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Study of Impact of Different Growth Regulators of Different Concentrations and Combinations Supplemented in MS Basal Medium on Hypocotyl and Internodal Explants of *Leonurus sibiricus*

KEYWORDS: Hypocotyl, internodal, callus, nodular, compact, *Leonurus sibiricus*

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ABSTRACT *Leonurus sibiricus is a wild species having immense medicinal value. It contains several alkaloids and diterpenes. Although they are extracted from the dried aerial parts but may also be extracted from the calli raised through tissue culture. In the present study, attempts has been made for high efficient callus induction from epicotyle and internodal explants an MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of 2,4-D alone and three different concentrations of BAP. MS basal medium supplemented with 1.0 mg /l 2,4-D + 2.0mg/l BAP was found most suitable because percentage of response was 94.4 in the hypocotyl explants while it was 91.6 in case of internodal explants of this species. Calli induced in the above cultures were sub-cultured in the same medium. The growth rate was excellent in both the calli in the above concentration that is MS +1.0 mg/l 2,4-D +2.0 mg/l BAP. Here, the calli were white, nodular and compact. MS +1.5 mg/l 2,4-D +2.0 mg/l BAP could induce callusing in 88.7 percent of the hypocotyl explants while it was 91.6 in case of internodal explants. The growth rate was best and the calli were white yellow and compact.*

INTRODUCTION:

Leonurus sibiricus is an annual herb, found growing on the marginal lands of the roadways on Government office. This plant belongs to the family Lamiaceae, and usually young plants can be noticed in late December, which starts flowering in February to March. During rainy season the plant become dry and seeds are dispersed near the main plant. It has dark green leaves and pink to red flowers in verticillaster inflorescence. Seeds are black and very small and produced in large numbers. The stem is rectangular and the dried stem is used as fuel by the local people.

This plant bears several medicinal properties and they are being used for the treatment of different diseases. The plant bears different alkaloids and diterpenes (Savona *et al.*,1982;Hon *et al.*,1991,1993;Li and Ca 2002). Because of the presence of above alkaloids and diterpenes, different parts of the plants are used for the treatment of hysteria and stomach diseases. Leaf extracts of the plant is used for the treatment of many disease related to females, i.e. disordered menstruation, excessive bleeding etc. Leaves are boiled in water and this is used for both which gives relief to muscularaches. Its juice is used to cure insect bites , roots are used in case of snake bites. The tribals used dried leaves as tea to cure obesity and haemorrhoids. It is also used to cure jaundice, chest ailments, bronchitis and epilepsy. It is also used to cure cough and cold, fever etc.

Above mentioned alkaloids and diterpenses are extracted from the plants harvested from its natural habitat. Because the content of alkaloid in wild plant is only 0.4% of its dried biomass , therefore, more and more plants are harvested for the required amount or to fulfil the demand both within and outside the country. This type of unplanned harvesting of the material is possing a threat on the survival of the species in its natural habitat. Therefore, it is essential to search an alternative so that the secondary metabolites should be supplied regularly to the pharmaceuticals companies at one hand while on the other hand the species be conserved in the natural conditions.

Plant tissue culture techniques appear to be a best alternative. The small part of the plant can be used as an explant and the callus raised from it can be sub-cultured. Then, the biomass can be used for the extraction of these secondary metabolites. There are different reports regarding induction of callus from different explants of medicinal plants. Sharma *et al.*, (1993) did tissue culture studies in *Gentiana kurroo* an indigenous threatened plant of medicinal value. Shudha and Seeni (1996) reported *in vitro* propagation of *Rauvolfia micrantha* a rare medicinal plant. Olivira *et al.*, (2001) induced callus in *Aspidosperma ramiflorum* and studied alkaloid production. Rashid *et al.*, (2012) reported *in vitro* propagation of *Orthosiphun stamineus* an important medicinal plant. Sharma *et al.* ,(2012) reported i*n vitro* conservation of *Bacopa monnieri* (L.,). Shahu and Khalkho (2012) reported induction of callus in *Boerhaavia diffusa*.

