Tissue culture studies of Heliotropium indicum an important medicinal herb for callus induction and micro propagation

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Abstract: Tissue culture studies of *Heliotropium indicum* (Boraginaceae) an important medicinal herb has been carried for induction of callus and multiple shoots from leaf, internodes and nodal explants. Internodal segments and leaves were inoculated in Murashige and Skoog (MS) (1962) medium, supplemented with 1.0 mg/l - 3.0 mg/l concentrations of 2,4-DichlorophenoxyAceticAcid(2,4-D), 3-Indole Butyric Acid (IBA) and α- Naphthalene Acetic Acid (NAA) separately, along with two different concentrations of BAP and Kinetin. Higher percentage of response for callusing in internodal explant was obtained in MS+2.0 mg/l BAP + 3.0mg/l NAA which was 78%. This was followed by similar concentration of KN+IBA which was 66%.However, the higher percentage of response for callusing was obtained in MS+2.0 mg/l BAP + 2.0 mg/l IBA which was 67% followed by similar concentration of BAP+NAA. Kinetin at this concentration revealed lower percentage of response. It was observed that similar concentration of 2, 4-D, either with BAP or KIN revealed poor response for callusing in both the leaf and internodal explants. For multiple shoot induction the apical shoot and nodal explants were inoculated in MS+BAP or KN alone or with various concentrations of NAA, IAA or GA_3. MS medium fortified with BAP alone could promote shoot bud induction in both the explants and it was 87% at 1.0 mg/l BAP, where the number of shoots was 5.4. The percent response was 92 at 1.0 mg/l concentration of Kinetin .When GA_3 0.5mg/l was added, the percent response was 92 and number of shoots 12.8. For apical shoot the percent response was 94, number of shoots 14.8 at the same concentrations. Well grown plantlets were used for rooting, in ½ MS medium + various concentrations of IAA, NAA and IBA. Here, 0.2 mg/l IBA was found more suitable where mean number of roots as 14.6 per plantlet. Survival rate in the field condition was 76%.

Keywords: *Callogenesis, Heliotropium indicum, multiple shoots, Boraginaceae, nodal and apical explants, plantlets*

Introduction:

Heliotropium indicum Linn. is commonly known as "Hathisura" due to its inflorescence, belongs to the family Boraginaceae. This medicinal herb is found on the bare lands, roadsides or in the marginal sides of Institution and Government offices. This plant can be located due to its deep green borage leaves and white flowers during April to July. However, plants in poor condition may also be found during rainy season or even in winter. Young plants can be located next year in the month of February. Leaf are opposite or alternate, petiolate, oblong-ovate, somewhat hairy, acute or acuminate, base decurrent along the petiole. Flowers are white in colour and born on one side of curved, terminal spikes which curves at the tip. Flowers all in one side. Fruits composed of 2, ovoid beaked nutlets.

Entire plants are used for the treatment of different diseases. The major alkaloids are heliotrine isolated from the seeds. In addition saponins, tannins, glycosides and flavonoids have been isolated.

The tribal use seeds to cure stomachic, scabies and other skin diseases. Decoction of leaves is used for washing cuts and sores and for the treatment of cholera. The poultice of leaves are applied to wounds and boils, in lemon. Juice of the leaves are used to curewounds,faciaacne,alongwithsaltforclearing vision. It is also used in herpes and other skin diseases. The decoction of leaves and inflorescence is used for gargle in sore throats and tonsillitis. Flowers in small doses are emmenagogue and in large doses may be abortive. Leaf juice is also used against insect bites and to cure cough of the infants. It is also used to cure eczema, dermatitis and wound healing. In addition, there are reports that the plant bears medicinal importance and are being used as gastro protective, wound healing, anti-tumour, anti-inflammatory, anti-tuberculosis, anti-microbial and as analgesic.

Due to the above properties and their prevalence in the wild habitat, the species has been brutally exploited by the agents of Vaidya or the labourers of the Pharmacompanies.

