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Research Article

Regeneration of *Hemidesmus indicus* (L.) R. Br. using *in vitro* nodes: an alternative method for efficient multiplication of shoots

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Abstract

In vivo nodes of *Hemidesmus indicus* (L.) R. Br. induced healthy multiple shoots with branching in our earlier studies and thus in the present study, potency of *in vitro* nodes to regenerate shoots was evaluated. *In vitro* nodes were excised from eight-week-old shoots and placed in Murashige and Skoog's (MS) medium fortified with sucrose (3%) and different concentrations of 6-benzyladenine (BA) and kinetin (Kn). After eight weeks, optimum of 5.42 ± 0.36 shoots with 100% response were regenerated in medium supplemented with BA (10 µM) and Kn (5 µM). These healthy shoots were placed in full, half and quarter strengths of liquid MS medium fortified with sucrose (1%) and α-naphthaleneacetic acid (NAA, 1-25 µM) for rooting. Among all the strengths of MS medium, full strength MS medium having 8 µM NAA formed maximum of 3.42 ± 0.55 roots (91.67% response) within four weeks. The protocol is in continuation with earlier study and it was confirmed that a single *in vivo* nodal explant can regenerate around 385 healthy elongated shoots within 4 months, which will help in mass-propagation of the species.

Keywords: axillary shoots; clonal multiplication; cytokinins; medicinal plant; tissue culture

Introduction

Hemidesmus indicus (L.) R. Br. (Asclepiadaceae), commonly known as 'anantmool', is a slender, laticiferous, semi erect shrub widely used in Indian Systems of Medicine (Anonymous, 1997). The plant is prescribed for its properties to give longevity, rejuvenation and strengthening the bones and tissues and hence considered as 'Rasayana' drug (Puri, 2003). Traditionally the plant is used to cure scorpion sting and rat bite poisoning (Sharma *et al.*, 1979) and also have uses to treat many ailments like blood diseases, diarrhoea, respiratory disorders, kidney and urinary disorders, skin diseases, syphilis and rheumatic fever (Nadkarni, 1989). The multipurpose medicinal attributes are because it has important secondary metabolites like lupeol, vanillin, rutin, 2-hydroxy-4-methoxybenzaldehyde and β-sitosterol (Chatterjee and Bhattacharya, 1955; Subramanian and Nair, 1968; Gupta *et al.*, 1992; Nagarajan and Rao, 2003). National Medicinal Plants Board (NMPB) has reported *H. indicus* as one of the priority species because its annual requirement is 500-1000 MT (Ved and Goraya, 2007). To fulfil this demand, *H. indicus* is extensively collected from natural habitats and its vegetative propagation is slow, due to which it has become endangered (Sreekumar *et al.*, 2000). Thus, since last two decades it is being imported from other countries like Myanmar, Mexico and Morocco (Planning commission, 2000).

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Biotechnological tool like plant tissue culture is known to regenerate large number of plants in short time duration; and to achieve this leaf and nodal explants are mostly utilized. Nevertheless, regeneration through leaf explant is known to induce soma clonal variants and hence nodes are best suitable explants when the aim is to produce true-to-type plants (Pathak and Joshi, 2018). There are reports on *H. indicus* regeneration using nodal explants (Patnaik and Debata, 1996; Sreekumar *et al*., 2000; Misra *et al*., 2003; Saha *et al*., 2003; Shekhawat and Manokari, 2016; Pathak *et al*., 2017). However, the plant is a slow growing and sparingly branched in wild (Sreekumar *et al*., 2000) and hence use of nodal explant for plant regeneration may increase the threat on its population. George (1996) has depicted that the explants derived from shoot cultures have an early and greater capacity for regeneration due to juvenile nature as compared to *in vivo* plants. The utilization of *in vitro* nodes as an alternative to *in vivo* nodal explants for generating clonal plants has been reported in many endangered species (Baskaran and Jayabalan, 2005; Loganathan and Bai, 2014), whereas in *H. indicus* only few reports are documented (Sreekumar *et al*., 2000; Nagahatenna and Peiris, 2007). In previous study on *in vivo* nodal explants it was observed that the axillary buds of *in vitro* shoots had a tendency to form branches when grown in cytokinin fortified media (Pathak *et al*., 2017).

Thus, the present study was focuses on the potency of *in vitro* nodes to form shoots through which clonal multiplication of *H. indicus* can be achieved and it will help in mass-propagation.

