INDUCTION OF CALLUS FROM DIFFERENT EXPLANTS OF CATHARANTHUS ROSEUS AND ENHANCED PRODUCTION OF CALLUS BIOMASS IN THE PRESENCE OF AMINO ACID IN SUBCULTURE

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Abstract

Tissue culture studies for induction of callus from different explants of *Catharanthus roseus* an important medicinal plant were performed in the Plant Biotechnology Laboratory ,University Department of Botany, B R Ambedkar Bihar University, Muzaffarpur.For induction of callus leaf and hypocotyl explants from the above plant were inoculated in MS basal medium supplemented with 3% sucrose, different concentrations of 2,4-D alone and with two concentrations of Kinetin and gelled with 0.8% Agar .MS + 2 4-D at various concentration induced calli in both the explants but with different percentage of response. MS + 2.0 mg/l 2,4-D alone could induce calli in 56% of hypocotyl explants and 44% in the leaf explants respectively. However, MS + 2.0 mg/l 2,4-D+ 1.0 mg/l KN induced calli in 93% of hypocotyl explant and 87% in the leaf explants . Here time taken for callus was12.6 days and 13.4 days respectively. The growth rate of the calli was excellent and colour white and texture compact nodular. This was followed by the explants inoculation in MS + 1.5 mg/l 2,4-D+ 1.0 mg/l KN where the percentage of response for callusing in hypocotyl explants was 90% and in the leaf explants 82% . Here time taken for callusing in hypocotyl was 13.8, while for leaf explant 14.6 days respectively, the growth of the calli was better, colour white and texture compact in both the cases. It was further noted that in MS + 2.0 mg/l 2,4-D+ 0.5 mg/l KN the percentage response for callus in hypocotyl was 88% and in leaf explants 80% respectively. Time taken for callusing in hypocotyl was 14.5 days and for leaf explants 15.2 days respectively. Growth was best and colour green yellow and texture compact .Similarly in MS + 1.5 mg/l 2,4-D+ 0.5 mg/l KN the percentage response for callusing in hypocotyl was 84% while in leaf explants 78% respectively. Time taken for callusing in hypocotyl explant was 14.4 day and growth better. For leaf explant time taken for callus induction was 15.4 days, growth good and colour was white while texture compact. MS + 2.0 mg/l 2,4-D+ 1.0 mg/l KN containing medium was supplemented with five different concentrations of amino acid tryptophan. After 50 days of incubation the weight of callus in MS + 2.0 mg/l 2,4-D+ 1.0 mg/l KN+ 300 mg/l tryptophan was 11 6.2 gm when the callus induced from the hypocotyl was sub cultured. In the same medium after similar period of incubation the fresh weight of callus of leaf explant was 110 .8 gm. The next highest biomass in hypocotyl based callus was when 200 mg/l tryptophan was supplemented. Here For both the explants the fresh biomass obtained was 11 2.6 gm and 104.2 gm respectively. Along with the increase in concentration of Tryptophan from 50 mg/l - 300 mg/l there was constant increase in the biomass of both the calli. While in control the fresh biomass of the raised callus from hypocotyl was 56.6 gm/l while 52.4 for callus derived from leaf explants.

Keywords: **Catharanthus roseus, hypocotyl ,explants ,callus ,fresh Biomass ,tryptophan**

Introduction

Catharanthus roseus commonly called as 'Sadabahar' because of it flowers in all the seasons, belongs to the family Apocynaceae, is a perennial herbaceous shrub. It is cultivated as an ornamental plants but the species is found growing as wild in different kinds of climatic conditions. The plant is sensitive to cold because during winter the flower and leaf size are reduced in comparison to summer and monsoon. This plant is popular among the common people as an important medicinal plant .Local people used to take 2-3 raw leaves in empty stomach to control diabetes. In the traditional medicines the extracts of different parts are used as an antidiabetic , antidysentric , antihelminthic, antihemorrhagic and for wound healing agent (Pahwa, 2009) . With the help of modern techniques two important alkaloids vincristine and vinblastine have been isolated from the leaves and stem materials of *Catharanthus roseus* L. However, due to low rate of synthesis of the alkaloids *in vivo* 1000 kg fresh leaves and the stem materials of *Catharanthus roseus* are required to produce 1 kg and 20mg of alkaloids (Taha *et al.*, 2009).Similarly due to low production of vinblastine and vincristine the cost of production is very high. One promising and alternative method for vinblastine and vincristine production is from the callus raised from the explants taken from *Catharanthus roseus*. Further, Gaines(2004), reported two fold and five fold increase in Serpentine and Ajmalicin production when methyl jasmonate was introduced to cell suspension culture.

