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RESEARCH ARTICLE

PRODUCTION OF INDICAN FROM THE CALLUS CULTURES OF *INDIGOFERA TINCTORIA* LINN.

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Abstract

Indigoferatinctoria Linn.has been extensively used as a source for the extraction of indigo, which is highly priced as a natural blue dye. The plant accumulates large quantities of indican, the colourless starting compound of indigo production. Indican content was analysed in calluses derived from leaf, petiole, cotyledons, stem and root explants cultured on MS medium fortified with 1mg/l 2,4-D and 0.5mg/l BA at three different phases of growth using HPLC. The stationary phase leaf callus showed a maximum of 910 µg/gm of indican. MS medium was found to be superior over WPM in inducing indican accumulation. Among different auxins used, IAA was found to induce maximum indican accumulation. Lower concentrations of cytokinins viz. BA (0.5 mg/l), KIN (1mg/l) and 2-ip (2mg/l) were found to be effective in the production of indican. Combinatorial effect of BA along with auxins (IAA, NAA, or IBA) was found to enhance the production of indican. 2, 4-D alone or in combination with cytokinins was found to inhibit indican production. Maximum accumulation of indican was recorded in presence of IAA (1.5mg/l) and BA (0.5mg/l) from stationary phase callus. L- tryptophan (100mg/l) was found to enhance intracellular accumulation of indican.

Introduction:-

Indigoferatinctoria Linn. (Indigo plant) belonging to the family Fabaceae is an important medicinal plant used for curing epilepsy, infantile convulsions, hysteria and amoenorrhoe (Iyer and Kolammal, 1992). Indigoferine, an important alkaloid is extracted from this species. The species is now widely used as a hair tonic and it forms the constituent of a number of ayurvedic preparations and has been extensively used as source of indigo (biindoline-3, 3'-dione). Indigo finds use in dying and printing cotton and rayon, and for dying the wool. It has been employed in the preparation of pigments for paints, lacquers, rubbers, and printing inks (Evans, 1990).

The actual colouring agent indigo is not present in the plant. Instead the plant contains indican, a colourless glucoside (Indoxylβ-D-glucoside), which serves as the starting material for indigo production. When the plant cells are broken, on hydrolysis, indican is degraded to glucose and indoxyl by the activity of β- glucosidase. Subsequent oxidation of indoxyl in air results in the precipitation of blue particles of indigo (Epstein *et al*., 1967).

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The indican content is maximum in *I. tinctoria* though few other species like *I. suffruticosa*, *I. sumantra*, *I. articulata*, *I. longerecemosa* and *I. arrecata*also contain the dye (Hass and Hill, 1913; Khazhakyan, 1986; Evans, 1990). The arial parts of the plants are usually used for the extraction of natural indigo. Destructive harvesting and indiscriminate use of the plant for medicinal as well as commercial purposes lead the species to the brim of extinction. *In vitro* methods are not so far standardized for the production of indican from *I. tinctoria*. Owing to the commercial importance of indican, the present study was undertaken to standardise protocols for the exploitation of indican from the callus cultures of this species.

Materials and methods:-

Callus culture

Explants, viz. leaf, petiole, stem and root from six-month-old green house grown plants and cotyledons from mature seeds were surface disinfected with 0.1% (w/v) mercuric chloride (Merck, India). After several rinses with sterile double distilled water, the explants were inoculated on to MS medium containing 1mg/l 2, 4-D and 0.5mg/l BA for developing stock callus. 250mg of stock callus was subcultured to MS medium and WPM containing 1mg/l 2, 4-D and 0.5mg/l BA or MS medium containing auxins viz. IAA (0.5-2mg/l), NAA (1-3mg/l) 2, 4- D (1-3mg/l) or IBA (1-4mg/l) alone or in combination with cytokinins, viz. BA (0.5-2mg/l), KIN (1-4mg/l) or 2-iP (2-6mg/l). Additives, Ads (25-100mg/l), deproteinised CW (50-200ml/l), PVP (25-100mg/l), PG (25-100mg/l), CH (50-200mg/l), or amino acids, L-glut (50-200), or TPN (50-150mg/l) were also added to the MS medium amended with 2 mg/l IAA and 0.5 mg/l BA to find out their role in indican production.30% sucrose was added to the medium and $_{P}$Hwas adjusted to 5.8 for MS medium and 5.6 for WPM. Before autoclaving, the media were gelled with 0.8% and 0.6% agar-agar respectively for MS and WPM. The cultures were incubated at 25 $\pm 1^{o}$ C on a 12/12 hour photoperiod at an intensity of 16µ Em^{-2} S^{-1}. The relative humidity was maintained between 70-80%. Sub culturing of the established cultures was done after every 30 days.

