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RESEARCH ARTICLE

CONSERVATION OF *HOLOSTEMMA ADA-KODIEN* SCHULT.-AN *IN VITRO* APPROACH.

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Abstract

The purpose of this study was to develop a new system of micropropagation for the conservation of *Holostemma ada-kodien* Schult., a valuable medicinal plant .The explants cultured on full strength Murashige and Schoog's (MS) medium supplemented with different combinations of growth regulators and also with additive ,adenine sulphate (AdS). MS medium with BAP (1.5 mgL^{-1}) and AdS (1.0 mg L^{-1}) the nodal explants showed faster initiation of shoots. Explants cultured on MS medium and BAP (3mgL^{-1}) in combination with AdS(1mgL^{-1}) was the best for shoot multiplication. The effect in nodal explant on MS medium fortified with BAP (2mg L^{-1}), NAA(1mg L^{-1}) shown well developed nodular compact pale green callus. The root tubers of this plant is medicinally important and it is useful to cough, opthalmopathy, orchitis, burning sensation, stomachalgia, fever and to cure 'tridosha'. The medicinal properties of *Holostemma* are due to the presence of terpenoid sugars and amino acids. This study deals with the analysis of *in vitro* propagation of shoot and induced callus of *Holostemma* with short duration of time.

Introduction:-

Holostemma ada-kodien Schult., is a rare and important medicinal plant of family Asclepiadaceae has been used a folk remedy for the treatment of diseases. The tuberous roots are medicinally important and have a huge demand in South Indian pharmacies. It is a rare medicinal plant distributed in the tropical rain forests of the world (Kolammal, 1979; Sivarajan and Balachandran, 1994). The plant is threatened by habitat destruction and over exploitation. This necessitates immediate attention for its conservation and cultivation. So the present work is focused on the standardization of the *in vitro* culture of *H.ada-kodien* explant collected from field grown plants. The plant is used for maintaining youthful vigour, strength and vitality (Guptha,1977).The terpenoid sugars present in the root tubers of the plant are responsible for the medicinal properties (Ramiah et al.,1981). The plant is used as an antidiabetic, rejuvenative, aphrodisiac, expectorant, galactogogue, stimulant, ophthalmic disorders and for maintaining youthful vigour, strength and vitality (Moming 1987; Warrier *et al.*, 1995 and Gupta 1997). *Holostemma ada-kodien* is listed as vulnerable and rare in Foundation for Revitalization of Local Health Traditions red list of medicinal plants (FRLHT, 1997). Immediate steps are therefore required for the conservation and cultivation of this plant. The present study *in vitro* propagation system provides an alternate method for rapid production of plants but it's ultimate success depends upon the successful transfer and establishment of this plant in field conditions. The medicinally important tuberous roots are utilized as a major ingredient of drug "jivanthi" (Kolammal, 1979) which has a huge demand in South Indian pharmacies. The cultivation of *Holostemma ada-kodien* is problematic due to the

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environmental and land pressure so it is difficulty to propagate. A simple and reliable protocol for an *in vitro* propagation of *Holostemma ada-kodien,* an important medicinal plant through axillary bud proliferation . The present work reports the study may be effectively used for the conservation of this rare and important medicinal plant. The study substantiates earlier reports that the basal nodal explants form ideal starting material for axillary bud proliferation for multiple shoot induction .Addition of Ads improved multiple shoot initiation along with healthy leaf and shoot development.

Materials and Methods:-

The plants were collected from the premises of a temple in Kollam district of Kerala and cultivated in the Department of Botany, Sree Narayana College, Kollam (Figure .1). The explants such as nodal, inter nodal, leaf and petiole were collected from these cultivated plants and used for investigating the effect of certain plant growth regulators on callogenesis in them. All glass-wares and instruments needed for the work were washed thoroughly, sterilized at 121 ºC and 15 lb pressure for 20 minutes. They were dried in Hot Air Oven (REMI) overnight. MS medium (Murashige ang Skoog , 1962) of Himedia Pvt. Ltd.(PT011) was used. The pH was adjested 5.8 using 1N HCl/1N NaOH. The final volume was made up to1000 ml with distilled water. The medium was boiled to dissolve agar completely. The medium was sterilized by autoclaving at 15lbs at 120 ºC for 15 minutes. The autoclaved medium was cooled to 45 ºC before adding the plant hormones. The basal media was supplemented with plant hormones such as Benzylaminopurine (BAP) , α-naphthalene acetic acid (NAA), Kinetin (Kn), 2,4-Dichlorophenoxy acetic acid (2,4-D), Indole butyric acid (IBA) and with additive Adenine sulphate(AdS).All the cultures maintained under at a light intensity of 4100 lux provided by cool, white fluorescent tubes (Philips,India).The temperature of the room was maintained at 24ºC.Explants such as node ,internode, leaf and petiole were first washed thoroughly under running tap water. Then they were cut into pieces having a size of 10-15 cm length and leaf segments of about 0.5-1.0 sq.cm.They were treated with 10% labolene (Qu aligens, India) liquid detergent for 5-10 minutes. For the removal of the detergent and other contaminants,explants were thoroughly washed under running tap water for 30 minutes and subsequently with double distilled water for 2-3 times. The explants were taken to the laminar air flow cabinet. Surface sterilization of the explants involved immersion in 0.1% mercuric chloride ($HgCl_2$) solution for 15 minutes. The explants were again washed with double distilled water 3-4 times to remove all traces of $HgCl_2$. The surface sterilized explants were excised to remove the sterilant, exposed regions cut into appropriate size using surgical blade and forceps into pre-sterilized petridish. The inoculated culture tubes were transferred to the inoculation room for development. The culture tubes were observed at regular intervals for any development.

