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RESEARCH ARTICLE

Studies on medicinal plants. 4. Micropropagation of Holostemma ada- kodien Schult.- a rare medicinal plant

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Abstract

In vitro culture of different explants of Holostemma ada-kodien on MS medium augmented with various concentrations of growth regulators were carried out. Caulogenesis was seen in leaves and shoot tips. Schizogenesis was observed in shoot tips and nodal segments, inoculated in MS medium fortified with NAA and BAP or KIN. Among these, shoot tips inoculated in MS medium supplemented with NAA (0.5mg L^{-1}) and KIN (1.5mg L^{-1}) showed highest percentage of regeneration. Rooted plantlets were transplanted.

Introduction

Holostemma ada–kodien Schult., belongs to the Family Asclepiadaceae is a rare and important medicinal plant. The tuberous roots are medicinally important and are utilized as a major ingredient of the drug " jivanthi" (Kolammal, 1979). The plant is a twining shrub with opposite leaves and flowers are developed in axillary umbellate cymes. The roots are used for cough, fever, ophthalmic diseases, stomachache, spermatorrhoea, emaciation, dysentery, tuberculosis, arrested urination, scorpion bite, kidney stones, goiter etc. The destructive and ruthless collection of tubers, in recent times, has led to acute scarcity of the plant and consequently it is listed out as vulnerable and rare in FRLHT red list (FRLHT, 1997). Commercial cultivation of H. ada-kodien is problematic due to increasing land pressure and its inherent difficulty to propagate. Hence, in vitro tissue culture method might be of great value as an alternate method to achieve rapid multiplication of this species. In the last few decades plant tissue culture has played an important role for production of large number of plants, independent of seasons, aiming at reestablishment of these plants in their natural habitat. Besides, they also serve as an alternate source of plant raw materials for commercial utilization, which lessens pressure on the plant population in wild habitat (Krishnan et al. 2011).In the present study an efficient protocol for rapid propagation of H. ada-kodien by using shoot tips and nodal explants is reported.

Material and Methods

The plants collected from Guruvayur and Mannuthy in Thrissur district were maintained in the green house. Leaves, shoot tips and nodal segments were collected from fresh, young, healthy mother plants for this study. The explants were excised and washed thoroughly in running tap water, followed by washes with Salvon for 5 minutes. The explants were treated with 5% teepol for 5-10 min and thoroughly washed with distilled water. For surface sterilization, the explants were immersed in 70% ethyl alcohol for 1 min and washed well with sterile double distilled water for three times. It was then treated with 0.1% $HgCl_2$ for 1 – 10 min and thoroughly washed to free of sterilants with autoclaved distilled water five times and the explants were cut into desired size 0.5 – 1.0 cm.

The surface sterilized explants were cultured on MS medium containing 3 % (w/v) sucrose and 0.8% agar-agar type I. In the present study, MS medium in full strength was used for callus induction and schizogenesis and half strength for subculture. Plant growth regulators (NAA, BAP and KIN) at different concentrations were incorporated in to the basal media. The P^H of the medium was adjusted to 5.8 by 1N NaOH or 1 N HCl before

autoclaving at 1.06 kg cm^{-2} pressure for 15 min. The cultures were incubated at 25 ± 2 ^{0}C in a culture room with 16 hours photoperiod from fluorescent tubes with a light intensity of 3000 lux and 55 ± 5% of relative humidity (RH).

Effect of growth regulators on shoot tips and leaves for callus induction

In order to study the combined effect of auxin and cytokinin for callus induction on shoot tips and leaves as explants, different concentrations of NAA (1.0 – 2.0 mg L^{-1}) and BAP (0.5 – 4.0 mg L^{-1}) or KIN (0.5 – 3.0 mg L^{-1} were used. Twenty explants were used per treatment.