Materials and Methods

Plant material and culture medium for shoot regeneration

Healthy twigs of *H. indicus* were collected from the Botanical Garden of The M.S. University of Baroda. Nodal explants were excised, washed under running tap water for 1 h, surface sterilized and inoculated in earlier optimized media for shoot culture establishment i.e. MS (Murashige and Skoog, 1962) medium fortified with 6-benzyladenine (BA, 10 µM) and kinetin (Kn, 5 µM) (Pathak *et al*., 2017). After eight weeks, these shoots were harvested from mother explant, all the leaves were trimmed off and *in vitro* nodes (2-3 cm long) were used as an explant for the present study. They were inoculated vertically into MS medium fortified with sucrose (3%) (SRL, Mumbai, India) and different concentrations of BA and Kn (5-20 µM) (SRL, Mumbai, India). pH of the medium was adjusted to 5.80 using NaOH/HCl (1 N) and agar (0.8%) (SRL, Mumbai, India) was used as the gelling agent. All the media were sterilized in autoclave at 121 °C (15 psi) for 20 min.

In vitro rooting

Healthy micro shoots (8 weeks old; >3 cm in length) were excised, leaves were removed and dipped in sterile distilled water and bavistin solution (0.01%) (1 min each). Different strengths (full, half, and quarter) of liquid MS medium fortified with sucrose (1%) and various concentrations of α-naphthaleneacetic acid (NAA; SRL, Mumbai, India) (1-25 µM) was used as rooting media.

Culture conditions

All the cultures were kept in culture room and maintained at 26 ± 2 °C under 16/8 h (dark/light) photoperiod at 40 µmol/m^2/s provided by cool-white fluorescent lights (Philips India Ltd., India).

Statistical analysis

The values are given as mean and standard error (SE) from twelve replicates. All the means were analysed using ANOVA (α = 0.05) and significant means were further analysed by Tukey's test using XLSTAT v2017.02.

Results and Discussion

Shoot regeneration

In vitro nodes derived from eight-week-old shoot cultures were used as an explant for clonal multiplication in MS basal medium, which evoked a meagre response as it formed only 0.58 ± 0.22 shoots (41.67% response) (Table 1). Supplementing the medium with different concentration of BA (5-20 µM) slightly increased the culture response and the shoot number was increased to 1.83 ± 0.16 (100% response) when BA concentration reached to 10 µM. Further increase in concentrations to 15 and 20 µM failed to enhance the number of shoots. When BA was replaced with Kn in the medium it also resulted in a similar response as only 1.58 ± 0.28 shoots (83.33% response) were recorded in media containing 10 µM Kn (Table 1). Thus, both the cytokinins individually formed healthy axillary shoots, but they failed to induce multiples.

The response in terms of number of shoots was increased as the MS medium was augmented with both BA and Kn as it induced multiple shoots in most of the combinations. Eight weeks observations depicted that optimum 5.42 ± 0.36 shoots in 100% cultures were formed in medium supplemented with BA (10 µM) and Kn (5 µM) (Table 1). In this combination the axillary bud beak was recorded within a week of inoculation (Figure 1A). This bud elongated and formed shoot during second week and simultaneously bud break from the opposite node was also observed (Figure 1B). These shoots started to elongate in third week and branching of shoots was occurred during fourth week (Figure 1C). At the end of four weeks the shoots were sub-cultured which further proliferated and multiplied till the end of eight weeks (Figure 1D). Thus, it was confirmed that *in vitro* buds, which were harvested after every eight weeks of culture period, retained the potency to form shoot cultures.

Table 1. Regeneration of shoots from *in vitro* nodes of *H. indicus* in presence of cytokinins (8 weeks)

PGR (µM)		No. of shoots/explant*	Response (%)
BA	Kn		
0	0	0.58 ± 0.22 f	41.67
5	0	1.17 ± 0.16 ef	91.67
10	0	1.83 ± 0.16 cdef	100
15	0	1.42 ± 0.18 def	91.67
20	0	1.08 ± 0.18 f	83.33
0	5	0.83 ± 0.16 f	75
0	10	1.58 ± 0.28 def	83.33
0	15	1.33 ± 0.30 def	83.33
0	20	0.83 ± 0.26 f	58.33
5	5	2.00 ± 0.51 bcdef	58.33
5	10	2.17 ± 0.47 bcdef	66.67
5	15	1.75 ± 0.47 cdef	66.67
5	20	1.08 ± 0.32 f	50
10	5	5.42 ± 0.36 a	100
10	10	4.67 ± 0.54 ab	100
10	15	4.33 ± 0.48 abc	91.67
10	20	3.83 ± 0.79 abcde	83.33
15	5	3.92 ± 0.61 abcd	83.33
15	10	3.25 ± 0.72 abcdef	75
15	15	3.17 ± 0.79 abcdef	75
15	20	2.58 ± 0.59 bcdef	75
20	5	3.00 ± 0.61 abcdef	83.33
20	10	3.25 ± 0.59 abcdef	75
20	15	2.83 ± 0.73 abcdef	66.67
20	20	2.25 ± 0.68 bcdef	50