Results and Discussion:-

The result of our investigation revealed that external concentration of plant growth regulators constituted an important factor affecting the rate of callus formation in *Holostemma ada-kodien.* Nodal explants cultured on MS medium supplemented with 6- benzylaminopurine (BAP) alone or in combination with α-naphthalene acetic acid (NAA)/ kinetin (KIN)/ indole butyric acid (IBA) showed swelling and enlargement of axillary buds, callus induction and or multiple shoot formation in 7-10 days after inoculation. However after 4 weeks it was evident that there was a great variation among the cultured nodal explants. In some of the explants no response was noted, while in others rapid proliferation of callus from cut ends or callus formation and subsequent axillary buds initiation and elongation was observed(Figure 2).The results of the preliminary culture studies led to the decision to analyse the effect of nodal position on axillary bud initiation in *Holostemma ada-kodien.*

The nodal explants on MS medium supplemented with BAP (2 mg L^{-1}) showed initial callus formation and proliferation from basal cut ends, followed by induction and elongation of multiple shoots in three weeks . The callus was creamish green, compact and nodular in texture. An average of 6 shoots was formed. Subculturing into the same media resulted browning of the callus and drying of shoots. It was found that the shoots when separated from the clump of calli failed to survive in subcultures. Subculturing could be done only as a clump. In the present work MS medium with BAP (2 mg L^{-1}) gives more basal callus production with very good response of large number axillary shoot formation.The nodal explants cultured on full strength Murashige and Skoog's (MS) medium fortified with different combinations of growth regulators and also with additive ,adenine sulphate (AdS).In MS medium supplemented with BAP (1.5 mg L^{-1}) and AdS (1.0 mg L^{-1}) the nodal explants showed faster initiation of shoots .MS medium supplemented with BAP (2 mgL^{-1}) and AdS (1.0 mg L^{-1}) gave an average of 4-5 shoots within 10 days. Nodal explants in MS media with BAP (3mg L^{-1}) and AdS (1.0 mg L^{-1}) showed rapid callus initiation and proliferation 10 days after inoculation but with time the callus formation(Figure 3). In MS medium supplemented

with BAP (1 mg L^{-1}) and IBA (0.5 mg L^{-1}) nodal explants showed initiation of axillary buds within 5 days after inoculation. Development of axillary shoot was slow due to the proliferation of basal callus. The calli were nodular, compact and pale green in colour (Table 1).

Internodal explant cultured on MS medium supplemented with BAP (1.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) the callus was semi nodular pale green initially but turned yellowish brown with passage of time and also when sub cultured on the same media(Figure 4). In MS medium fortified with BAP (1.5, 2.0 & 3.0 mg L^{-1}) in combination with NAA (0.5 &1.0 mg L^{-1}) the callus formation was slower and less profuse when compared to the response to BAP alone. Shoot differentiation was not observed from the proliferating calli formed from leaf, internodal or petiole explant in this study. (Table 2).

Petiole explants in MS medium fortified with PGRs gave good response in terms of callus induction with all the combination tried in this work .The petiole explants gave a good callus initiation from cut ends .The creamish green and friable calli proliferated rapidly and covered the entire explant. The best response in terms of callus initiation and proliferation was in MS medium with BAP (1.0 mg L^{-1}),NAA (0.5 mg L^{-1}) and KIN (0. 5 mg L^{-1}) (92%)(Figure 5). The effect of different PGRs on the callus induction from leaf explants of *Holostemma ada-kodien* inoculated in MS medium augmented with different combination of BAP,NAA,KIN and IBA alone and in combinations also showed good callus initiation and proliferation from cut ends. (Table 3).

