. Unlike BA, the better response at low concentration of TDZ was also in agreement with the findings in *D. hamiltonii* (Singh *et al*., 2012a) who reported TDZ to be superior over BA and Kinetin (Kin).

Effect of TDZ on multiplication

Explants containing more than 2 shoots as well as single shoot were transferred to liquid MS medium supplemented with 3 mg l^{-1} BA found effective for multiplication. Liquid media was chosen for multiplication as it has been found effective for multiplication in *D. asper* (Banerjee *et al.,* 2011) and *D. brandisii* (Kavitha and Kiran , 2014). At three weeks intervals, the plants were transferred to fresh medium to prevent browning. Strikingly, like two shoots bearing explants, significant multiplication was found when single shoot bearing explants from TDZ containing medium were transferred to liquid MS with 3 mg/l BA (Fig. 2). This finding is rarely possible in case of BA treated explants as reported by earlier workers. Single shoots were not suitable for multiplications, reported for several bamboo species including *D. strictus* (Pandey and Singh, 2012), *B. tulda* (Pratibha and Sarma, 2013), *D. hamiltonii* (Godbole *et al*., 2002), *B. nutans* (Mudoi *et al*., 2014). But we found the single shoot bearing plants, grown in MS solid medium supplemented with TDZ during shoot initiation, induced multiple shoots if transferred to liquid MS with 3 mg l^{-1} BA (Fig. 2). Being very active cytokinin, TDZ promotes pseudoshoots due to which liquid MS medium with BA was found to be suitable for further shoot multiplication and maintenance of bamboo culture (Kavitha and Kiran, 2014). It otherwise, limits the use of TDZ in multiplication media (Lin *et al*., 2007). Though both single or two shoots was found suitable for multiplication but high multiplication rate was observed in single shoot plants (~6.5 folds) in comparison to plants having two shoot (~4.45 folds) (Fig. 2) after third cycle of multiplication.

At three weeks intervals, the plants were transferred to fresh media to prevent browning. Despite low branching, TDZ treatment in explants prior to multiplication stage was found effective, which in other words increases the survivality of plant and reduce the explants loss. Though initially response was quite slow but successive transfer to liquid medium enhanced the rate of multiplication. The delayed response might be due to the shock in plant after transfer from TDZ to BA. Moreover, TDZ treated plants having high shoot length may prevent loss of explants during multiplication at liquid media. Subculture was done at three weeks interval to increase the mass of culture.

Rooting

Since plants with spontaneous rooting were transferred to rooting medium, there was no significant variation found in rooting percentage (Fig. 3a). Significant variation was observed in root numbers plant^{-1} and root length plant^{-1} under various concentrations of IBA (Fig 3.b, c). Among all treatments, IBA at lowest concentration (1 mg l^{-1}) was found most suitable to induce the maximum root numbers/plant (10.75) and root length plant^{-1} (5.67 cm). This finding was in conformity to the findings for *D.asper* (Kumar and Banerjee, 2014) and *B. balcooa* (Gantait *et al*., 2018). Increase of IBA in medium leads to reduction of root length as well as root length (Fig 3 b,c). Among all the concentrations, ½MS containing 1 mg l^{-1} IBA was found best in terms of root length and root numbers per plant

