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RESEARCH ARTICLE

HIGH FREQUENCY SOMATIC EMBRYOGENESIS FROM COTYLEDONARY EXPLANTS OF *INDIGOFERA TINCTORIA* LINN.

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Abstract

Protocol was standardized for the rapid clonal propagation through high frequency indirect somatic embryogenesis from the cotyledonary explants of a medicinally and commercially important plant, *I. tinctoria* Linn. Maximum embryogenic callus was induced from explants cultured on MS medium amended with 2, 4-D (0.5 mgl^{-1}) and BA (0.5 mgl^{-1}). Abnormal somatic embryos were developed from NAA and BA containing cultures. Among different additives or amino acids used, L-glutamine enhanced maximum high frequency embryogenic callus formation. Embryogenic calluses, when subcultured to ½ MS basal medium induced maximum embryo conversion. BA or KIN along with MS or ½ MS medium also enhanced the development of embryos. Maturation and rooting of somatic embryos took place in ½ MS solid medium enriched with 5% sucrose. Plantlets derived from somatic embryos were hardened in sugar free liquid ½ MS basal medium. Subsequent transfer of hardened plantlets to vermiculite and then to soil recorded maximum survival.

Abbreviations:-
MS- Murashige and Skoog medium, 2,4-D- 2,4-dichlorophenoxy acetic acid, NAA- α-naphthaleneacetic acid, IAA- Indole-3-acetic acid, BA- benzyladenine, KIN- 6-furfuryl aminopurine, CH- casein hydrolysate, MIL- myoinositol, Ads- adenine sulphate, L-glut- L-glutamine and CW-coconut water.

Introduction:-

Indigofera tinctoria Linn. (Indigo plant) belonging to the family Fabaceae is an important medicinal plant, used in indigenous systems of medicine as well for the extraction of chemical substances of high therapeutic value. It is used as a remedial agent in epilepsy, infantile convulsions, hysteria and amenorrhoe. The commercially important indigo ($C_6H_{10}N_2O_2$), the blue pigment and an alkaloid indigoferine are commonly extracted from *I. tinctoria*. Several Spp. of *Indigofera* became enlisted as rare or endangered. *I. tinctoria* is now a threatened species in India. Since *I. tinctoria* contains more indigo content than other members of the genus (Kazhakyan *et al*., 1986), the present study was undertaken to standardize protocols for rapid clonal propagation through indirect somatic embryogenesis for conservation as well as for large scale cultivation. *In vitro* studies in *Indigofera* species are very few. However

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Bharal and Rashid (1979) succeeded in the regeneration of plants from tissue cultures of *I. ennaephylla*. Evans (1986) reported shoot organogenesis from *in vitro* cultures of *I. patoninii*

Materials and methods:-

Pods were harvested from six-month-old green house grown mother plants during August of 2000. The seeds were excised from the pods and washed thoroughly with running tap water for one hour and with a neutral detergent (Labolene; Qualigens, India). The seeds were disinfected with 0.1% mercuric chloride (Merck, India) for 5 minutes. After several rinses with sterile double distilled water, seeds were dissected and cotyledons were released. The cotyledonary explants of $1mm^2$ size, were excised aseptically and inoculated on to MS (Murashige and Skoog, 1962) medium amended with 2,4-D (0.5-1 mgl^{-1}), NAA (1-2 mgl^{-1}) or IAA (0.5-1 mgl^{-1}) along with BA (0.1-0.5 mgl^{-1}), or KIN (0.5-1 mgl^{-1}). Additives and amino acids (50-200 mgl^{-1}) viz. casein hydrolysate, myoinositol, adenine sulphate, L-glutamine and coconut water (50-200mll^{-1}) were incorporated to the medium containing 2,4-D (0.5mg/l) and BA (0.5mg/l). 3% sucrose was added to the medium and the P^H was adjusted to 5.8 before autoclaving. MS medium was gelled with 0.8% agar- agar. The cultures were incubated at 25 ± 1^o C on a $^{12}/_{12}$-hour photoperiod at intensity of 16 $\mu E\ m^{-2}\ S^{-1}$ light. The relative humidity was maintained between 70 and 80%.