Quantitative analysis of indican

The indican content of calluses derived from different explants, media or hormonal combinations was analysed separately at three different stages of growth, viz. lag phase (10^{th} day), log phase (20^{th} day) and stationary phase (30^{th} day).

100mg of callus was homogenized in liquid nitrogen and suspended in 0.5ml of methanol- chloroform solution (CH_3OH: $CHCl_3$: H_2O=12: 5: 3, v/v) and the suspension was centrifuged at 26,000 x g for 15 min. The upper layer was saved and the lower layer was extracted twice in the same manner. The combined upper layers were mixed with o.35ml of chloroform and 0.5 ml of H_2O and were again centrifuged. The aqueous layer was used for the quantitative analysis of indican. The samples were filtered using 0.2micron nitrocellulose filters and injected to a Shim-pack C-18 column (4.6x 150mm, Shimadzu, Japan) and the excitation was measured at 290nm using an UV-detector (SPD- 10A*i*, Shimadzu, Japan). The chromatography was carried out with 10mM MES-KOH, $_{P}$H- 5.5 and 20% (v/v) methanol at a flow rate of 1.0 ml^{-1} min^{-1} on an HPLC system (LC-10AS, Shimadzu, Japan). The peaks of the samples of indican from different calluses were compared with that of the authentic standard indican (Sigma, USA). Ten replicates each were kept for all the experiments and the data were analysed statistically.

Results:-

Callus induction and indican production from different explants in MS medium

Indican content was analysed in calluses derived from leaf, petiole, cotyledons, stem and root explants cultured on MS medium fortified with 1mg/l 2,4-D and 0.5mg/l BA at three different phases of growth (lag, log and stationary phases). Leaf derived callus in the stationary phase recorded a maximum of 910µg/gmFwt. of indican. Stationary phase callus initiated from petiole, stem and cotyledonary explants accumulated 480µg/gmFwt., 514µg/gmFwt., and 697µg/gmFwt. respectively. Minimum indican was extracted from root callus at the three phases of growth (Fig.1).

Indican production in MS and WPM

Since indican production was maximum in leaf callus, it was used for raising the stock callus for further experiments. This stock callus was subcultured to MS medium and WPM amended with 1mg/l 2,4-D and 0.5mg/l BA. The callus growth as well as indican content was found higher in MS medium than in WPM. 921.4µg/gmFwt. and 842.3µg/gmFwt. of indican were detected from calluses at the stationary phase.

Effect of auxins and cytokinins on indican production

The stock callus was subcultured to MS medium containing different concentrations of auxins (2,4-D, NAA, and IAA) alone or in combination with cytokinins (BA, KIN and 2-iP) to find out their role in the synthesis of indican. Among different auxins used, IAA (1.5mg/l) was found to be more effective than others. 918.4µg/gmFwt., 936.7µg/gmFwt., and 982.5µg/gmFwt. of indican were extracted from calluses at lag, log and stationary phases respectively. 2,4-D (1mg/l), IBA (1mg/l), and NAA (3mg/l) induced the deposition of maximum quantities of indican (Table. 1). Lower concentrations of cytokinins viz. BA (0.5 mg/l), KIN (1mg/l) and 2-ip (2mg/l) used alone or together with auxins (IAA or IBA) were found to be effective in the production of indican (Table.2).