The leaf explant showed faster initiation and the proliferation of callus. The calli were nodular and the proliferation of calli occurred on both adaxial and abaxial sides (Figure 6). Callus formation was observed on the veins and vein tips of leaves also. In MS medium fortified with BAP (1.5, 2.0 &3.0 mg L^{-1}) in combination with NAA (0.5&1.0 mg L^{-1}) the callus formation was slower and less profuse when compared to the response to BAP alone. Shoot differentiation was not observed from the proliferating calli formed from leaf, internodal or petiole explant in this study .Among the different explants the basal nodal explant was the best source explant for multiple shoot initiation. Increase the callus proliferation was coupled to lowered number of multiple shoots.
Callus formation was observed on the veins and vein tips of leaves also(Figure 7).
(Table 4).

The maximum number of shoots per explant was achieved in MS medium fortified with BAP (3mg L^{-1}) in combination with adenine sulphate (AdS) was the best for axillary bud sprouting and initiation(8-9 shoots per explant).Subculturing of the initiated shoots to the same medium increased the number of shoots (Table 5).*In vitro* experiment using nodal explants of *H.ada-kodien* were conducted for clonal propagation and axillary bud proliferation. For rooting of the *in vitro* generated shoots ,the shoots were excised and individual shoots inoculated on half strength MS media supplemented with IBA at different concentrations (0.1,0.2,and 0.3 mg L^{-1}).The best response was in MS media containing IBA(0.2 mgL^{-1}) which formed an average of 5-6 roots. Well rooted plantlets were transferred to paper cups covered with polythene covers and kept in the green house. After 12 weeks 69% survival was achieved. The study substantiates earlier reports that the basal nodal explants form ideal starting material for axillary bud proliferation for multiple shoot induction and also the nodal explant is the best explant source for callogenesis in *Holostemma ada-kodien*. Addition of Ads improved multiple shoot initiation along with healthy leaf and shoot development. Rooting from the micro shoots was achieved using in vitro rooting techniques. The multiple shoots formed were transferred to rooting media (1/2 MS+IBA).Rooted plantlets were potted and acclimatized in growth chamber and transferred to field with 69% survival after 12 weeks in the field conditions. Thus the production of plantlets along with hardening was achieved.

Table 1:- Effect of PGR's on callus induction from nodal explants of *H. ada-kodian*

Plant Growth Regulators				**Intensity of callus induction**	**Response %**	**Nature of callus**
BAP	**NAA**	**IBA**	**KIN**			
0.0	**-**	**-**	**-**	-	-	-
0.5	**-**	**-**	**-**	+	21	*nodular pale green*
1.0	**-**	**-**	**-**	+	24	*nodular pale green*
1.5	**-**	**-**	**-**	+ + ++	**84**	*Nodular compact pale/dark green*
2.0	**-**	**-**	**-**	+ + +	81	*Nodular compact Creamish green*
3.0	**-**	**-**	**-**	+ +	75	*Nodular compact pale green*

1.5	**0.5**	**-**	**-**	+ + +	78	*Nodular compact pale green*
1.5	**1.0**	**-**	**-**	+ +	67	*Nodular compact pale green*
2.0	**0.5**	**-**	**-**	+ +	58	*Nodular compact pale green*
2.0	**1.0**	**-**	**-**	+ + + +	**85**	*Nodular compact pale green*
3.0	**1.0**	**-**	**-**	+ +	76	*Nodular compact pale green*
1.0	**0.5**	**-**	**0.5**	+ + + +	81	*Nodular compact pale green*
1.0	**-**	**0.5**	-	+ + + +	78	*Nodular compact pale green*

Table 2:- Effect of PGR's on callus induction from internodal explants of *H. ada-kodian*

Plant Growth Regulators				**Intensity of callus induction**	**Response %**	**Nature of callus**
BAP	**NAA**	**IBA**	**KIN**			
0.0	-	-	-	-	-	-
0.5	-	-	-	+	34	*Pinkish white friable*
1.0	-	-	-	+	32	*Pinkish white friable*
1.5	-	-	-	+ + +	47	*Pinkish white friable*
2.0	-	-	-	+ + +	52	*Greenish white friable –distal end Creamish white nodular*
3.0	-	-	-	+ +	36	*Pinkish white nodular*
1.0	**0.5**	-	-	+ + + +	**43**	*pale green mixed callus*
1.0	**1.0**	-	-	+++	42	*Creamish yellow compact semi nodular*
1.5	**0.5**	-	-	+ + +	66	*Creamish yellow compact semi nodular*
1.5	**1.0**	-	-	+ + +	73	*Creamish yellow compact semi nodular*
2.0	**0.5**	-	-	+ + +	67	*Creamish yellow compact nodular*
2.0	**1.0**	-	-	+ + +	63	*Creamish yellow compact nodular*
3.0	**1.0**	-	-	+ + +	58	*Creamish yellow compact nodular*
1.0	**0.5**	-	**0.5**	+ + + +	**87**	*Light pink semi nodular*
1.0	**-**	**0.5**	-	+ + +	74	*Light pink semi nodular*