Percentages of embryogenic calluses were calculated (Berthouly and Ferriere, 1996). The embryogenic friable calluses initiated after 20 days were transferred to fresh MS medium with same hormonal milieu or to ½ strength or full strength basal MS medium (regeneration medium) alone or with BA (0.1-0.5 mgl^{-1}) or KIN (0.5-1 mgl^{-1}). Subculturing was done after every 20 days. The rate of conversion of somatic embryos was noted. The bipolar somatic embryos were transferred to fresh ½ MS solid medium with 5% sucrose. The plantlets were hardened on filter paper bridges in sugar free ½ MS liquid medium. The hardened plantlets were transplanted to vermiculite in plastic cups, covered over by moistened plastic bags and nourished with $^1/_{20}$ th the strength of Hoagland's solution (Epstein, 1972) after every 5th day. The plantlets, after 20 days of growth in vermiculite were transferred to soil in green house conditions. 21 replicates were used for each experiment and the results were analysed statistically.

Results:-

Within about 8-10 days after placement on the culture medium, all the explants began to exhibit callus formation primarily from the cut ends and occasionally from the midrib portion. The embryogenic calluses varied from white friable to yellowish green friable with embryoids. The most efficient callus induction was obtained in presence of 2, 4-D (0.5 mgl^{-1}) and BA (0.5 mgl^{-1}). Even though the callus was embryogenic, the stages of somatic embryogenesis were not observed. However, certain greenish globular or torpedo stage embryos were observed, when the calluses along with the explants were transferred to fresh solid medium with same hormonal composition (Fig. 1). Further stages of somatic embryogenesis were not observed in subsequent subcultures in the same fresh medium. The callus derived in presence of 2, 4-D (0.5 mgl^{-1}) and KIN (1 mgl^{-1}) was white loosely arranged and soft. But it became beady and proliferative covering the entire surface of the explant in further subcultures. IAA along with BA or KIN induced friable yellowish green proliferating callus after first subculture. Compact white nodular structures were developed from the callus derived in presence of NAA and BA. These nodular structures became anomalous somatic embryos with a well-developed root pole and a fused hypocotyl region in subsequent subcultures. The callus derived from NAA (2mg/l) and KIN (0.5 mgl^{-1}) showed signs of root morphogenesis after first subculture.

Percentage of high frequency embryogenic calluses was obtained 20 days after first subculture varied from 0 to 100 according to different auxin-cytokinin ratios (Table.1). The callus induced by 2,4-D (0.5mg/l) and BA (0.1 mgl^{-1}) recorded a maximum of 100% high frequency embryogenic callus induction from the cotyledonary explants 20 days after first subculture.

White friable highly proliferating callus was found in cultures enriched with myoinositol. While high frequency embryogenic percentage was maximum for L-glutamine containing cultures (Table 2). Slightly yellowish nodular proliferating callus was initiated from L-glutamine containing cultures. Coconut water also induced nodular callus from the explant and recorded 87-98% of high frequency embryogenic callus induction, where as adenine sulphate showed only 59- 89%. Among different additives and amino acids used, casein hydrolysate was found inhibiting the embryogenic callus induction.

Successive stages of somatic embryogenesis from all the embryogenic calluses were observed only when transferred to regeneration medium with or without growth regulators. Globular structures occurred at the surface of the

embryogenic calluses one week after inoculation. Heart and torpedo shaped structures appeared after 20 days of inoculation. Cotyledonary embryos developed after a period of 30 days in all the cultures. The morphology of the somatic embryos was quite similar to that of zygotic embryos. White unpigmented somatic embryos were initiated from the calluses cultured on ½ MS medium devoid of phytohormones (Fig. 2). Slightly green embryos derived in MS medium containing BA (Fig. 3). Distinct bipolar structures were noticed from cultures with ½ MS medium fortified with 0.5 mgl^{-1} BA after 25 days (Fig. 4).