Incorporation of 0.5mg/l BA along with 1.5mg/l IAA induced a maximum of 996.5µg/gmFwt. of indican, while KIN (2mg/l) and 2-ip (2mg/l) induced only 972.1µg/gmFwt. and 927.4 µg/gmFwt. of indican respectively. Combinatorial use of cytokinins along with 2,4-D and NAA was less effective in the synthesis of indican.

Effect of additives/ amino acids on indican production

A slight increase in the indican content was noticed when additives/ amino acids were added to the medium. However L-TPN (100mg/l) enhanced maximum intracellular accumulation of indican (1182.1µg/gmFwt.) from stationary phase callus. Calluses subcultured to higher concentrations of Ads (100mg/l), CH (150mg/l) and L-glut (200mg/l) and lower concentrations of CW (50ml/l), PG (50mg/l), and PVP (25mg/l) induced maximum accumulation (Table. 3).

Discussion:-

A number of secondary metabolites in the higher plants are stored in cells as energy sources, plant hormones, toxic products for defense, detoxificated products, metabolic wastes etc. These metabolites accumulate in several intracellular compartments, viz. vacuole, cell wall, cystol, and other organelles (Rhodes, 1994). A large number of metabolites are stored in the form of glycosides (Luckner, 1990). The major secondary metabolite in the indigo plant is indican, a glucoside (Indoxyl β-D-glucoside), which serves as the starting material for indigo production.

In the present study, callus cultures were raised from different explants, viz. leaf, petiole, stem, cotyledons and root of *I. tinctoria* using different media, hormonal concentrations, additives or amino acids for the *in vitro* exploitation of indican. The optimum concentrations and combinations of these components for maximum accumulation of indican were standardized. Among different explants used for initiating callus cultures of *I. tinctoria*, maximum accumulation of indican was noticed from callus derived from leaf explants followed by cotyledons. Stem, petiole and root explants recorded only lower levels of indican. Among different plant parts analysed for indican content, Minami *et al*. (2000) reported maximum indican from the young leaf of *Polygonum tinctorium*. Since leaf tissues are more potent sites of indican production than the tissues of other parts, the callus tissue derived from leaf explants may have more or less similar capabilities to synthesiseindican. MS medium was proved to favour more indican production than WPM, which presumably is due to the higher levels of macronutrients available for indican production in the medium.

Incorporation of IAA to MS basal medium induced maximum indican production from callus cultures. Luckner (1972) traced the path of certain indole derivatives and found out that the IAA formed from different precursor compounds may be converted to respective glycosides or other indole compounds. The IAA supplemented in the callus cultures may act as an intermediate compound for the synthesis of indoxyl β-D-glucoside. Presence of NAA and IBA in the medium showed significant levels of indican production. 2, 4-D inhibited the production of indican considerably as reported by Hagendoorn *et al*. (1994) in the cell cultures of *Morinda citrifolia*. Among the aminopurine derivatives (BA, KIN and 2-ip), BA was found to be effective in the formation of indican. According to Xu *et al*. (1999), cytokinins induce differentiation of cells and influence secondary metabolite production. Lower levels of phytohormones help in the biomass production by triggering the primary metabolism and secondary metabolism starts at a later phase of growth. Rhodes *et al*. (1986) explained the increase in the rate of secondary metabolism by the exogenous supply of hormones. Higher levels of auxins or cytokinins inhibited the secondary metabolite production. Auxins when used along with lower concentrations of cytokinins, especially BA, were found to stimulate the biosynthesis of indican significantly.