*Values represents mean ± SE. Means (n = 12) were subjected to ANOVA and means followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test

Beneficial effect of cytokinins in axillary shoot regeneration is mainly attributed due to their involvement in processes like promotion of cell division, expansion, regulation of shoot formation and its multiplication (Mok and Mok, 2001). Synergistic combination of BA and Kn evoked optimum regeneration in the present study and the shoot number obtained in this protocol was higher as compared to previous report on same plant (Nagahatenna and Peiris, 2007). The combined effect of BA and Kn is also reported for maximum axillary bud multiplication in *H. indicus* (Shekhawat and Manokari, 2016). Similarly, in other medicinal plants like *Eclipta alba* (Baskaran and Jayabalan, 2005), *Vanilla planifolia* (Abebe *et al*., 2009), *Pogostemon cablin* (Swamy *et al*., 2010) and *Enicostema axillare* (Loganathan and Bai, 2014) BA with Kn induced optimum shoots from *in vitro* nodes. Moreover, the potency of *in vitro* nodes to regenerate shoots was less as compared to *in vivo* nodal explants of the same plant (Patnaik and Debata, 1996; Sreekumar *et al*., 2000; Misra *et al*., 2003; Saha *et al*., 2003), it is also in contradiction to the earlier report which states that the potency of *in vivo* and *in vitro* nodes is the same (Sreekumar *et al*., 2000). But this study is in continuation of previous work in which from single node around 11 shoots were formed within eight weeks, each shoot had an average 7 nodes (Pathak *et al*., 2017). Thus approximately 77 *in vitro* nodes were obtained which were taken up for clonal multiplication of shoots and transferred to the media optimized in the present study. Total 5 shoots were regenerated with 100% response at the end of eight weeks which confirmed that around 77 × 5 = 385 shoots can be regenerated within 4 months from single *in vivo* nodal explant. Therefore, this method of using *in vitro* nodes for shoot regeneration is more efficient as compared to transferring of shoot clumps as reported for the same plant (Shekhawat and Manokari, 2016). The use of *in vitro* nodes will also resolve the problems reported for *in vivo* nodal explants of *H. indicus* such as leaf fall during culture multiplication, decrease in shoot number over successive passage, excessive callusing at the base of node and seasonal effect on regeneration frequency (Patnaik and Debata, 1996; Misra *et al*., 2003; Shekhawat and Manokari, 2016).

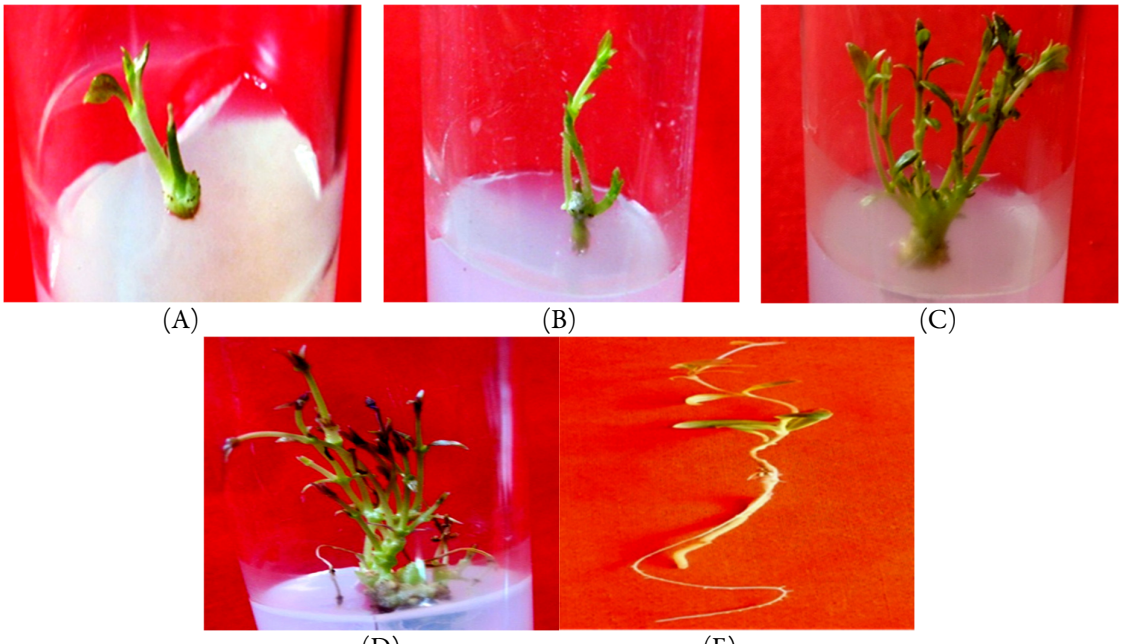

Figure 1. Shoot regeneration from *in vitro* nodes of *H. indicus* in MS + sucrose (3%) + BA (10 µM) + Kn (5 µM)
(A) bud break after one week; (B) elongation of bud into shoots after two weeks; (C) multiple shoots with branching at the end of four weeks; (D) healthy elongated shoots after eight weeks; (E) rooting of microshoots in MS + sucrose (1%) + NAA (8 µM) after four weeks