Among different regeneration media used, ½ MS basal medium showed a higher rate of embryo conversion than full strength MS medium. Incorporation of BA/ KIN in the regeneration medium induced somatic embryo conversion (Table. 3). The bipolar somatic embryos, when transferred to ½ MS solid medium enriched with 5% sucrose, became rooted plantlets and grew to a height of 5-7cms after two weeks of culture (Fig.5). Well- rooted plantlets were harvested after 20 days of culture (Fig. 6). The plantlets, after hardening in liquid ½ MS medium on filter paper bridges and nourishing with ½oth Hoagland's solution for 15 days, were transferred to vermiculite in plastic cups, where they grew successively. The plantlets derived from ½ MS basal medium showed maximum survival in the soil (Fig.7).

Discussion:-

Protocol was standardized for a fast reproducible initiation of high frequency somatic embryogenesis from cotyledonary explants of *I. tinctoria*. This is the first successful report of plant regeneration through somatic embryogenesis for this medicinally and commercially important species. Venkataiah *et al.* (2016) reported somatic embryogenesis from leaf and cotyledonary explants of *Capsicum bacatum.* The induction and expression of somatic embryos was dependant on the type and concentration of growth regulators in the primary medium, followed by successive transfer to a medium adequate for each critical step of the process. Etiennae *et al*. (1993) established, in *Hevea brasiliensis*, that the expression of somatic embryogenesis is not just related to the establishment of a specific balance between different endogenous hormones or exogenously applied growth regulators but perhaps to the particular levels of hormones or growth regulators in action.

The first signs of callogenesis were observed after 8-10 days of culture. The embryogenic calluses showed variation in the embryogenic potential and morphology in accordance with variation in the medium, concentration of auxins, cytokinins, additives or amino acids. Among the auxins used in the primary medium for the induction of embryogenic callus, 2, 4-D seems to be critical for the embryogenic callus induction of *I. tinctoria*. According to Vasil (1982) embryogenic competence is acquired during the first stages of culture of several plants in the presence of 2,4-D. High frequency somatic embryogenic callus induction percentage was maximum in presence of 2,4-D along with BA. The need of BA in conjunction with 2,4-D for embryogenic callus induction was also well documented in *Panax ginseng* (Tang, 2000). But the embryoids fail to attain successive stages of somatic embryogenesis in presence of auxin. Komamine *et al*. (1992) observed that auxin is only necessary for the formation of meristamatic cell clusters and is inhibitory to subsequent developmental phases of somatic embryogenesis. Certain abnormal embryos with well developed radicle and highly condensed plumule was observed from cultures containing NAA and BA. Strandberg (1993) observed such embryo like structures with abnormalities in the *in vitro* cultures of *Ophiopogon japonicus* in presence of NAA after several subcultures.

Stuart and Strickland (1984) have investigated the stimulatory effect of adding amino acids to cultures of alfa alfa undergoing *in vitro* somatic embryogenesis. Additives and amino acids were also employed in the embryogenic cultures of *I. tinctoria*. L-glutamine induced more high frequency somatic embryogenic callus than the other additives or amino acids used. Arcioni (1990) found out that the number and structural quality of somatic embryos has been enhanced by the stimulatory effect of glutamine.

Conversion of somatic embryos took place only in an auxin free medium. The transference of 2,4-D derived callus to an auxin free medium is also necessary for the development of somatic embryos of flax (Gomes da Cunha and Ferreira, 1996). Presence of BA or KIN in the medium stimulated the conversion of somatic embryogenesis similar to the reports in cassava (Sofiari *et al.*, 1997).

The germination and rooting of somatic embryos are more prominent in ½ MS medium as reported by Xie and Hong (2000) in the somatic embryogenesis of *Acacia mangium*. In our experiment relatively high concentration of sucrose was necessary for embryo conversion and maturation. The precise role of high levels of sucrose in enhancing

somatic embryogenesis is not evident. However, many previous studies suggest that sucrose has both a nutritive and an osmotic effect on somatic embryogenesis (Parrot *et al*., 1992).