The enrichment of the culture medium with additives (Ads, CW, PVP, PG or CH) or amino acids (L-glut or TPN) was highly beneficial for the intracellular accumulation of indican. L-tryptophan was the most effective organic supplement for the *in vitro* generation of indican. Since tryptophan is considered as one of the compound from

which the indole ring originates in several systems (Sundberg, 1970), tryptophan can enter the pathway of the biosynthesis of indican. Such an over accumulation of carboline alkaloids was observed in the feeding experiments using L- tryptophan in *Cinchona ledgeriana* (Harkes *et al*., 1986). Higher concentration of Ads was found to enhance the production of indican similar to the reports on stimulation of vasicine content in the *in vitro* cultures of *Adhatoda vasica* (Jose *et al*., 2000). The cytokinin activity of Ads favoured the differentiation process and thereby the biosynthesis of indican. Lower levels of CW enhanced the glucoside production due to the presence of cytokinins, which induce differentiation and secondary metabolite production similar to the reports in *Gardenia jasminoides* (Ueda *et al*., 1981).

Stationary phase callus accumulated higher quantities of indican than lag or exponential phases of all the cultures. During rapid cell division and growth, the utilization of intermediary metabolites in primary metabolism predominates and the metabolites are available for the synthesis of secondary metabolites only when the rate of cell division decreases (Kurz and Constabel, 1979). On the other hand the rate of cell growth directly regulates secondary metabolism by affecting the kinetic partitioning of precursors between primary and secondary metabolic pathways. If the primary pathways are blocked, the common precursors can be diverted to secondary pathways (Yeomman, 1987), thereby enhancing the secondary metabolite synthesis.

The protocol developed is a fruitful method for exploitation of indican from callus cultures of this valuable medicinal plant without affecting its natural population.

Table 1:-Effect of auxins and cytokinins on indican production from callus cultures of *I. tinctoria* during different phases of growth.

Hormones (mg/l)							Indican (µg/gm. F. wt.) Mean ± SE		
2,4-D	NAA	IAA	IBA	BA	KIN	2-ip	Lag Phase	Log phase	Stationary Phase
1	-	-	-	-	-	-	801.4±0.14	827.5±0.04	810.5±0.18
2	-	-	-	-	-	-	671.7±0.13	694.3±0.17	703.4±0.29
3	-	-	-	-	-	-	593.2±0.23	601.1±0.19	618.1±0.39
-	1	-	-	-	-	-	887.1±0.16	901.5±0.21	914.8±0.41
-	2	-	-	-	-	-	861.9±0.31	881.5±0.28	908.3±0.32
-	3	-	-	-	-	-	832.4±0.08	852.3±0.31	885.8±0.16
-	-	0.5	-	-	-	-	893.2±0.21	911.2±0.36	951.3±0.17
-	-	1.5	-	-	-	-	918.4±0.27	936.7±0.41	982.5±0.12
-	-	2.0	-	-	-	-	863.3±0.32	880.3±0.25	932.4±0.15
-	-	-	1	-	-	-	811.2±0.15	882.2±0.21	903.6±0.13
-	-	-	2	-	-	-	782.3±0.23	826.7±0.18	891.7±0.42
-	-	-	4	-	-	-	691.8±0.34	723.6±0.16	841.5±0.31
-	-	-	-	0.5	-	-	881.8±0.18	921.4±0.14	941.4±0.21
-	-	-	-	1	-	-	818.4±0.13	863.1±0.19	917.4±0.12
-	-	-	-	2	-	-	804.3±0.27	853.7±0.31	879.4±0.16
-	-	-	-	-	1	-	869.1±0.21	897.8±0.17	931.9±0.12
-	-	-	-	-	2	-	813.1±0.26	853.2±0.15	878.4±0.31
-	-	-	-	-	4	-	804.3±0.15	841.3±0.21	861.3±0.29
-	-	-	-	-	-	2	831.3±0.19	868.7±0.41	921.1±0.24
-	-	-	-	-	-	4	739.1±0.21	768.5±0.37	848.7±0.16
-	-	-	-	-	-	6	632±0.35	731.5±0.17	821.4±0.12

