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Regeneration of *Invitro* Plantlets in *Hemidesmus indicus* (L.) R. Br. through Nodal and Leaf Explants

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Abstract

Invitro plantlet regeneration of *Hemidesmus indicus* (L.) R.Br, through nodal and leaf explants were attempted by culturing on MS medium supplemented with various PGRs. Nodal and leaf explants induced callus on media containing 2,4-D, 2,4,5-T, 2ip, IAA, NAA, BAP and KIN. The highest frequency of callus was observed in the leaf explants supplemented with 1.0, 3.0, 5.0 mgL^{-1} concentration of IAA, NAA, IBA. Rhizogenesis was observed in leaf explants highly in Auxin treatment like 2,4-D and 2,4,5-T with the concentrations of 0.2 mgL^{-1} and 0.5 mgL^{-1} respectively. Shoot differentiation was obtained from the callus of nodal explants on MS-medium with 1.0, 3.0, 5.0 mgL^{-1} of 2,4,5-T, KIN and BAP. Regenerated shoots showed rhizogenesis in MS-medium supplemented with 0.5 mgL^{-1} of IBA and KIN. Eight week old rooted *invitro* plantlets of *H.indicus* were removed from the culture vessels and transplanted into plastic pots. The plantlets were exposed to a relative humidity of 80%-90% and temperature 28±2ºC during day time, and 24 ±2ºC during night time. The percentage of surviving plantlets were recorded after four weeks of acclimatization. More than 95% of acclimatized plantlets grew vigorously without any morphological abnormalities.

Keywords: *Hemidesmus indicus;* Asclepiadaceae; *invitro* propagation; nodal explants; leaf disc explants

Abbreviations: BAP- Benzyl Amino Purine; 2,4-D- 2,4- Dichloro phenoxy Acetic Acid; 2,4,5-T – 2,4,5- Trichloro phenoxy Acetic Acid; IAA – Indole-3- Acetic Acid; IBA- Indole Butyric Acid ; KIN – Kinetin; M- Molar; NAA – Napthalene Acetic Acid.

INTRODUCTION

Tissue culture has been successfully used for the commercial production of pathogen-free plants (Debergh and Maene, 1981), to conserve the germplasm of rare and endangered species (Fay, 1992). The application of biotechnology especially tissue culture provides an important tool to propagate the selected genotypes (Campbell et al., 2003). The regeneration of plants under aseptic and controlled environmental conditions is referred to as micropropagation because very small pieces of plant tissue organs are used as starting vegetative tissue (Davis and Becwar, 2007). Techniques such as meristem culture (Hu and wang, 1983) and hot-water treatment of explants before *invitro* culture (Langens - Gerrits et al., 1998) have been used to produce plants free from pathogens. Plant tissue culture technology may help to conserve rare and endangered medicinal plants.

Many important Chinese medicinal herbs have been successfully propagated

invitro, either by organogenesis (Erdei et al.,1981) or by somatic embryogenesis (Sagare et al., 2000). *Invitro* propagated plants of many important medicinal species were found to be uniform, showing less variation in their content of secondary metabolites than their wild cultivated counterparts (Yamada et al*.,* 1991). Making health care and medical facilities available to the people is now a major concern of a large number of countries (Ghani, 2000). In addition, *invitro* propagation methods offer powerful tool for conservation of germplasm and mass-multiplication of threatened plant species (Murch et al., 2000a).

Hemidesmus indicus R.Br. (Anantamul) belongs to the family Asclepiadaceae, is one of the most widely used medicinal plant in India, well known for its medicinal values. The plant is used to cure Leprosy, Leucoderma, Itching, Skin disease, Asthma, Bronchitis, Leucorrhoea, Dysentery, Piles, Syphilis, Paralysis, promotes health and cures all kinds of diseases caused by vitiated blood (Kirtikar and Basu, 1987). The plant is rare and getting endangered, (Rahman, 2001). It is climbing slender plant. The root is long, rigid, cylindrical, little branched, a brownish corky bark, furrowed with annular cracks. Externally it has been applied as a poulitice to boils, swellings and other painful parts. The roots are harvested in autumn and dried for later use. Huge quantities of plant materials were imported for the manufacture of Ayurvedic, Unani and Homeopathic medicines (Chatterjee and Sastry, 2000).