MATERIALS & METHODS:

Desired amount from the stock solutions for macronutrients, micronutrients and vitamins of MS basal medium were taken. Similarly, prescribed amounts of additive viz., sugar plant growth regulators and agar solution was added. The volume was made one litre with the help of glass distilled water. pH of the medium was adjusted to 5.8 with

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the help of NaOH or HCl. 20 ml of the medium was then poured into each of the test tubes. 60 ml of the medium was taken into the culture jar. Two plant growth regulators such as 2,4-D and BAP were used. Stock solutions of all growth regulators were prepared by weighing100 mg of each component that was dissolved in few drops of 1 M NaOH (Auxin) and in few drops of ethyl alcohol (Cytokinin). The volume was then made 100 ml by adding doubled distilled water. Above culture medium containing tubes were autoclaved at 15 lb pressure for 20 min. The mouth of the tubes were closed with non-absorbent cotton plug and wrapped with aluminium foil before autoclaving. The medium was stored in freeze for inoculation.

Preparation of the explant:
Hypocotyl was obtained after germinating the seeds in the laboratory. Seeds were collected from the plants growing in natural population. These seeds were surface sterilized and were germinated in petriplates , lined with pre-soaked filter paper. The internodal segment was obtained from the healthy branches of *Leonurus* which was collected from the field growing plants. Above materials were treated with 0.1% $HgCl_2$. Hypocotyl for one minute and internodal segment for 2 minutes, then in 75% alcohol, 10 sec for hypocotyl and 30-40 sec for internodal explants followed by three sequential rinses for 1 min in sterile distilled water. Innoculation was done in Laminar flow air chamber and were incubated in the the culture room having controlled temperature 26 ± 1^0 and light. The light period was given for 12 hours. Observations were made on alternate day. Cultures showing necrosis or infection were discarded.

Inoculation of callus:
2,4-D as auxins and BAP as cytokinins were added into the MS medium to observe their impacts on callus formation. The concentration of phytohormones varied from 0.5 to 2.5 mg/l each for auxin and 0.5 to 2.0 mg/l for cytokinin. Each experiment contained 20 replicates and the experiments were repeated three times. Data were collected from the five weeks of the culture and have been depicted in table 1.

Result and Discussion:
Hypocotyl explants taken from the germinated seeds after 18^{th} day of the germination and internodal segments prepared from the plant growing in nature, when inoculated in MS +2.0 mg/l 2,4 D alone, gave the highest percentage of response which was 74.2 in case internodal explants and 48.6 in the hypocotyl. It may be noted that there was increase in the induction rate along with the increase of the concentration of 2,4-D up to 2.0 mg/l. MS +2.0 mg /l 2,4-D +0.5 mg/l BAP, promoted the rate of induction of callusing. Here, the percentage of response was 51.4 in hypocotyl and 78.8 in the internodal explants. When the concentration of BAP was raised to 1.0 mg/l and 2.0mg/l the percentage of response in hypocotyl explants was 56.4 and 94.8 and in the internodal explants it was 56.8 and 91.6 respectively. However, here the highest percentage of response was observed in MS+ 1.0 mg/l 2,4-D+2.0 mg/l BAP.

Growth rate of calli, raised from both the explants, was also excellent. The colour and the texture of the calli were also noted. The calli were white and loose, white and compact, however, some calli were white nodular and compact also.

Induction of callus in different medicinal plants have been reported by Thomas and Maseena (2006) in *Cardiospermum halicacabum*, Shrivastava and Dubey (2007) in *Withenia somnifera*, Azimi *et al*; (2008) in *Catharanthus roseus*; Yang et al; 2008 in *Leonurus heterophyllus*; Dasilva *et al*; (2009) in *Withenia somnifera* (L.,); Kalidas *et al*., (2010) *Catharanthus roseus* (L.,). All these workers evaluated effect of auxins and cytokinin for induction of callus. Present findings are in agreement with the findings of the above authors.

Light brownish nodulated callus in *Tinospora cordifolia* was induced by Khalilsarai *et al*., (2011) in MS + 2.0 mg/l 2,4-D+1.0 mg/l BAP. Rishi(2011) induced callus in *Gloriosa superba* Linn., with the help of different concentrations and combinations of auxins and cytokinin.