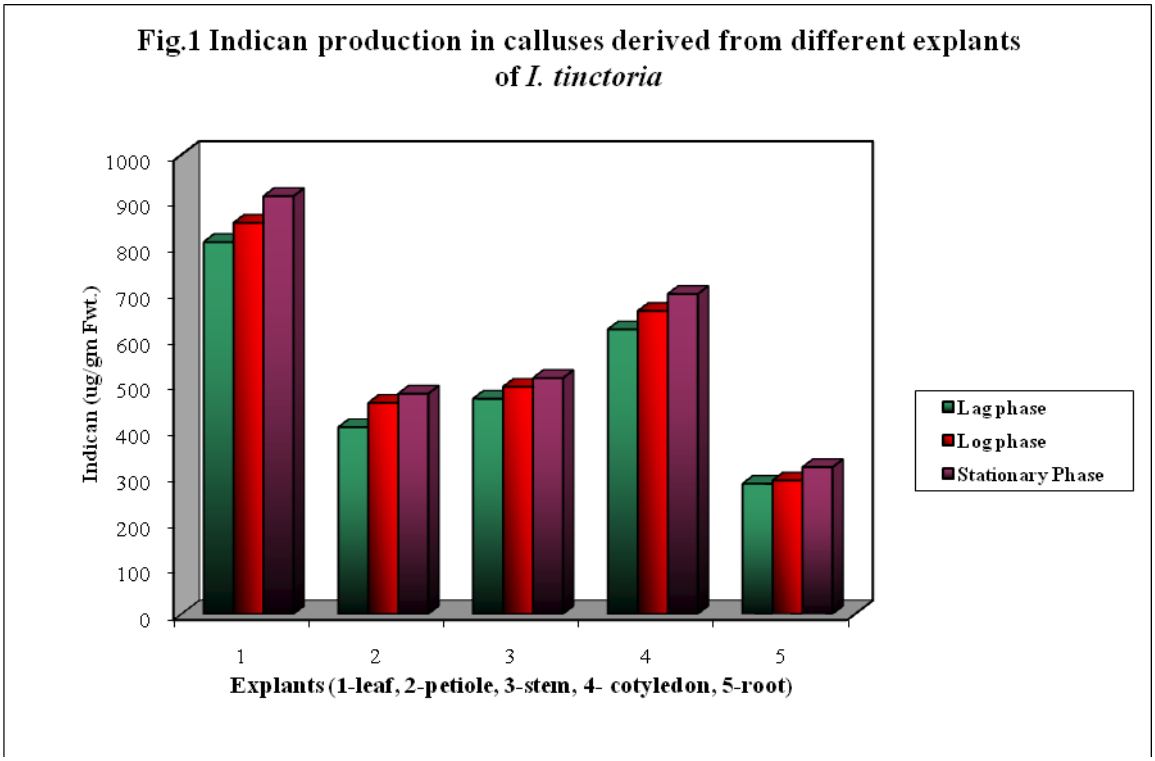
Table 2:-Combinatorial effect of auxins and cytokinins on indican production from callus cultures of *I. tinctoria* during different phases of growth.