Effect of growth regulators on shoot tips and nodal segments for schizogenesis

For the study of the combined effect of plant growth regulators on shoot tips and nodal segments for schizogenesis, explants were inoculated in MS medium supplemented with various combinations of NAA (0.5mg L^{-1}) and KIN (0.5 – 3.0 mg L^{-1}**)** or BAP(1.0-2.5 mg L^{-1}**).**

Results and Discussion

During surface sterilization, it was seen that among the various treatments attempted (1 – 10 min) leaves, shoot tips and nodal explants treated with 0.1 per cent mercuric chloride for 6 min duration showed the highest percentage of survival. Hence it was identified as the best treatment for surface sterilization in explants of H. ada-kodien. Increasing the duration of treatment resulted in high rate of mortality in the explants.

Various stages of shoot development from shoot tips of *Holostemma ada-kodien*, inocu in MS medium with NAA (0.5mgL^{-1}) & KIN (1.5mgL^{-1})

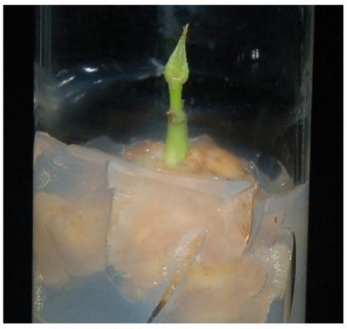

Fig.1. 25 days after inoculation

Fig. 2. Multiple shoot development (One month after inoculation)

Fig. 3. Two months after inoculation

Fig. 4. 70 days after inoculation

Fig. 5. Three months after inoculation

Fig. 6. Hardening of plantlet in sterile soil

Effect of growth regulators on shoot tips and leaves for callus induction

In caulogenesis, the combined effect of NAA (1.0 and 2.0 mg L^{-1}) and BAP (0.5 – 4.0 mg L^{-1}) or KIN (0.5 – 3.0 mg L^{-1}) on shoot tips and leaves was studied .The results are presented in tables 1and 2.

During this study leaf segments and shoot tips showed callus initiation in MS media with various combinations of NAA (1.0 and 2.0mg L^{-1}) and BAP (0.5- 4.0 mg L^{-1}) and NAA (1.0 and 2.0 mg L^{-1}) and KIN (0.5– 3.0 mg L^{-1}). The callus initiation was observed after 14 days of inoculation. Callus initiation started from lower cut ends. The callus was pale green in colour at initial stage and pale brown after 35 days of inoculation. Among these, shoot tips inoculated in MS medium with NAA (1.0mg L^{-1}) and KIN (2.5mg L^{-1}) showed highest percentage of callus induction (59.3%), followed by 55.3 per cent response in leaf segments in the same medium.

Mohanty et al.(2013) have also reported similar results while inoculating rhizome buds of Hedychium coronarium in MS medium fortified with NAA (0.5mg L^{-1}) and BAP (2.0mg L^{-1}). Subsequently Senapathi et al.(2013) have also observed highest percentage of caulogenesis, while inoculating nodal explants of Celastrus paniculatus in MS medium fortified with NAA (0.1mg L^{-1}) and BAP (0.5mg L^{-1}).

Effect of plant growth regulators on shoot tips and nodes for schizogenesis

The shoot tips and nodal segments were inoculated in MS medium supplemented with various concentrations of auxin (NAA) and cytokinins (KIN, BAP). The results are presented in tables 3 and 4. The shoot tips and nodal segments showed varying degrees of responses towards schizogenesis (figs. 1- 4). The shoot initiation was started after 19 days of inoculation. The percentage of shooting response, mean number of days taken for schizogenesis and mean length of shoots after three months of inoculation were also recorded.

In the present study bud proliferation was observed on shoot tips, inoculated in MS medium fortified with different combinations of NAA (0.5 mg L^{-1}) and KIN(0.5, 1.0, 1.5,2.0,2.5 and 3.0 mg L^{-1})or BAP(1.0, 1.5, 2.0 and 2.5 mg L^{-1}) and nodal segments inoculated in MS medium augmented with different concentrations of NAA (0.5 mg L^{-1}) and BAP(1.0, 1.5,2.0 and 2.5 mg L^{-1}) . Among these, shoot tips inoculated in MS medium fortified with NAA (0.5mg L^{-1}) and KIN (1.5mg L^{-1}) showed the highest percentage of response (68.4%) followed by 58.8 per cent in MS medium augmented with NAA (0.5mg L^{-1}) and KIN (1.0mg L^{-1}) and 45.7 per cent in MS medium fortified with NAA (0.5mg L^{-1}) and KIN(2.0mg L^{-1}). In the above medium ie, MS medium with NAA (0.5mg L^{-1}) and KIN (1.5mg L^{-1}), the shoots attained maximum length of 10.0 ± 0.8 cm(fig.5) after 90 days of culture and multiple shoot development was also seen. Hence this medium is identified as the best medium for schizogenesis in H.ada-kodien.