Praveena *et al*., (2012) in *Coleus forskohlii*, Princy (2012) in *Tolyphora*; Sahu and Khalkho (2012) in *Boerhaavia diffusa*, Sharma *et al*., (2012) in *Bacopa monnieri*, Rashid *et al*., (2012) in *Orthosiphun staminea*, Sheeba *et al*., (2013) in *Physalis minima* induced callus in MS basal medium supplemented with 2,4-D + BAP at various concentrations and combinations. Thus our findings are in corroboration with the findings of the above.

Conclusion:
The presence of 2,4-D in all the experiments performed, was found to be effective in inducing callus proliferation. The result obtained here indicate that callus proliferation was affected by the concentration of 2,4-D in the media. Most of the calli were none embryonic.Nodular structure may be an indication of somatic embryogenesis. The change in colour in the older callus may be due to pigmentation. The green colour of the calli may be due to the chlorophyll development. It became gradually yellow due to loss in the pigment. These calli may be used for the *in vitro* production of secondary metabolites. Enhanced production of bioactive compounds in *in vitro* culture system is possible using cheap and nutritive elicitors. This can help in the conservation of the medicinal herbs in their natural habitat and they may not forced to become endangered, because in the conventional methods the bioactive compounds are extracted from the plants after collecting it from their natural habitat (Sandhya2014)

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Table:1, Impact of different growth regulators at different concentration in MS basal medium on hypocotyl and nodal explants of *L.sibricus*.

S.N.	Growthregulators (mg/l) 2,4-D	BAP	% response of explants		Growth rate	Texture & colour
			Hypocotyl	Internodal		
	0.5	-	18.6±1.16	21.2±1.28	+	WL
	1.0	-	34.8±1.52	38.4±1.82	++	WL
	1.5	-	42.2±2.24	46.6±2.46	+++	WC
	2.0	-	48.6±2.58	74.2±2.66	++++	WNC
	2.5	-	36.8±1.66	44.6±1.48	+++	WC
	0.5	0.5	22.4±1.26	26.4±1.28	+	WL
	1.0	0.5	37.6±1.32	44.6±1.36	++	WL
	1.5	0.5	46.2±1.38	51.5±1.42	+++	WC
	2.0	0.5	51.4±1.48	78.8±1.54	++++	WYC
	2.5	0.5	36.8±1.32	33.4±1.34	++	WL

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	0.5	1.0	28.6±1.34	31.8±1.32	+	WL
	1.0	1.0	41.8±1.38	48.2±1.44	++	WC
	1.5	1.0	53.4±1.44	56.8±1.48	+++	WY
	2.0	1.0	56.8±1.48	82.6±3.28	++++	WYL
	2.5	1.0	31.4±1.31	38.5±1.38	+	WL
	0.5	2.0	84.6±2.62	80.4±2.60	++++	WYL
	1.0	2.0	94.8±2.66	91.6±3.72	+++++	WNL
	1.5	2.0	88.7±2.63	82.2±2.62	++++	WYL
	2.0	2.0	81.8±2.64	78.7±2.42	++++	WYC
	2.5	2.0	78.8±2.42	71.6±2.31	++++	WYL

+ = Average WL= White loose

++ = good WC=White compact

+++ = better WY=White yellow

++++ = best WNC= White nodular compact

+++++ = excellent WYC=White yellow compact

Fig A : Curling of explant

Fig B : Initiation of callus formation

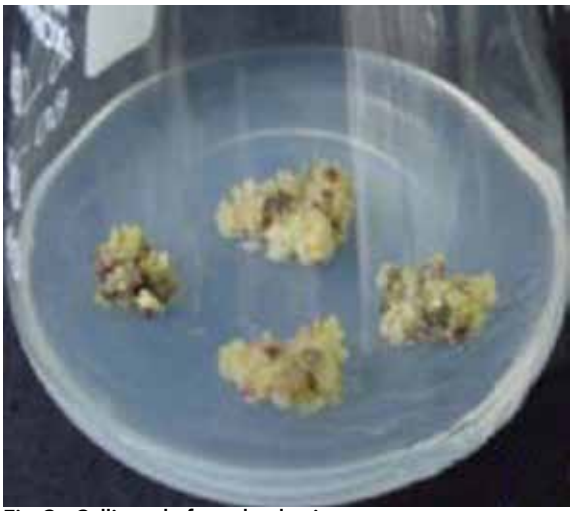

Fig C : Calli ready for subculturing

Fig D : Well developed callus

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