Hormones (mg/l)							Indican (µg/gm. F. wt.) Mean ± SE		
2,4-D	NAA	IAA	IBA	BA	KIN	2-ip	Lag Phase	Log Phase	Stationary Phase
1	-	-	-	0.5	-	-	831.7±0.1	877.5±0.1	910.5±0.2
1	-	-	-	1	-	-	791.3±0.4	832.6±0.3	904.3±0.3
1	-	-	-	-	1	-	819.8±0.3	841.5±0.5	893.9±0.1
1	-	-	-	-	2	-	807.1±0.2	851.6±0.2	889.5±0.5
1	-	-	-	-	-	2	821.4±0.2	837.5±0.3	910.5±0.3
1	-	-	-	-	-	4	801.7±0.3	818.2±0.4	902.5±0.2
-	2	-	-	0.5	-	-	907.3±0.2	941.1±0.4	961±0.2
-	2	-	-	1	-	-	896.5±0.1	921.7±0.1	957.6±0.1
-	2	-	-	-	1	-	917±0.1	923.5±0.3	948.3±0.6
-	2	-	-	-	2	-	898.4±0.1	914.3±0.3	933±0.3
-	2	-	-	-	-	2	895±0.1	928.5±0.2	964.1±0.5
-	2	-	-	-	-	4	890.2±0.2	911.5±0.1	944.3±0.2
-	-	1.5	-	0.5	-	-	949.1±0.3	976.8±0.3	996.5±0.5
-	-	1.5	-	1	-	-	938.4±0.4	956.7±0.4	985.1±0.1
-	-	1.5	-	-	1	-	923.2±0.3	954.1±0.5	972.7±0.5
-	-	1.5	-	-	2	-	905.9±0.2	919.5±0.4	932.3±0.3
-	-	1.5	-	-	-	2	909.4±0.3	916.2±0.4	927.3±0.6
-	-	1.5	-	-	-	4	917.9±0.4	931.3±0.3	912.6±0.2
-	-	-	1	0.5	-	-	834.3±0.4	892.7±0.3	914.1±0.3
-	-	-	1	1	-	-	813.7±0.1	843.5±0.1	908.5±0.1
-	-	-	1		1	-	821.1±0.3	868.2±0.1	931.6±0.5
-	-	-	1	-	2	-	821.3±0.4	842.3±0.3	913.5±0.4
-	-	-	1	-	-	2	809.2±0.1	872±0.2	906.6±0.3
-	-	-	1	-	-	4	809.2±0.3	869.5±0.3	897.4±0.3

Table 3:-Data on indican production from callus cultures of *I. tinctoria* amended with additives/ amino acids**.**

Additives/ amino acids (mg/l)					(ml/l)		Indican (µg/gm. F. wt.) Mean ± SE		
Ads	PVP	PG	CH	L-glut	TPN	CW	Lag phase	Log phase	Stationary phase
25	-	-	-	-	-	-	932.3±0.23	981.5±0.32	998.6±0.21
75	-	-	-	-	-	-	989.3±0.43	1002.4±0.4	1065.2±0.5
100	-	-	-	-	-	-	992.1±0.31	1023 ±0.17	1087.243±0.
-	25	-	-	-	-	-	872.3±0.18	982.1±0.42	995.336±0.
-	75	-	-	-	-	-	831.5±0.14	883.9±0.23	978.4±410.
-	100	-	-	-	-	-	805.8±0.18	873.6±0.42	961.3±017.
-	-	25	-	-	-	-	871.2±0.21	904.7±0.51	987.7±0.18
-	-	75	-	-	-	-	786.4±0.32	891.3±0.13	976±0.14
-	-	100	-	-	-	-	879.3±0.41	926.1±0.19	959.6±0.15
-	-	-	50	-	-	-	913.2±0.18	948.9±0.25	993.7±0.13
-	-	-	150	-	-	-	947.3±0.15	986±0.36	1002.6±0.1
-	-	-	200	-	-	-	910.8±0.31	949.7±0.42	988.6±0.16
-	-	-	-	50	-	-	918.1±0.27	964.4±0.38	1003.8±0.1
-	-	-	-	100	-	-	961.5±0.36	993.8±0.29	1024.5±0.1
-	-	-	-	200	-	-	990.4±0.25	1003 ±0.21	1047.2±0.3
-	-	-	-	-	50	-	981.5±0.15	992.3±0.41	1082 ±0.12
-	-	-	-	-	100	-	1005.7±0.2	1102 ±0.3	1182.1±0.5
-	-	-	-	-	150		993.9±0.16	1093.7±0.1	1131.2±0.3

-	-	-	-	-	-	50	985. ±0.42	994±0.37	1072.3±0.4
-	-	-	-	-	-	100	979.4±0.28	1002.4±0.2	1037.6±0.3
-	-	-	-	-	-	200	984.3±0.24	1009.3±0.3	1012.5±0. 2