Tissue culture studies and production of callus from different explants of medicinal plants have been reported by different workers. Tissue culture study of medicinal plant has been done by different workers for the production of important secondary metabolites and for resistant against abiotic or biotic stresses. For example, Schlatmann *et al.*,(1995) studied the impact of different concentrations of glucose on the production of ajmalicine in cell culture of *Catharanthus roseus*. Zong *et al.*, (1998), studied effect of Nitrogen sources on the production of Ginseng saponin by cell culture of *Panax quinquefolium.* Chaudhary and Gupta (1999), reported production of biomass and alkaloid in *Catharanthus roseus* under *in vitro* conditions in the presence of abiotic stresses.Silva(2004), studied effect of carbon source on *in vitro* organogenesis of *Chrysanthemum* and production of secondary metabolites. Taha *et al.*, (2008), reported production of callus from different explants of *Catharanthus roseus* and detected different alkaloids *in vitro*. Hussan *et al.*,(2009) reported callus induction in *Abrus precatorius* and screened phytohormones *in vitro*. Amir *et al.,(*2014) reported quantification of rosamarinic acid in *Satureja* spp. Callus induction in *C. roseus* has been reported by Negi (2011).Reshi *et al.*,(2013) reported callus induction in *Orthosiphon aristatus*. Begum and Mathur(2014) reported tissue culture study of *C.roseus* and *Bacopa monnieri*. Meenakshi *et al.*,(2014) reported induction of callus in *Heliotropium indicum*. Ritika and Shukla (2014) reported callus induction in *Bacopa monnieri*.

Thus, we see that different workers have reported callus induction and production of secondary metabolites in the cell suspension or callus culture of this species. Keeping all the ideas in mind the present tissue culture study of *Catharanthus roseus* was done to induce callus and enhance production of callus biomass in the presence of Tryptophan.

Material and Methods

Plant Material:

The leaf explants were prepared from the healthy leaves of *Catharanthus roseus* collected from the plant growing in the campus of University Department of Botany, B.R.Ambedkar Bihar University. The collected healthy leaves were washed in running tap water for 1 hour. Leaf segments were treated with 0.1% $HgCl_2$ for 2 minutes. They were rinsed thrice in distilled water to remove even a trace of the chemical. Treated leaves were stored in mist clothes at low temperature.

The hypocotyl explants were prepared in the laboratory. Healthy seeds were collected from the ripe fruits. They were surface sterilized and rinsed with distilled water. These seeds were placed on the presterilized moist filter paper, lined in the petri plates. In the aseptic conditions these seeds were thus germinated, and the hypocotyles were used for explants. Murashige and Skoog basal medium was supplemented with five different concentrations

of 2,4-D alone and with two different concentration of Kinetin

MS basal medium was supplemented with 3% sucrose and gelled with 0.8% Agar powder. Five different concentrations of 2,4-D(0.5 – 2.5 mg/l) where supplemented in the above medium. The pH was adjusted to 5.8 before autoclaving. 20 ml medium was dispersed in the culture tube and tubes where plugged with suitable cotton plug. The plugs were wrapped with aluminium foil to avoid wetting during autoclaving. Autoclaving was done for 20 minutes at 15 lb pressure. All these cultures tubes were taken out and stored at room temperature. Other set of medium was prepared with different concentrations of 2,4-D + two concentrations of KN. Both the above culture media were inoculated with leaf and hypocotyl explants in the aseptic conditions of laminar air flow chamber.These cultures where incubated in the culture room maintained at 26 ± 1° C temperature, 66% relative humidity and 3,000 lux of light generated by white fluorescence tubes. The photo period was maintained at 16 hours light and 8 hours dark conditions. Observation was made on an alternate day and culture showing any contamination where discarded after autoclaving. Observation was made for percentage response for callusing, time required for callus induction, texture and colour of the callus and growth rate. All the experiments were done in triplicate and mean of the data was tabulated in table 1

Well grown calli were selected and 25 gram of the callus was subcultured in the MS liquid medium in the presence of five different concentrations of tryptophan. The fresh weight of the callus culture in the presence of different concentrations of tryptophan was taken after 50 days of incubation and difference from the initial weight was calculated. Mean of the data obtained was presented in table -2.