. Reduction in shoot length was observed when shoot tips were cultured on MS medium with NAA and BAP. The shoots reached a maximum height of 4.6±0.8 cm only in MS medium fortified with NAA (0.5mg L^{-1}) and BAP (2.0mg L^{-1}). Shooting response was observed to be 29.1 per cent in shoot tips inoculated in MS medium fortified with NAA (0.5mg L^{-1}) and BAP (2.0 mg L^{-1}), followed by 23.2 per cent in MS medium supplemented with 0.5 mg L^{-1} NAA and 1.5 mg L^{-1} BAP and 19.3 per cent in MS medium augmented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP.With shoot tip explants, KIN was found to be more efficient than BAP in respect of shoot initiation. Shoot tip buds showed better shoot induction than nodal explants.

Promotion of shoot induction has been reported in other medicinal plants like Dianthus caryophyllus and D. barbatus (Kundu and Maity ,2006) by using MS medium fortified with NAA (0.2 mg L^{-1}) and KIN (1.0 mg L^{-1}) and Andrographis alata (Nagaraja et al.,2003), Utleria salicifolia (Gangaprasad et al., 2003), Psoralea carylifolia (Anis and Faisal, 2005) and Vitex negundo (Vadewale et al., 2006) by using NAA and BAP.

In rhizogenesis, the plantlets were transferred to ½MS medium supplemented with NAA (0.5, 1.0 mg L^{-1}). Among these, the highest percentage (56.07%) of root initiation was observed in plantlets subcultured in ½MS medium augmented with NAA (0.5 mg L^{-1}). Raomai et al.(2013) have reported similar results in Homalomena aromatica while using ½MS medium fortified with NAA (0.5 mg L^{-1}).

The in vitro produced plants were taken from the culture tubes and planted in plastic cups (10cm x 8 cm) with sterile soil and sand mixed in the ratio 1:1 (fig. 6). These were then covered by plastic covers. The plastic covers were opened after 7–15 days and temperature was gradually increased to 28 ± 2ºC. The plants survived were then transplanted to pots kept in green house showed 71.8 per cent success rate. The established plantlets did not show any variation in morphological or growth characteristics when compared to the mother plant. Thus, this efficient in vitro procedure of regeneration from shoot tips and nodal explants could be used to maintain clonal fidelity to this valuable genotype and mass propagation.

Table 1. Effect of NAA and BAP on callus induction from shoot tip and leaves of H. ada-kodien

Treatment No.	Explants used		Growth regulators (mgL^{-1})		Percentage of response(%)	
			NAA	BAP	Leaf	Shoot tip
1	Leaf	Shoot tip	1.0	0.5	11.2	19.0
2	Leaf	Shoot tip	1.0	1.5	17.0	23.4
3	Leaf	Shoot tip	1.0	2.0	49.6	30.0
4	Leaf	Shoot tip	1.0	2.5	45.6	52.6
5	Leaf	Shoot tip	1.0	3.0	38.7	35.7
6	Leaf	Shoot tip	1.0	4.0	27.2	31.5
7	Leaf	Shoot tip	2.0	2.5	24.1	29.2
8	Leaf	Shoot tip	2.0	3.0	17.3	26.0

Table 2. Effect of growth regulators on callus induction from shoot tip and leaves of H. ada- kodien