In vitro rooting

Elongated shoots were transferred to full, half and quarter strengths of liquid MS medium fortified with different NAA concentrations (1-25 µM). In all the strengths, basal MS medium failed to induce roots and further fortifying it with NAA resulted in induction of roots. In full strength MS medium, lower levels of NAA (1-4 µM) evoked poor response. The number of roots increased as the NAA level increased to 6 and 8 µM, and within four weeks optimum 3.42 ± 0.55 roots/shoot (91.67% response) were formed at 8 µM concentration in which 100% shoot survived (Figure 1E). Further increase in concentration adversely affected shoot survival as well as rooting. It was noted that reducing the strength of MS medium to half and quarter couldn't evoke a better rooting response (Table 2).

Table 2. Effect of different strengths of MS medium and concentrations of NAA on root formation in *H. indicus* shoots (4 weeks)

NAA (µM)	No. of roots/shoot*	Response (%)	Shoot survival (%)
MS			
0	0 d	0	0
1	0.33 ± 0.14 cd	33.33	25
2	0.83 ± 0.26 bcd	50	33.33
4	1.17 ± 0.28 bcd	66.67	66.67
6	2.08 ± 0.30 b	83.33	83.33
8	3.42 ± 0.55 a	91.67	100
10	1.33 ± 0.40 bc	50	83.33
15	1.08 ± 0.38 bcd	41.67	66.67
20	0.83 ± 0.39 bcd	33.33	50
25	0 d	0	25
½MS			
0	0 d	0	0
1	0.33 ± 0.18 cd	25	25
2	0.42 ± 0.22 cd	25	25
4	0.50 ± 0.22 cd	33.33	33.33
6	0.67 ± 0.30 cd	33.33	41.67
8	0.75 ± 0.17 cd	66.67	41.67
10	0.92 ± 0.36 bcd	41.67	50
15	0.58 ± 0.25 cd	33.33	33.33
20	0.17 ± 0.11 cd	16.67	16.67
25	0 d	0	0
¼MS			
0	0 d	0	0
1	0.17 ± 0.16 cd	8.33	16.67
2	0.25 ± 0.17 cd	16.67	25
4	0.33 ± 0.18 cd	25	25
6	0.50 ± 0.25 cd	33.33	33.33
8	0.42 ± 0.18 cd	33.33	41.67
10	0.17 ± 0.11 cd	16.67	33.33
15	0 d	0	0
20	0 d	0	0
25	0 d	0	0

*Values represents mean ± SE. Means (n = 12) were subjected to ANOVA and means followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test.

The MS media composition and auxins are two main factors which affects *in vitro* rooting (George and Sherrington, 1984). NAA has been reported to induce roots in *Decalepis hamiltonii* (Anitha and Pullaiah, 2002) and *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012). Similarly, in the present study rooting was achieved in full strength MS medium fortified with NAA (8 µM). It was also observed that increase in its concentration adversely affected the rooting response and this may be due to biosynthesis of ethylene by higher concentration of auxins that hinders root formation (Riov and Yang, 1989; Taiz and Zeiger, 2003). However, the number of roots formed in presence of NAA is less as compared to indole-3-butyric acid (IBA) fortified medium (Pathak and Joshi, 2017). Martin (2002) documented that superiority of IBA is due to its slow movement and better degradation as compared to NAA, which makes it more available in the medium and increases the rooting. There are reports which stated the superiority of IBA over NAA in *H. indicus* (Shekhawat and Manokari, 2016) and other plants like *Tylophora indica* (Thomas and Philip, 2005) and *Rubia cordifolia* (Khadke *et al.*, 2013).

Conclusions

In vitro nodes of *H. indicus* have potency to regenerate multiple shoots within eight weeks. Hence present study along with earlier developed protocol can generate around 385 shoots from single *in vivo* nodal explant within span of four months. Utilization of *in vitro* nodes from shoot cultures will help in continuous multiplication of shoots without hampering the wild plants. This protocol is efficient for mass-propagation and conservation of this endangered species as well as extraction of valuable secondary metabolites.

Authors' Contributions

AGJ and ARP: Designed and conceptualized the research. ARP: Performed the experimental work, analysed data and wrote the paper. AGJ: Supervised the work and revised the manuscript for important intellectual content. Both authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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