Hemidesmus indicus is of economic interest for its wide-ranging pharmacological activity and one of the major constraints in utilizing natural population in the existence of plant-to-plant chemo variability. The fragrance of roots contains 2-hydroxy-4-methoxy benzaldehyde (91%) and ledol (4.5%), which are isolated in pure form, as the major constituents. Well identified endangered medicinal plant *H. indicus* deserve research endeavored for establishing tissue culture protocol towards their conservation (Ghani, 1998). Secondary metabolites of pharmaceutical values can be harvested from medicinal plants by tissue culture and hemidesmic acid, Smilasperic acid from the cell suspension culture (Nagarajan et al*.,* 2001; Ramachandra Rao., 2002; Broun, 2004). It is hoped that a standard protocol to induce multiple shoots in culture may provide a more homogenous source of plants.The present study was made to establish efficient *invitro* propagation for the production of secondary metabolites in

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H.indicus.

MATERIALS AND METHODS

Plant materials

Hemidesmus indicus (L.) R. Br was collected from the Botanical Garden, Department of Botany, Annamalai University. Young, healthy and disease free portion of the branches were selected and used as explants. Healthy explants like leaf disc, nodal explants axillary buds were selected and washed thoroughly under running tap water to wash off the microbes present on the surface. The explants were transferred into a double distilled water, then treated with few drops of surfactant for 10 -20 mins with constant shaking. These explants were treated with 0.5% of sodium hypochlorite solution for 3-5 mins. The surface sterilized explants were washed thoroughly with sterile distilled water. After sterilization, the explants were trimmed to appropriate sizes and inoculated on surface of the culture medium.

Culture media and conditions for plant regeneration

The basal medium consisted of MS mineral salts and organics (Murashige and skoog, 1962) supplemented with 30 gL^{-1}sucrose and solidified with 6.5 gL^{-1} of agar. pH was adjusted to 5.8 prior to autoclaving at 121ºC with pressure of 15lbs for 20 mins. For experimental trials the basal medium was supplemented with 1, 3, and 5 mgL^{-1} of α–Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and Indole-3-Acetic acid (IAA), in combination with 1, 3 mgL^{-1} of kinetin,
6-benzylaminopurine (BAP). Medium without plant growth regulators served as control. The similar sterilization techniques were reported by Evans et al (1983) and Pierik (1987). Callus from these primary cultures were transferred to MS medium containing different concentration and combinations of BAP, KIN, NAA and IAA for shoots. Cultures were incubated at 25±2ºC under the cool-white fluorescent light with intensity varying from 2000-3000 lux. Each experiment consisted of five replicates, with five leaf and nodal explants in each culture bottle. After complete morphogenesis the rooted plantlets were transferred to a mixture of soil and vermiculite (1:1) in portrays for further development and hardening. During Hardening, the plantlets were irrigated with one-fourth strength of MS basal medium (without sugar and vitamins) for one week. This helped the plantlets to recover the shock resulting from a change of environment.The porous pot that allows adequate drainage and aeration has been recommended for fast acclimatization of *invitro* regenerated plants (Dunstan and Turner, 1984). Earlier (Kar and sen, 1985) reported maintenance of plantlets in half strength of MS medium, prior to their transfer to the soil. Plantlets were transferred to pots under natural conditions. Experiments were repeated to obtain healthy clones.

RESULTS AND DISCUSSION

In this experiment, the first two expanded leaves (0.5 -1 cm) at the apex of vigorous shoots were used as explants. Callus induction was observed in leaf disc explants inoculated in MS media containing different concentrations and combinations of 2, 4-D, 2,4,5-T, IAA, NAA, BAP, KIN, 2IP. Depending upon the concentration and combination of hormones the callus get induced. The highest percentage of callus induction was observed in leaf disc explants with the hormone IAA, 2,4-D, 2,4,5-T with 1.0 , 3.0 , 5.0 mgL^{-1} with various combinations. Callus initiation on cut ends of *invitro* cultured explants could be observed in all 2, 4-D levels after 12- 15 days, Fig. A and B. Moreover, many researchers observed 2,4-D as the best auxin for callus induction as common in monocot and even dicot (Hol and Vasil 1983; Jaiswal and Naryan 1985; chee, 1990; Mamun et al., 1996). Similar findings were reported by Fiegert and Vorlop, 2000 ; yasmin et al., 2003. Color of the calli was light green to dark green. Indirect rhizogenesis from leaf disc explants were groomed on MS-medium supplemented with 2,4-D , 2,4,5-T and IBA with 0.2 &0.5 mgL^{-1} Fig C and D.