Somatic embryogenesis offers promise for rapid multiplication of plants and for genetic transformation. A combination of an efficient regeneration system through somatic embryogenesis with biolistic or *Agrobacterium*-mediated transformation will provide a valuable alternative breeding method for introducing desirable commercial traits to *I. tinctoria*.

Table 1:-Embryogenic callus formation from cotyledonary explants of *I. tinctoria* in MS media after 20 days after culture.

Concentration of auxins and cytokinins (mgl^{-1})					% of embryogenic callus*
NAA	IAA	2,4-D	BA	KIN	
1	-	-	0.1		58.4d
1	-	-	0.5	-	61.8d
2	-	-	0.1	-	47e
2	-	-	0.5	-	44e
-	0.5	-	0.1	-	56.2d
-	0.5	-	0.5	-	67.2d
-	1	-	0.1	-	63.1d
-	1	-	0.5	-	51.5e
-	-	0.5	0.1	-	100a
-	-	0.5	0.5	-	98.3a
-	-	1	0.1	-	91b
-	-	1	0.5	-	89b
1	-	-	-	0.5	47.1e
1	-	-	-	1	58.7d
2	-	-	-	0.5	43e
2	-	-	-	1	41.5e
-	0.5	-	-	0.5	58d
-	0.5	-	-	1	57.8d
-	1	-	-	0.5	52e
-	1	-	-	1	47.3e
-	-	0.5	-	0.5	69.1d
-	-	0.5	-	1	74.2
-	-	1	-	0.5	65.2
-	-	1	-	1	60.3

*Number of explants for each experiment = 21 Mean value followed by the same letter denotes no significant difference by LSD (0.05) test.

Table 2:-Effect of additives and amino acids on embryogenic callus formation from cotyledonary explants of *I. tinctoria* in MS media after 20 days of inoculation.

Concentration of additives and amino acids (mgl^{-1})					% of embryogenic callus *
CH	MIL	Ads	L-Glut	CW (ml/l)	
50	-	-	-	-	67.4d
100	-	-	-	-	65.8d
150	-	-	-	-	63.4d
200	-	-	-	-	61.7d
-	50	-	-	-	98.6a
-	100	-	-	-	97.2a
-	150	-	-	-	83.1b
-	200	-	-	-	81.5b

-	-	50	-	-	89b
-	-	100	-	-	78c
-	-	150	-	-	67d
-	-	200	-	-	59e
-	-	-	50	-	87.1b
-	-	-	100	-	100a
-	-	-	150	-	100a
-	-	-	200	-	97a
-	-	-	-	50	98a
-	-	-	-	100	97.8
-	-	-	-	150	92b
-	-	-	-	200	87.3b

*Number of explants for each experiment = 21. Mean value followed by the same letter denotes no significant difference by LSD (0.05) test.