References:-

1. Epstein, E., Nabores, M. W. and Stowe, B. B. 1967. Origin of indigo of woad. *Nature* 216: 547-549.
2. Evans, P.K. 1990. *Indigofera*spp. In: Biotechnology in Agriculture and Forestry-10 Legumes and Oilseed Crops I, ed. Y. P. S. Bajaj. Springer-Verlag, London. pp. 645-656.
3. Hagendoorn, M. J. M. , van der Plas, L. H. W. Segers, G. J. 1994. Accumulation of anthraquinones in *Morindacitrifolia* cell suspensions. Plant Cell Tiss. Org. Cult. 38: 227-234.
4. Iyer, K. N. and Kolammal, M. 1992. Pharmacognosy of Ayurvedic drugs. Pharmacognosy unit, Ayurveda college, Thiruvananthapuram, Kerala. Pp: 61-71.
5. Harkes, PAA., De Jong, P. J., Wijnsma, R., Verpoorte, R. and Van Der Leer, T. 1986. Influence of production media on *Cinchona* cell cultures; Spontaneous formation of B- carbolines from L- tryptophan. *Plant Sci*. 47: 71.Hass and Hill, 1913
6. Jose**,** A. S. R., Geetha, M. and Nair, G. M. 2000. Production of vasicine alkaloid from *Adhatodavasica*Nees. through tissue culture. *Proc. Nat. Sem. Phytochemicals and nutrition.* 1: 73-79.
7. Khazhakyan, Kh. K., Egibian, KV. andDevedzhyan, AG. 1986. Effect of day lenghth on growth, development and productivity of indigo in soil less culture. *Doklady- AkademiiNauk-Arminskoi-SSR*. 2: 84-88.
8. Kurz, W. G. N. and Constabel, F. 1979. Plant cell suspension cultures and their biosynthetic potential. *Microb. Technol*. 1: 389-416.
9. Lloyd, G. and McCown, B. 1981. Commercially feasible micro propagation of mountain laurel, *Kalmia latifolia* by the use of shoot tip culture. *Proc. Int. Plant Prop. Soc*.30: 421-427.
10. Luckner, L, 1972. Secondary metabolism in plants and animals. Chapman and Hall, London. pp.312-316.
11. Luckner, M. 1990. Structure, biosysnthesis, and metabolism of secondary products in microorganisms, plants, and animals. In: Secondary metabolism in Microorganisms, Plant, and Animals. (ed)Luckner, M. pp. 100-444. 3rdedn. Spinger, Berlin.
12. Murashige, T. and Skoog, F. 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures; *Physiol Plant* 15: 473-497.

13. Paul Hass and Hill, T. G. 1913. An introduction to the chemistry of plant products. Longmans Green and Co, London. pp. 188-189.
14. Rhodes, M. J. C. 1994. Physiological role of secondary metabolites in plants: some progress, many outstanding problems. *J. Plant Physiol*. 118: 237-249.
15. Rhodes, M. J. C., Payne, J. and Robbins, R. J. 1986. Cell suspension cultures of *Cinchona ledgeriana* II. The effect of a range of auxins and cytokinins on production of quinoline alkaloids. *Planta Med*. 40: 226-229.
16. Sundberg, R. J. 1970. The chemistry of indoles. Academic Press. London. pp. 431-447.
17. Ueda, S., Kobayashi, K., Muramatsu, T., and Inouye, H. 1981. Studies on MonoterpeneGlucosides and related natural products. *PlantaMedica* 41: 186-191.
18. Xu, J. F., Ying, P. Q., Han, A. M. and Su, Z. G. 1999. Enhanced salidroside production in liquid cultivated compact callus aggregates of *Rhodiolasachalinensis*: manipulation of plant growth regulators and sucrose. *Plant Cell Tiss. Org. Cult*. 55: 53-58.
19. Yeoman, M.M. 1987. Bypassing the plant. *Annals of Botany* 60, Supplement **4**: 157-174.