Table 3:- Effect of PGR's on callus induction from petiole explants of *H. ada-kodian*

Plant Growth Regulators				**Intensity of callus induction**	**Response %**	**Nature of callus**
BAP	**NAA**	**IBA**	**KIN**			
0.0	-	-	-	-	-	-
0.5	-	-	-	++	28	*Pale green semi nodular*
1.0	-	-	-	++	26	*Pale green semi nodular*
1.5	-	-	-	+ + +	41	*Pale green semi nodular*
2.0	-	-	-	+ + +	47	*Pale green semi nodular*
3.0	-	-	-	+ +	21	*Pale green semi nodular*
1.5	**0.5**	-	-	+ + + +	79	*Creamish green & friable*
1.5	**1.0**	-	-	+ + + +	**92**	*Creamish green & friable*
2.0	**0.5**	-	-	+ + +	64	*Creamish green & friable*
2.0	**1.0**	-	-	+ + +	76	*Creamish green & friable*
3.0	**1.0**	-	-	+ +	33	*Creamish green & friable*
1.0	**0.5**	-	**0.5**	+ + + +	**81**	*Creamish green & friable*
1.0	-	**0.5**	-	+ + +	70	*Creamish green & friable*

Table 4:- Effect of PGR's on callus induction from leaf explants of *H. ada-kodian*

Plant Growth Regulators				Intensity of callus induction	Response %	Nature of callus
BAP	**NAA**	**IBA**	**KIN**			
0.0	-	-	-	-	-	-
0.5	-	-	-	+	43	*Green nodular*
1.0	-	-	-	+	47	*Green nodular*
1.5	-	-	-	+ + +	93	*Green nodular*
2.0	-	-	-	+ + + +	**91**	*Green nodular*
3.0	-	-	-	+ + + +	**31**	*Pale green Nodular*
1.5	**0.5**	-	-	+ + +	87	*Pale green Nodular*
1.5	**1.0**	-	-	+ + +	72	*Pale green Nodular*
2.0	**0.5**	-	-	+ + +	77	*Pale green Nodular*
2.0	**1.0**	-	-	+ + +	74	*Pale green Nodular*
3.0	**1.0**	-	-	+ +	64	*Pale green Nodular*
1.0	**0.5**	-	**0.5**	+ + + +	**96**	*Pale green friable*
1.0	-	**0.5**	-	+ + +	88	*Pale green friable*

Table 5:- Effect of PGRs and adenine sulphate (AdS) on induction of multiple shoots from nodal explants of *H. ada-kodian*

Plant Growth Regulators (mg L^{-1})				No of Shoots /explant	Response %
BAP (mg L^{-1})	**NAA (mg L^{-1})**	**IBA (mg L^{-1})**	**AdS (mg L^{-1})**		
0.5	-	-	-	1	14
1.5	-	-	-	2	21
1.5	0.5	-		3	28
1.5	-	-	1.0	3	42
2.0	1.0	-	-	2	21
2.0	-	-	1.0	5	57
3.0	1.0	-	-	-	-
3.0	-	-	1.0	9	71
1.0	-	0.5	-	-	-

Conclusion:-

The study substantiates nodal explants form ideal starting material for axillary bud proliferation for multiple shoot induction and also the nodal explant is the best explant source for callogenesis in *Holostemma ada-kodien*. The internodal, petiole and leaf explants showed good callus initiation and proliferation but no organogenesis was achieved in the time frame of this work. Only the nodal explants showed *in vitro* morphogenetic potential. Addition of Ads improved multiple shoot initiation along with healthy leaf and shoot development.

Figure 1:- Habit of *H. ada-kodian*

Figure 2:- Axillary buds from nodal explant

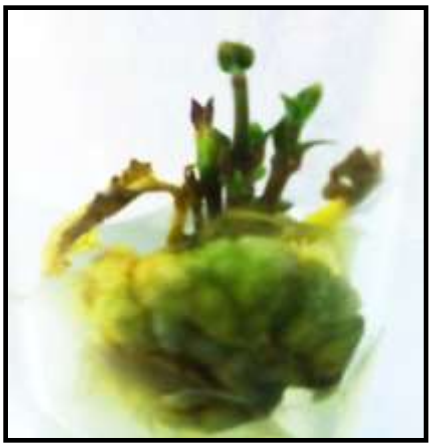

Figure 3 :- Rapid callus initiation and proliferation of nodal explant with multiple shoot induction

Figure 4:- Callus initiation from inter nodal explant

Figure 5:- Good callus initiation and proliferation from cut ends of petiole.
Figure 6:- Calli occurred on both adaxial and abaxial sides of leaf explant.
Figure 7:- Callus formation was observed on the veins and vein tips of leaves explant.

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