Hardening

After one month of rooting, the plantlets were transferred to green house for hardening. Primary hardening was done on cocopeat (100%). The pots were covered with transparent polyethylene to protect from sunlight. Watering was done to maintain more than 97% humidity. After 20 days, plantlets were transferred to plastic pots having soil and were kept in green house. Soil (100%) was found effective secondary hardening material and after one month, the healthy hardened plantlets were transferred to field. Sterilized cocopeat reported as hardening material for *B. balcooa* (Gantait *et al*., 2018), *D. asper* (Banerjee *et al.,* 2011). The use of non-sterilzed cocopeat for the purpose of primary hardening makes the protocol easier, faster and cheap. The use of non-sterilized cocopeat has been reported for *D. asper* (Kumar and Banerjee, 2014). Cocopeat (100%) was found better than sand: soil (100%) for *Sinningia speciosa* (Kashyap and Dhiman, 2011) and Perlite (100%) for *Garcinia indica* Chois (Chabukswar and Deodhar, 2005) respectively in terms of plants survivality. Cocopeat is considered as effective hardening material may be due to capable of sufficient aeration to roots of developing plants, also have capacity to absorb water and release it slowly (Kamle *et al*., 2016). Secondary hardening was done on soil for the plantlets. There is no report till now soil (100%) as secondary hardening material for *D. asper.* Several growth supporting materials were used for secondary hardening of *D. asper* i.e. dune sand and vermi compost (3:1) (Singh *et al*., 2012b), sand, soil and farm yard manure (1:1:1) by several workers (Arya *et al*., 2001; Kumar and Banerjee, 2014; Banerjee *et al*., 2011; Arya *et al*., 1999). This is again possibly first successful report of secondary hardening in soil (100%) with high survivality.

Field trial

Sixty hardened plantlets were transferred to the field for examining their performance at field conditions. After 18 months of transplanting, high survival rate (93%) was observed. The significant increase in plant height (133 cm to 375 cm), culm circumference (1.17 cm to 11.67