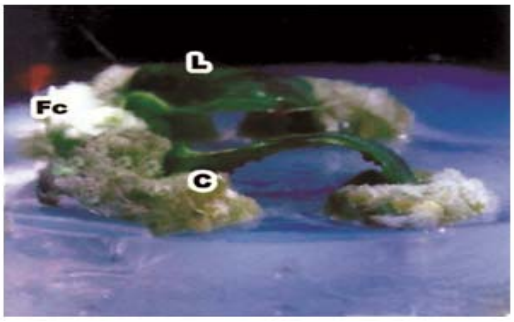

Fig. A

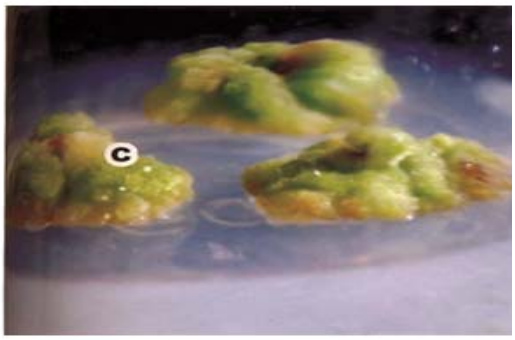

Fig. B

Fig. A and B Callus initiation on cut ends observed in 2, 4-D after 12- 15 days.
Fc – Fraiable callus ; L- Leaf ; C- Callus; R- Root; Sh- Shoot

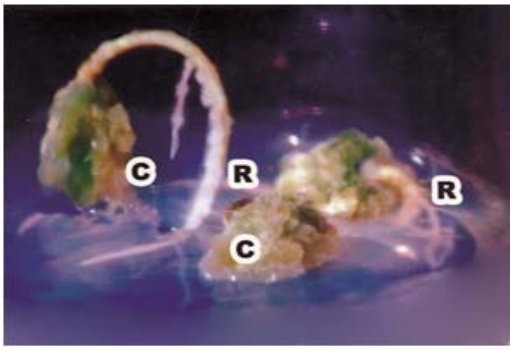

Fig.C

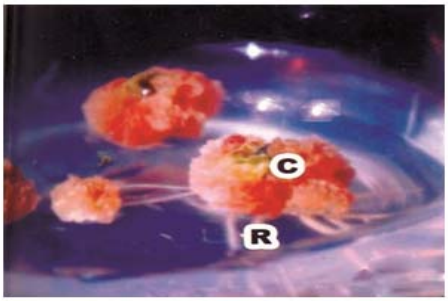

Fig.D
Fig. C and D shows Indirect Rhizogenesis from leaf explant with 2,4-D and IBA
Fc – Fraiable callus ; L- Leaf ; C- Callus; R- Root; Sh- Shoot

The percentage of rooting response was average. These results are in accordance with those of certain plants like *Kaempteria rotunda* (Mustafa and Molly, 1997), *Withania somnifera* (Indian ginsens) (Manickam et al., 2000) and *Anthemis robilis* (Segio et al., 2000). Yang (1977) reported *Asparagus* rooting on a medium containing NAA. Good rooting response was obtained for *chlorophytum* in auxin- free liquid medium and also with auxin (Purohit et al., 1994). However in the present investigation, a combination of IAA, NAA with 2,4-D was proved as the most efficient medium composition for better callusing in Hemidesmus indicus, Table 1&2.

TABLE:1 Effect of different concentrations and combinations of Hormones on induction of callus from leaf disc explants of *Hemidesmus indicus* (L).R.Br.

Treatments(mgl^{-1})	Days to callus initiation	% of callus formation	Color	Texture
MS+ 2,4-D 1.0	12	75.00	LG	Friable
MS+ 2,4,5-T 3.0	12	21.00	DG	Compact
MS+ IBA 1.0	12	30.00	LG	Friable
MS+ NAA 3.0	12	45.00	DG	Compact
MS+ IAA 1.0	12	70.00	LG	Friable
MS+ BAP 5.0	12	65.00	LG	Friable
MS+ 2,4D 0.1+IAA0.3+NAA0.5	12	72.00	LG	Friable
MS+ 2,4-D+2,4,5-T 0.2	12	55.00	LG	Friable

TABLE:2 Effect of different concentrations and combinations of Hormones on induction of root from leaf explants of *Hemidesmus indicus*(L).R.Br.

Treatments(mgl^{-1})	Days to root initiation	% of root formation	Root morphology
MS+ 2,4-D 1.0	20	21.00	Friable, long
MS+ 2,4,5-T 3.0	18	11.00	Friable, long
MS+ IBA 1.0	23	30.00	Thin, long

TABLE:3 Effect of different concentrations and combinations of Hormones on induction of shoot from nodal explants of *Hemidesmus indicus* (L).R.Br.