Treatment No.	Explants used		Growth regulators (mgL^{-1})		Percentage of response (%)	
			NAA	KIN	Leaf	Shoot tip
1	Leaf	Shoot tip	1.0	0.5	8.5	22.6
2	Leaf	Shoot tip	1.0	1.5	30.9	27.0
3	Leaf	Shoot tip	1.0	2.0	42.0	35.8
4	Leaf	Shoot tip	1.0	2.5	55.3	59.3
5	Leaf	Shoot tip	1.0	3.0	48.4	43.7
6	Leaf	Shoot tip	2.0	2.5	29.1	18.6
7	Leaf	Shoot tip	2.0	3.0	23.3	8.5

Table 3. Effect of different combinations of NAA and KIN or BAP on schizogenesis in shoot tips of H.ada-kodien

Treatment No.	Basal media	Plant growth regulators	Concentration (mgL^{-1})	Percentage of shooting response (%)	Mean number of days taken for schizogenesis	Mean length of shoots after3months (cm)
1.	MS	NAA+KIN	0.5+0.5	23.3	21.0 ± 1.3	7.4 ± 1.0
2.	MS	NAA+KIN	0.5+1.0	58.8	23.0 ± 2.4	9.3 ± 1.1
3.	MS	NAA+KIN	0.5+1.5	68.4	19.0 ± 2.0	10.0 ± 0.8
4.	MS	NAA+KIN	0.5+2.0	45.7	26.0 ± 2.1	8.2 ± 0.9
5.	MS	NAA+KIN	0.5+2.5	26.3	31.0 ± 2.7	7.8 ± 0.7
6.	MS	NAA+KIN	0.5+3.0	13.6	38.0± 1.1	5.1± 0.8
7.	MS	NAA+BAP	0.5+1.0	19.3	29.0 ± 2.2	3.1± 0.6
8.	MS	NAA+BAP	0.5+1.5	23.2	27.0 ± 2.1	4.3 ± 1.0
9.	MS	NAA+BAP	0.5+2.0	29.1	26.0 ± 2.3	4.6 ± 0.8
10.	MS	NAA+BAP	0.5+2.5	17.7	32.0 ± 2.5	2.2 ± 0.6

Table 4. Effect of different combinations of NAA and KIN on schizogenesis in nodal segments of H.ada kodien

Treatment No.	Basal media	Plant growth regulators	Concentration (mgL^{-1})	Percentage of shooting response (%)	Mean number of days taken for schizogenesis	Mean length of shoots after 3 months (cm)
1.	MS	NAA+KIN	0.5+1.0	17.4	27.0 ± 2.1	3.9 ± 1.0
2.	MS	NAA+KIN	0.5+1.5	24.8	25.0 ± 1.9	5.0 ± 1.1
3.	MS	NAA+KIN	0.5+2.0	18.3	28.0 ± 2.0	4.4 ± 0.9
4.	MS	NAA+KIN	0.5+2.5	11.9	32.0 ± 2.5	2.8 ± 0.7

Abbreviations used:

NAA - α- Naphthalene Acetic Acid
KIN - Kinetin
BAP - 6- Benzyl Amino Purine
MS - Murashige and Skoog (1962)
min - minutes
cm - centimetre

Conclusions

In vitro culture of shoot tips and leaves of H. ada-kodien in MS medium fortified with NAA (1.0 – 2.0 mg L^{-1}) and BAP (0.5 – 4.0 mg L^{-1}) or KIN (0.5- 3.0 mg L^{-1}), shoot tips inoculated in MS medium supplemented with NAA (1.0 mg L^{-1}) and KIN (2.5 mg L^{-1}) showed highest response in callus induction. Nodal segments and shoot tips inoculated in MS medium augmented with NAA (0.5 mg L^{-1}) and KIN (0.5- 3.0 mg L^{-1}) or BAP (1.0- 2.5 mg L^{-1}), shoot tips inoculated in MS medium supplemented with NAA (0.5 mg L^{-1}) and KIN (1.5 mg L^{-1}) showed maximum percentage of schizogenesis. In this medium, the shoots attained a maximum length of 10.0± 0.8 cm and showed multiple shoot development. The plantlets were transferred to natural habitat with 71.8 per cent success rate. This efficient protocol could be used for rapid propagation of this rare medicinal plant H. ada-kodien from shoot tips and nodal segments.

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