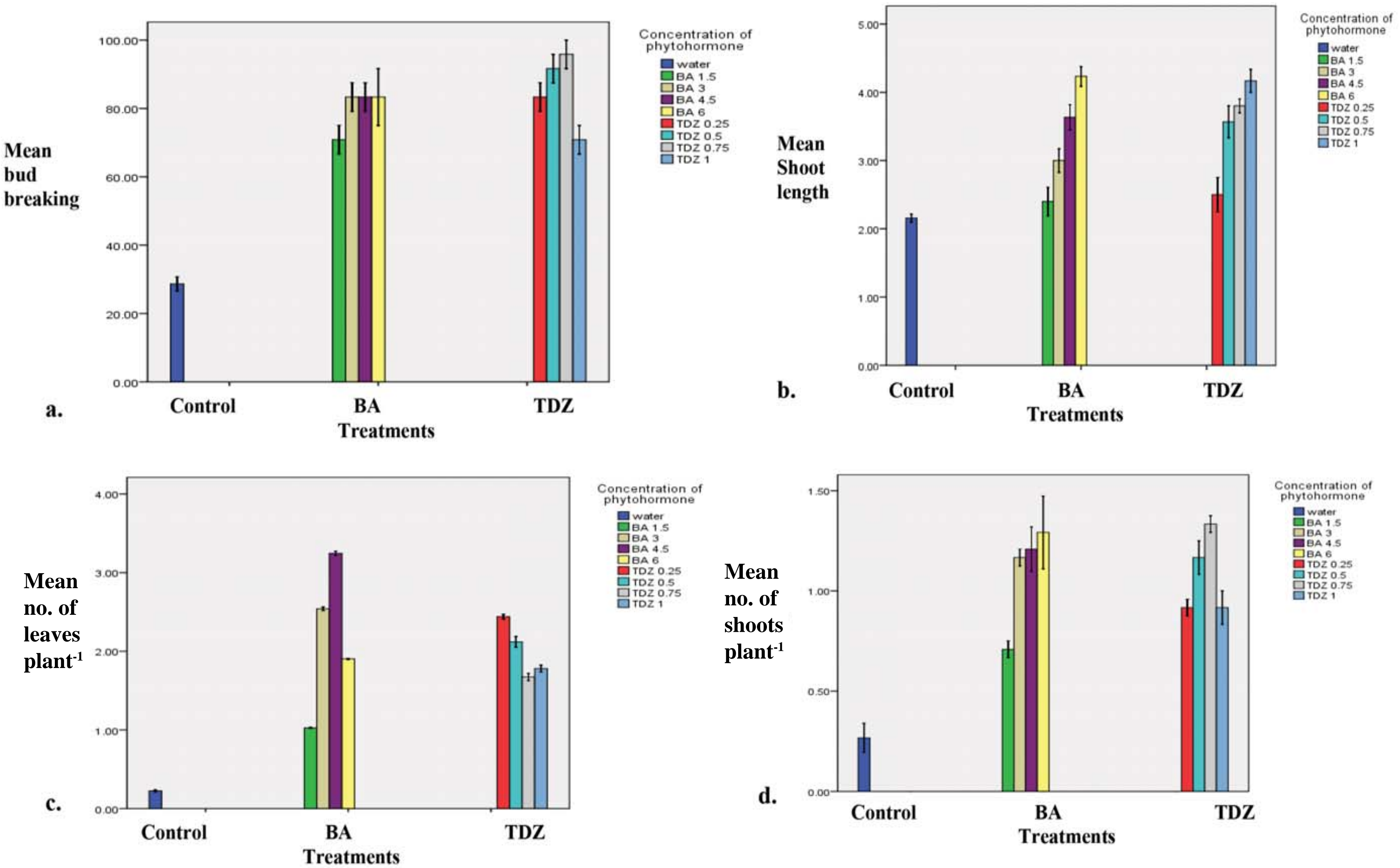

Fig. 1: Affect of difference doses of TDZ and BA on different variables such as a) bud breaking, b) shoot length, c) No. of leaves plants^{-1}, and d) No. shoots plant^{-1} during micropropagation of *D.asper*

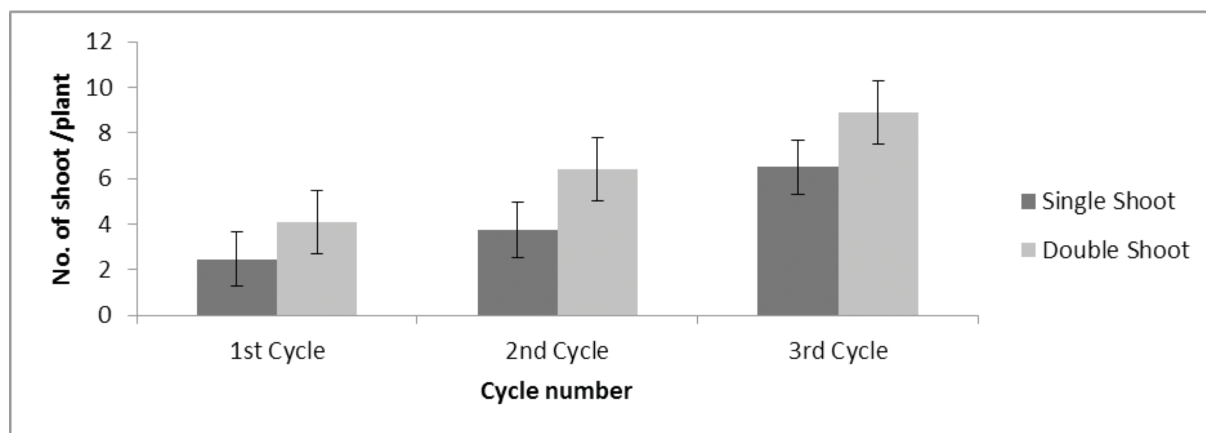

Fig. 2: Shoot multiplication during *in vitro* propagation of *D. asper* upto 3rd cycle (10 plants having single shoots and double shoots were transferred from TDZ to liquid MS + 3 mg/l).