Treatments(mgl^{-1})	Days to Shoot proliferation	% of shoot formation	Root
MS+ 2,4-D 1.0	20	11.00	+
MS+ 2,4,5-T 3.0	20	21.00	+
MS+ IBA 1.0	20	30.00	+
MS+ NAA 3.0	20	45.00	-
MS+ IAA 1.0	20	70.00	-
MS+ BAP 5.0	20	75.00	-
MS+ 2,4D 0.1+IAA0.3+NAA0.5	20	72.00	-
MS+ 2,4-D+2,4,5-T 0.2	20	55.00	-

Proliferation of shoot buds were observed in nodal explants cultured in MS-medium supplemented with 2,4-D, 2,4,5-T, BAP, 2IP, NAA, IAA alone or in combinations with 1, 3 and 5 mgL-1 concentrations. The nodal segments shows an initial enlargement of the dormant axillary buds. The explants first appeared as a nodular growth within 3-4 weeks of culture and at the end of the 4th week this nodular growth increased in size and produce leaf primordial. Proliferation of shoots were observed in 2,4,5-T, BAP and KIN with 2mgL-1concentrations alone or in combinations. Dodd and Robert (1995) reported that 2, 4-D is a herbicide and a powerful suppressant of organogenesis. BAP 2mgL-1 showed the highest shoot induction ability Fig.E and F.

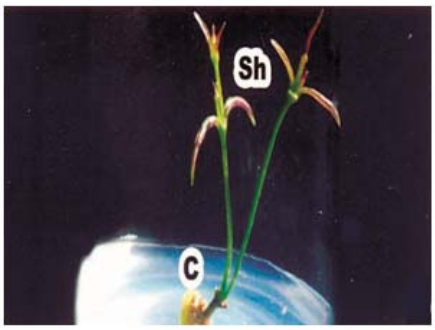

Fig.E

Fig.F
Fig. E and F shows highest shoot induction with BAP.
Fc – Fraiable callus ; L- Leaf ; C- Callus; R- Root; Sh- Shoot

Thus, BAP was found to be the most effective plant growth regulator. Indicating, the cytokinin specificity of nodal explants of *H.indicus* for shoot formation. These results are in consonant with shoot induction in *Ceropegia jainii* and *C.bulbosa* (Patil, 1998), *C.bulbosa var. bulbosa* (Britto et al., 2003; Goyal and Bhadauria, 2006), *C.sahyagrica* (Nikam and savanth, 2007). In contrast to the synergistic effect of BAP in combination with an auxin has been reported in *C.candelabrum* (Beena et al., 2003) and other Asclepiad *Holostemma annulare* (Sudha et al., 1998), *Hemidesmus indicus* (Sreekumar et al., 2000), *Holostemma ada- kodien* (Martin, 2003). It was observed that mostly light green calli produced shoot buds. Similar results were observed on the medium for shoot multiplication of *K.galanga*, (Vincent et al., 1992; Shirin et al., 2000; Rahman et al., 2005). An alternative approach to increase the number of shoot formation has been reported in ginger and turmeric in MS medium containing BAP with NAA used to multiply shoots (Haque et al., 1999 ; Rahman et al., 2004) incubated explants showed shoot initiation.Table: 3 reveal the effect of various concentrations and combinations of hormones in Nodal explants. The endogenous levels of growth regulators in the members of Asclepiadaceae might be responsible for the observation of variation in the response and growth regulator requirement for *invitro* shoot regeneration.

In this investigation, the morphogenetic potential of various explants were attempted using appropriate medium, hormone and culture condition required to derive plantlets. Shoot tip, nodal stem, leaf and root were cultured on MS-medium supplemented with various PGRs. Among the explants used, nodal and leaf disc explants shows *invitro* morphogenetic potential. It was observed that BAP and NAA alone or in combinations in MS- medium were most effective for rooting of shoots in *Hemidesmus indicus* (L.)Br. These results confirm that some plant species have enough levels of endogenous hormones and does not require high levels of exogenous growth regulators for plant regeneration, (Hussey, 1982).

Conclusion

In conclusion, an efficient protocol for micro propagation of the endangered medicinal plant (*Hemidesmus indicus).* This protocol ensures a successful and rapid technique that can be grown and cultivated in fields. The application of this protocol can help to minimize the pressure on wild populations and contribute to the conservation of valuable flora in India.

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