For the conservation of germplasm of such medicinal plants which are considered to be rare or threatened, different strategies are being followed. Conservation through seeds or cutting are not applicable in all the species. However, tissue culture techniques and in vitro multiplications for the production of large number of planting material are getting much importance for different plant species including medicinal plants. (Ajith Kumar and Seeni,1998; Prakash et al.,1999). Micropropagation is being

considered an effective approach to conserve germplasm of such medicinal plants. In vitro propagation have proven as a potential techniques for mass scale production of genetically alike planting materials of medicinal plants.(Lui and Li,2001; Wawrasch et al.,2001; Martin,2002 and 2003; Azad et al.,2005; Faisal et al.,2003;Hassan and Roy,2004,2005). Usually the acting ingredients of the medicinal plants are extracted from the whole plants or its roots, for which they are harvested from their natural habitat. Callus culture or cell suspension culture may be exploited for the production of secondarymetabolites.

This will enable to supply the constituents all the year round without any interruption because the callus or suspension culture can be utilized for a longer periods, if once the protocol is established. This will also promote us to conserve the species in their natural habitat. In the present study attempts have been made to initiate callus from the various explants, and to developed protocol for efficient micropropagation from the various explants of *H.indicum* an important medicinalherb.

Material and Methods:

All the experiments were carried at the University Department of Botany, B.R. Ambedkar Bihar University in the Plant Biotechnology Laboratory. Healthy and young branches of *H.indicum* were collected from the campus of the Department and from the marginal land of the road. Branches were collected in the beaker containing water. They were brought in the laboratory, to be used for the explant. Both the lower mature and the upper young parts of the branch were removed. Now leaves were detached with the help of a sharp edged blade. The branch was cut into pieces in such the each segment had at least one node with single pre-existing meristem in the form of axillary buds. Above explants were washed with running tap water, in a 500 ml Erlenmayer flask. The mouth was closed with muslin cloth to avoid, run off the materials along with the circulating tap water. These explants were treated with 1% Savlon for 30 min. It was rinsed with glass distilled water and treated which 0.1% mercuric chloride for 2-3 minutes. The flask was shaken vigorously for uniform treatment. They were rinsed thrice with sterile distilled water to remove even a trace of chemical attached on its surface.

MS medium supplemented with (Murashige and Skoog, 1962) basal medium was supplemented with 3% sucrose and agar was used as gelling agent. The medium was also supplemented with seven different concentrations (0.1-3.0 mg/l) of BAP and KN, separately, and in combinations of BAP + KN + IAA, or IBA or NAA, for multiple shoots induction from nodal and apical buds, while MS medium supplemented with basal medium was supplemented with various concentration of 2,4-D and IBA, either alone or in combinations for induction of callus .MS basal medium with above composition was dispensed into 150X15 mm culture tubes(20 ml) and 250 ml conical flask(40ml). Before this the pH was adjusted to 5.8. The cotton plugs were covered with Aluminium foil and tubes and flasks were autoclaved for 20 min at 15 lb pressure. The culture tube and flasks were taken out and allowed to cool at room temperature. Inoculation was made on 3rd day and all the culture showing contamination were discarded. The cultures were incubated in culture room at 26± 1^0C temperature and fluorescent light for 16 hours photoperiod. Observation were made on alternate day and data were collected after 36days for per cent response, number of axillary shoot, shoot length etc. multiplication of shoot was done on the same medium.

For induction of callus, leaf and internodal explants were inoculated in MS+ 2,4-D or IBA or NAA. Here also data were collected after 36 days for percentage of response, callus growth rate, morphology of the callietc.

Well grown tissues cultured plants were used for in vitro rooting in half strength MS medium supplemented with IBA and NAA. Rooted plants were transferred in poly bag containing 1:1:1 ratio of soil, sand and coir peat. The temperature and moisture were maintainedartificially.

Result and Discussions:-

Induction of callus

For callusing MS medium was supplemented with five different concentration of 2,4-D, IBA and NAA alone and two different concentration (1.0-2.0 mg/l) of KN &BAP.

Data were collected after 36th day of inoculation and have been presented in table1.

From the table it is apparent that 2,4-D at all its concentration revealed poor percentage response as well as slow growth rate in comparison to the similar concentration of IBA or NAA. Here the higher percentage of response was 50 at 3.0 mg/l and growth rate was average (Fig: A &B).