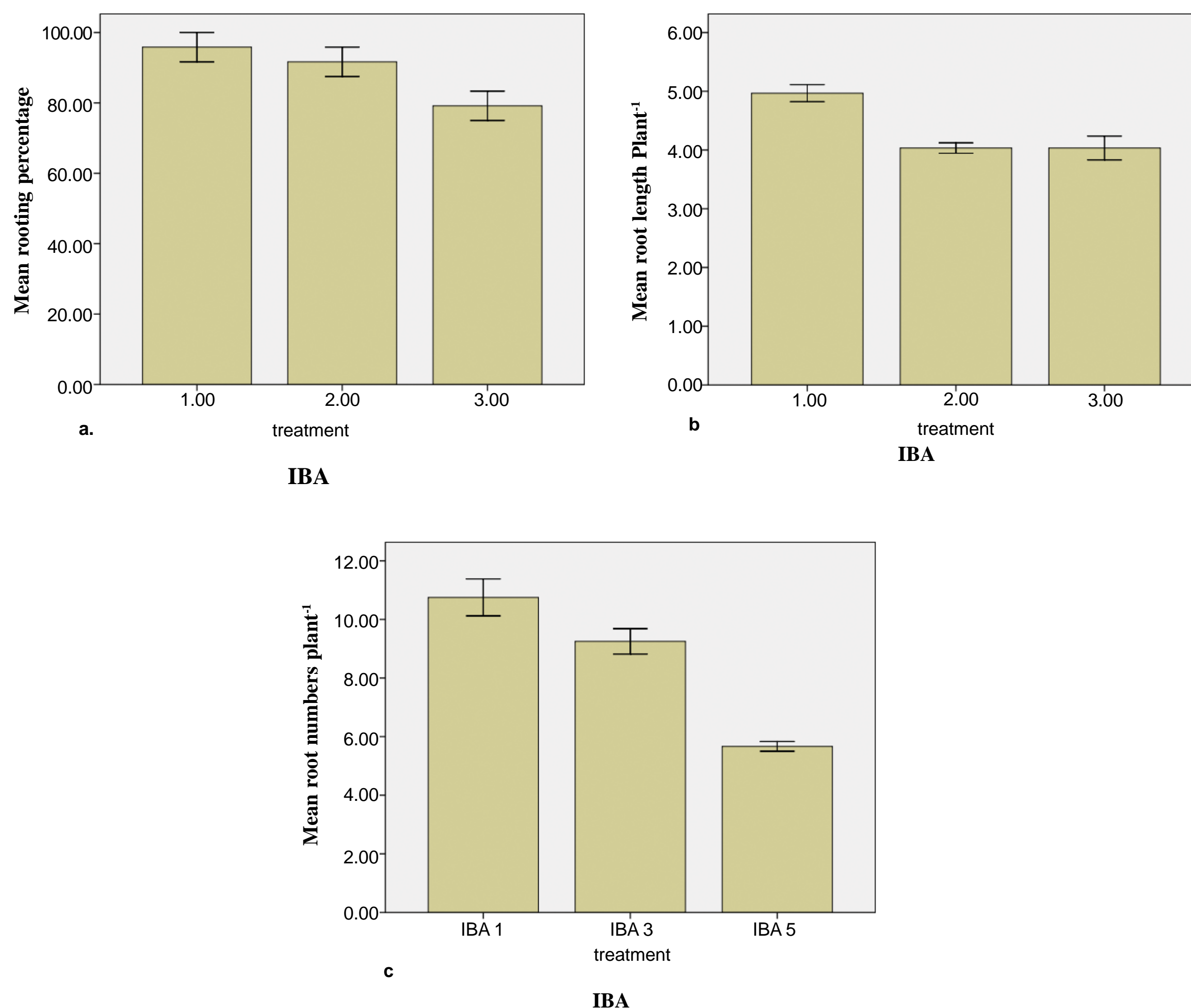

Fig. 3: Affect of difference doses of IBA on different variables such as a) rooting percentage , b) mean root length plant^{-1} length, c) root numbers plant^{-1} during micropropagation of *D.asper*