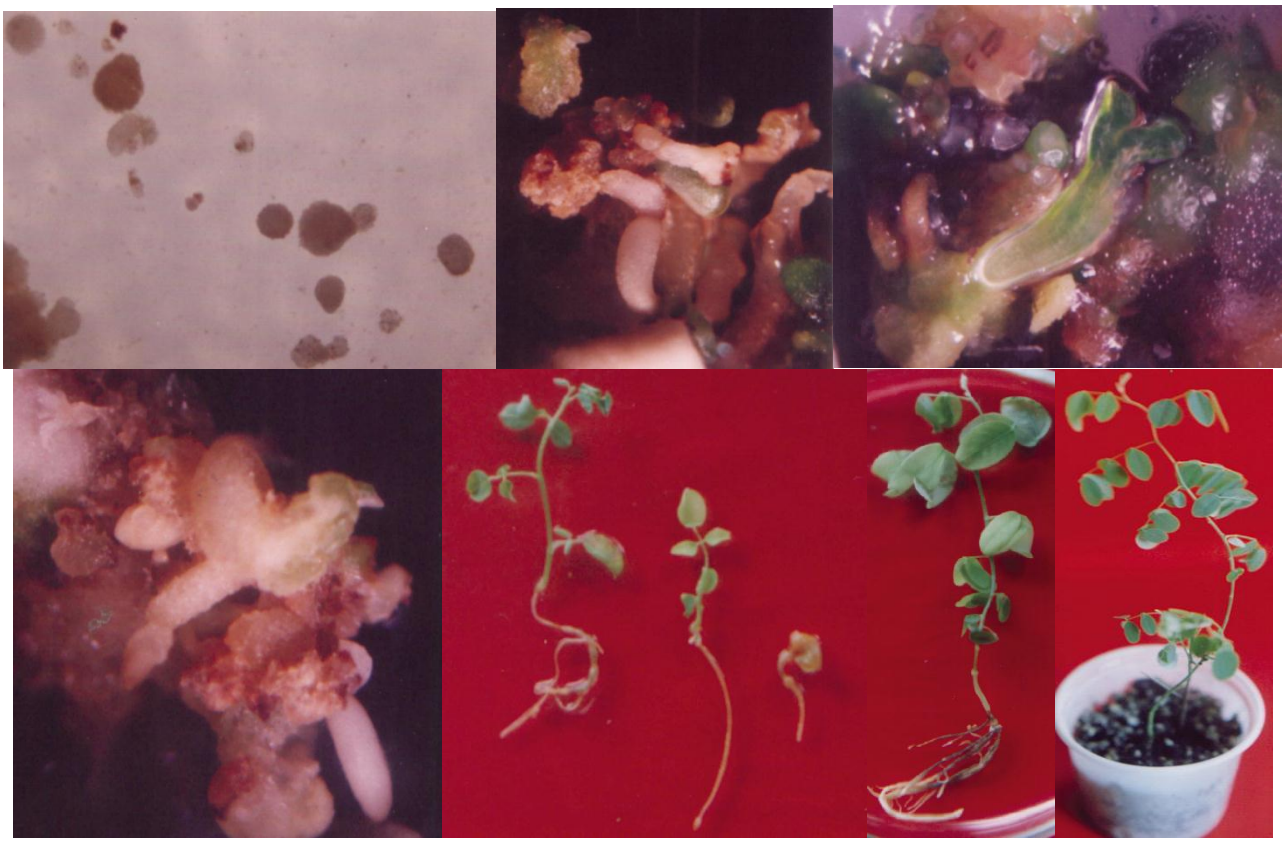
Table 3:-Germination of somatic embryos from embryogenic calluses derived from cotyledonary explants of *I. tinctoria* in MS or ½ MS media with/ without cytokinins after first subculture (after 40 days).

Medium	Conc. of BA(mgl^{-1})	Conc. of KIN (mgl^{-1})	% of embryo conversion(after I subculture)**	**Embryos survived after hardening ±SE** *
MS	-	-	84.2b	43.2± 0.02
MS	0.1	-	67.1d	21.5± 0.05
MS	0.5	-	46e	12.4± 0.03
MS	-	0.5	59d	15.7± 0.14
MS	-	1.0	38.4e	10.2± 0.06
½ MS	-	-	97.8a	54.1± 0.09
½ MS	0.1	-	85.3b	45.6± 0.03
½ MS	0.5	-	74.2c	41.2± 0.13
½ MS	-	0.5	71c	38.4± 0.07
½ MS	-	1.0	67.6d	27.3± 0.02

Data represents mean ± SE of 21 replicates.
Mean value followed by the same letter denotes no significant difference by LSD (0.05) test.

Explanation of figures:-

1. Photomicrograph of greenish globular and torpedo stages of somatic embryos derived in presence of 2, 4-D (0.5 mgl^{-1}) and BA (0.5 mgl^{-1}).
2. White unpigmented somatic embryos induced from the calluses cultured on ½ MS basal medium.
3. Greenish bipolar somatic embryos induced in presence of BA.
4. Cotyledonary stage somatic embryos initiated in ½ MS medium amended with 0.5 mgl-^{1} BA after 25 days.
5. Plantlets derived from ½ MS medium containing 5% sucrose after two weeks of culture.
6. Well- rooted plantlets after 20 days of culture in ½ MS medium containing 5% sucrose.
7. Hardened *I. tinctoria* plantlets in the soil after five weeks.



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