From the table it is further observed that at the similar concentration i.e., 3.0 mg/l of IBA the percentage response was 66 (Fig: C) and the growth rate was good while that of in case of NAA it was 62 (Fig: D) and the growth rate was also good. However, when 1.0 mg/l KN or BAP was added in 3.0mg IBA the percentage response increased that was 57 and 68 respectively and with similar concentration of NAA it was 64 and 68. Similarly, when 2.0 mg/l KN were added with 2.5 mg/l NAA the per cent response was 66 and when the same concentration of KN added with 2.5 mg/l or 3.0 mg/l IBA the percentage of response and growth rate increased further which was 54 and 67 respectively. Here it may be concludedthat 3.0 mg/l IBA + 2.0 mg/l BAP induced callusing in maximum explants and the best growth rate in the callus was also found here which was followed by similar concentration of NAA and BAP (Fig: E & F).

Multiple Shooting:

From the table 2, it is obvious that nodal gave different response when it was cultured in MS-basal medium supplemented with 0.1 mg/l – 3.0 mg/l BAP or kinetic alone. Highest % of response for shoot induction was noted on MS+ 1.0 mg/l BAP that was 87.0. This was followed by MS+ 0.5 mg/l BAP which was 82.0. on the same concentration of kinetic the % response was 92.0 and 85.0 % respectively. It may be further noted that addition of IAA 0.5 mg/l with 0.5 mg/l BAP + 1.0 mg/l kinetic had no further promising impact. Here also the % response was 92.0 only. The average shoot number per explant was 5.4. In case of MS+ 1.0 mg/l BAP. This number was 6.8, at the same concentration of kinetin. Similarly, 1.5 mg/l KN supplemented MS medium, the mean number of shootsperexplantwas6.2.ItmaybenotedthatMS+ 0.5 mg/l BAP + 1.0 mg/l KN + 0.5 mg/l IAA produced 12.8 mean number of shoot. Here, the per cent response was similar but the number of axillary shoot was equivalent to double of the KN alone. This number was followed by 0.5 mg/l BAP + 0.5 mg/lKN +0.2mg/lIAAwhichwas96.When0.5mg/lBAP+ 1.0 mg/l KN + 0.5 mg/l NAA was supplemented in MS medium, the mean of shoots was 10.6 (Fig: G & H).

From the table, it may be noted that the shoot length was also influenced by the concentration of the growth regulators. The maximum shoot lengthwas 3.8 cm on MS+ 1.0 mg/l KN or 1.5 mg/l KN.

The shoot length was 4.7 cm in the shoot developed in 0.5 mg/l BAP + 1.0 mg/l KN+ 0.5 mg/l IBA.

From the table 3, it may be noted that MS basal medium + 1.0 mg/l BAP induced shoot bud formation in 92% of the explants. At the same concentration of KN, the % response was 94%. Similarly, when KN 0.5 mg/l + GA_3 0.5 mg/l supplemented in MS medium induced shoot bud formation in 94% of the explant. Likewise, MS+ 1.0 mg/l BAP +1.0 mg/l KN + 0.1 mg/l GA_3 induced shoot buds in 92% of the explants. It may further be noted that the mean number of the shoot per explant was 14.8 in MS+ 0.5 mg/l KN + 0.5 mg/l GA_3. This was followed by the medium containing 1.0 mg/l BAP + 1.0 mg/l KN + 0.1 mg/l GA_3 which was 10.8. The mean length of the shoot was 4.8 cm in the shoot developed in MS medium supplemented with + 0.5 mg/l KN + 1.0 mg/l GA_3. However,theshootinitiatedinMS+1.0mg/lBAP+ mg/l KN + 0.1 mg/l GA_3 which was 5.2 cm (Fig: I &J).

Root initiation:

From the table 4, it may be noted that maximum % response for rooting in tissue culture raised plantlet was 88 in MS+ 0.2 mg/l IBA. Here, the mean number of roots per shoot was 14.6. This was followed by shoots cultured in MS+ 2.2 mg/l IAA which was 78%. Here, the mean number of roots per explant was 12.4. The maximum mean root length was 1.8 among the shoots cultured in MS+ 0.2 mg/l IAA. This was followed by the shoots cultured in MS+ 0.2 mg/l IBA which was 1.6 cm respectively. Rooted plants were transferred in poly bag containing 1:1:1 ratio of soil, sand and coir peat. The temperature and moisture were maintain artificially. The survival rate were 71%. The present finding corroborates with the finding of Kumar and Rao, 2007, who also obtained similar pattern of results in *H.indicum.* The proliferation of shoots and their elongation were noted simultaneously in the similar concentration of the growth hormones. Multiple shoot induction in MS medium containing different combination and concentration of Cytokinin and auxins have also been reported by (Dutta *et.al.*,2003; Gawde and Paratkar, 2004; Bhaskaran and Jayabalan, 2005; Hussain and Anis, 2006; Han *et.al.,2007;* Usha *et.al.*,2007; Hassan *et.al.*,2008; Afroj *et.al.*,2008; Priyadarshni *et.al.*,2013) (Fig: K &L).

Conclusion:

From table 1, it is concluded that MS medium supplemented with IBA (3.0 mg/l) along with BAP (2.0 mg/l) induced callusing in maximum explants and become the best growth medium for callus induction.

From the table 2 & 3, it is clear that among the explants, the apical bud was superior to the nodal explants with respect to percentage response induction of shoot buds.

Similarly, KN was found superior with respect to BAP because the % response, mean number of shoot buds and mean length was superior at all the concentration supplemented in BAP. Table 4 indicates for rooting IBA was most promising than the NAA and IAA at all the four concentrations used for rooting. The survival rate of the above shoot were 71%.

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Table: 1 *Showing impact of different concentrations of growth regulators on callusing in H.indicum nodal explants.*

2,4-D(mg/l)	IBA	NAA	KN	BAP	% Response	Growth rate
	-	-	-	-	22	+
	-	-	-	-	32	+
	-	-	-	-	38	++
1.0	-	-	-	-	46	++
1.5	-	-	-	-	50	++
2.0		1.0			46	+
2.5		1.5			48	+
3.0		2.0			52	+
-		2.5			60	++
-		3.0			62	++
-	1.0	--	-		25	+
-	1.5		-	-	36	++
-	2.0	-	-	-	43	++
-	2.5	-	-	-	54	++
-	3.0	-	-	-	66	+++
-	-	1.0	-	2.0	24	+
-	-	1.5	-	2.0	33	+
-	-	2.0	-	2.0	41	++
-	-	2.5	-	2.0	52	++
-	-	3.0	-	2.0	72	+++
-		3.0	1.0		64	++
-	2.5		1.0	-	54	+++
-	3.0	-	1.0	-	57	++
-	2.5	-	2.0		54	++
-	3.0	-	2.0	-	67	+++
-	-	2.5	2.0	-	66	++
-	-	3.0	2.0	-	56	++
-	-	-	-		62	+++
-	2.5	-	-	1.0	61	+++
	3.0	-	-	1.0	68	+++
	2.5	-	-	2.0	64	+++
	3.0	-	-	2.0	78	++++

Poor- +
Average - ++
Good- +++
Best - ++++

Table-2 *Impact of various concentration of growth regulators supplemented with MS basal medium onnodal explant of H. indicum on shoot induction.*

Growth regulators Mg/l		**Nodal explants**		
BAP +	Kin	% response	Average shoot number pea explant	Shoot length
00	00	56	2.4±0.22	1.6±0.18
0.1	00	68	3.6±0.24	1.5±0.22
0.2	00	72	3.8±0.26	2.2±0.23
0.5	00	82	4.6±0.30	2.6±0.22
1.0	00	87	5.4±0.32	2.8±0.22
1.5	00	80	4.6±0.28	2.2±0.24
2.0	00	73	4.4±0.30	2.2±0.24
2.5	00	64	3.8±0.26	2.4±0.25
3.0	00	58	2.6±0.22	2.2±0.24
00	0.1	64	3.4±0.24	2.6±0.22
00	0.2	74	4.2±0.30	2.8±0.26
00	0.5	85	4.8±0.32	3.2±0.28
00	1.0	92	6.8±0.36	3.8±0.32
00	1.5	90	6.4±0.28	3.8±0.32
00	2.0	80	4.8±0.32	3.4±0.30
00	2.5	76	4.2±0.28	2.8±0.24
00	3.0	63	3.4±0.26	2.4±0.22
BAP+KIN+IAA				
0.5 + 0.5+0.2		86	8.6±0.32	4.6±0.30
0.5+1.0+0.5		92	12.8±0.28	4.8±0.28
0.5+1.0+1.0		80	6.4±0.24	4.2±0.24
BAP+KIN+IBA				
0.5+0.5+0.2		80	9.6±0.34	4.6±0.28
0.5+1.0+0.5		86	12.2±0.36	4.7±0.30
0.5+1.0+1.0		74	7.6±0.28	4.2±0.26
BAP+KIN+NAA				
0.5+0.5+0.2		78	8.2±0.24	4.4±0.28
0.5+1.0+0.5		84	10.6±0.25	4.6±0.32
0.5+1.0+1.0		72	7.8±0.30	4.4±0.32