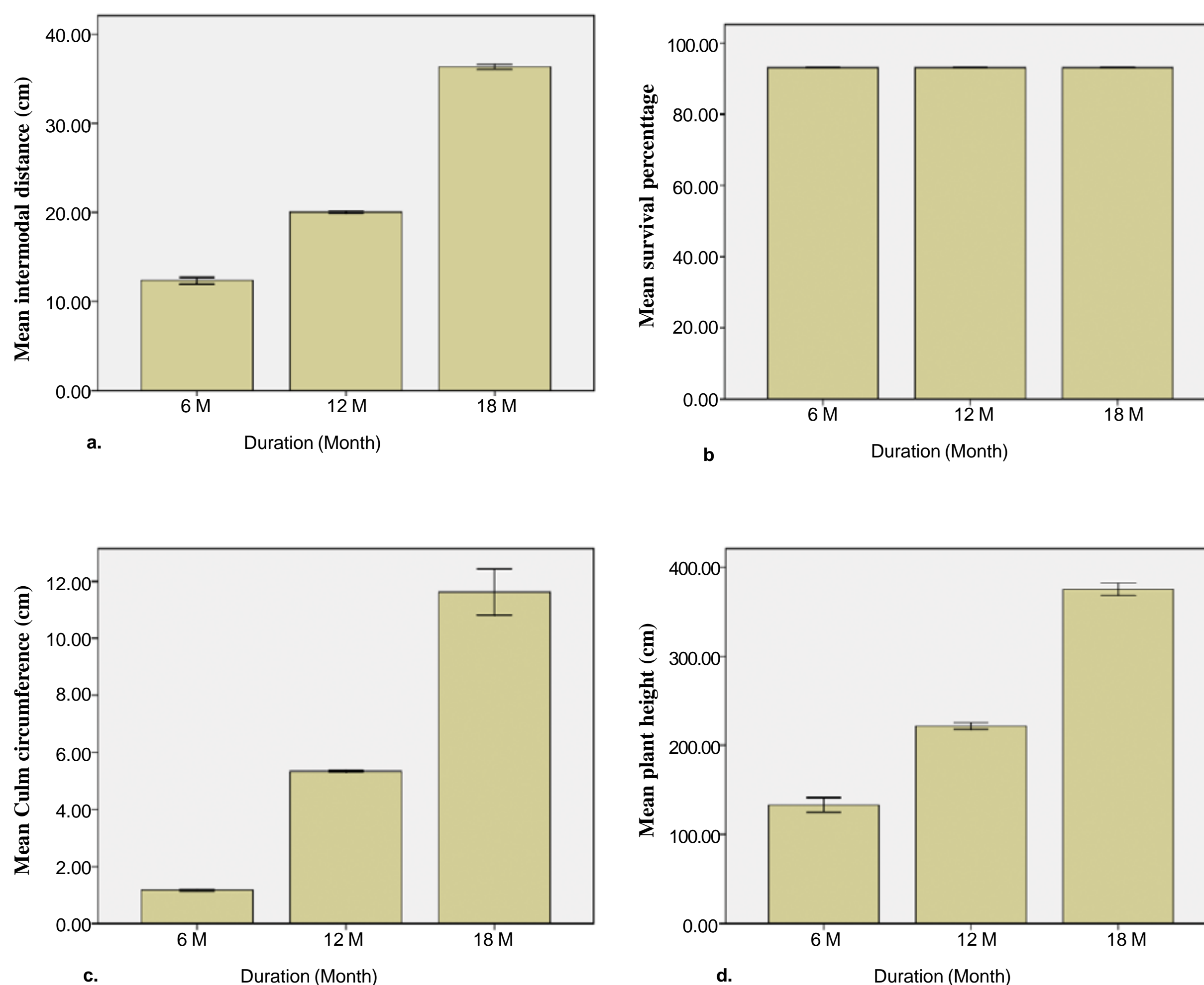

Fig. 4: Growth performances of *D. asper* at 6, 12 and 18 months intervals with respect to different variables such as a) Internodal distance , b) survivality rate , c) culm diameter and d) plant height during field trial.

cm) and intermodal distance (12.33 cm to 36.33 cm) were recorded (Fig. 4 a, b and c). Previously, field experiment were carried out in *Thamnocalamus spathiflorus* (Trin.) Munro (Bag *et al*., 2000); *Musa spp* (Vuylsteke and Ortiz, 1996) and sugarcane (Sood *et al*., 2006) and reported high survivability. Unlike the field experiment of *D. asper* (Banerjee *et al*., 2011) the present report recorded not only the survivality of the plants in field condition but also included several other morphological descriptors.

Among both cytokinins, TDZ found more effective than BA in terms of *in vitro* propagation of *D. asper* using nodal explants. Minute dose of TDZ were found effective for bud breaking and shoot growth. This will also make this protocol cost effective. High shoot length prevents the submergence of explants in liquid medium during multiplication and also prevents use of Filter Bridge reported by several authors at this stage. Single shoot multiplications will definitely help in reducing the loss of explants during *in vitro* propagation.

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