Table-3 *Impact of various concentration of growth regulators supplemented in MS basal medium on apicalbud explant of H. indicum on shoot induction.*

Growth regulators Mg/l		**Apical bud**		
BAP +	Kin	% response	Average shoot number /explant	Average shoot length (cm)

0.1	00	70	3.8±0.20	2.24±0.18
0.2	00	78	4.2±0.24	2.6±0.22
0.5	00	88	5.4±0.32	3.2±0.24
1.0	00	92	6.2±0.30	3.4±0.26
1.5	00	86	6.8±0.34	3.8±0.32
2.0	00	74	4.8±0.26	3.2±0.24
2.5	00	66	3.2±0.22	2.8±0.20
00	0.1	74	2.8±0.18	1.8±0.16
00	0.2	80	3.8±0.22	2.15±0.20
00	0.5	86	4.4±0.26	2.8±0.22
00	1.0	94	5.8±0.32	3.2±0.26
00	1.5	88	7.6±0.34	3.4±0.28
00	2.0	82	5.8±0.32	3.3±0.26
00	2.5	77	4.6±0.28	2.4±0.24
BAP	**+ KIN**	**+GA_3**		
0.5	+0.00	+0.1	6.4±0.30	3.8±0.24
0.5	+0.00	+0.5	8.6±0.36	4.4±0.32
0.5	+0.00	+1.0	8.2±0.32	4.2±0.28
00	+0.5	+0.1	6.6±0.34	4.2±0.28
00	+0.5	+0.5	14.8±0.38	4.8±0.32
00	+0.5	+0.1	7.8±0.34	3.6±0.28
0.5	+0.5	+0.1	7.2±0.36	3.8±0.26
1.0	+1.0	+0.1	10.8±0.38	5.2±0.26
1.0	+1.0	+0.5	8.0±0.34	4.8±0.24

Data were collected after 28 days of induction

Table-4 *Response of Tissue culture of derived shoots of* ***H.indicum*** *with respect to root induction on* ***half strength MS basal medium*** *supplemented with various concentration of Auxins.*

Growth regulators Mg/l			Apical bud		
IAA	NAA	IBA	% response for rooting	Mean number of roots/shoots	Mean root length (m)
00	00	00	48	2.5±0.22	1.2±0.18
0.1	-	-	66	4.8±0.26	1.4±0.24
0.2	-	-	78	12.4±0.32	1.8±0.22
0.5	-	-	71	8.6±0.34	1.5±0.24
1.0	-	-	52	3.8±0.26	1.2±0.22
-	0.1	-	54	3.4±0.18	0.8±0.15
-	0.2	-	62	3.8±0.22	0.8±0.18
-	0.5	-	bc++		
-	1.0	-	bc+++		
-	-	0.1	72	8.4±0.28	1.4±0.16
-	-	0.2	88	14.6±0.32	1.6±0.18
-	-	0.5	74	9.2±0.34	1.2±0.24
-	-	1.0	58	4.8±0.24	0.8±0.28

Data were collected after 28 days of inoculation.
Average values ± SE = Replica of 25 cultures
bc++ = induction of callus at the callus with slow growth bc+++ = induction of callus with fast growth rate


Fig A: Curling in explant


Fig B: Swelling for callusing


Fig C: Initiation of callusing


Fig D: Enlargement in callus


Fig E: Developing callus


Fig F: Well developed callus


Fig G: Initiation of shooting


Fig H: Initiation of shooting


Fig I: Multiple shooting


Fig J: Multiple shooting


Fig K: Initiation of root


Fig L: Development of root

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