Result and Discussion

In the present work ,experiments were done to induce callus on leaf and hypocotyl explants of *Catharanthus roseus*. The mean of the data was presented in table 1. From the table it may be noted that maximum percentage of response for callusing in leaf and hypocotyl explants 44 and 56 was observed in MS + 2.0 mg/l 2,4-D alone. Here times taken for callusing were 16.2 and 14.4 Days respectively for leaf and hypocotyl explants. Lowest percentage of response 28 and 32 and maximum periods 18.4 and 16.6 days for callusing in both the explants were observed in MS + 0.5 mg/l 2,4-D medium. However when MS + 2.0 mg/l 2,4-D+ 1.0 mg/l KN was used for inoculation of the above explants, the highest percentage of response 87 and 93, minimum period for callusing 13.4 and 12.6 days were noted for leaf and Hypocotyl explants respectively.

Here the growth of callus was excellent in hypocotyl and best in the leaf explants, while the texture was compact to nodular and the colour white. Here minimum percentage of response 42 + 56, maximum periods for callusing 18.2 + 16.2 days where noted when the leaf and hypocotyl explants where inoculated in MS + 0.5 mg/l 2,4-D + 1.0 mg/l KN. Here the growth rate was average.

It may be noted from the table that MS basal medium with 3% sucrose was also supplemental four different concentrations of 2,4-D along with 0.5 mg/l KN. Here highest percentage of response for callusing in both the above explants 88 and 80, time taken for callus induction 14.2 + 15.2 Days respectively were found in hypocotyl and leaf explants. This was followed in MS + 1.5 mg/l 2,4-D + 0.5 mg/l KN which were 84 and 78 and times taken where 14 .4 and 15.4 days respectively for callus induction in hypocotyl and leaf explants. Here again lowest percentage of response 58 and 44, maximum periods for callus induction 16.4 and 18.2 days were taken for hypocotyl and leaf segment explants when inoculated in MS + 0.5 mg/l 2,4-D+ 0.5 mg/l KN. Here the growth was also average while the calIi had loose texture, white to white green colours. From the table 1 it was noted that both the hypocotyl and leaf explants when inoculated in MS + 5 different concentrations of 2,4-D alone or four different concentrations of 2,4-D and two concentrations 0.5 mg/l and 1.0 mg/l of KN, callusing was there in both the explants at all the concentrations of the growth regulators.

However there was discrepancy in the percentage of response, time taken for callusing, growth rate, colour and texture of the calli induced. Best results were obtained for both the explants when they were inoculated in MS + 2.0 mg/l 2,4-D+ 1.0 mg/l KN. Here percent of response was the highest, the time taken for callus induction was lowest and growth was

excellent. Tissue culture study of different medicinal plants have been done by different workers. Here either the callus was initiated or there were experiments for micropropagation. Tissue culture of different medicinal plants have been done for callus induction such as Sen *et al*.,(2012) in case of *Achyranthus aspera*,Upadhyay *et al*.,(2012) in *Sauropus androgynous*, Guruchandran *et al.,(*2013) in *Stevia rebaudiana*, Hussan *et al.,(*2009) in case of *Abrus*, Lim *et al.,(*2009) in case of *Ocimum*, Negi *et al.,(*2011) in case of C*atharanthus* , Khan *et al*.,(2011) in *Catharanthus*, Thirupathy *et al.*,(2014) in case of *Tephrosia hookeriana*, , for have reported that callus induction was more efficient in presence of MS + 2 4-d and KN at different concentrations. Therefore present findings are in agreement with the findings of the above workers.

In the present experiment the callus raised from the leaf and hypocotyl explants were subcultured in MS + 2.0 mg/l 2,4-D + 1.0 mg/l KN + (50 – 300) mg/l concentration of tryptophan. The initial weight of callus inoculated was 25gm and when they were cultured in different concentration of tryptophan, there was many fold increase in the fresh biomass of the calli in comparison to the control. Here higher biomass of the calli raised from hypocotyl was 116.2 gm/l and for the leaf callus 110 .8 gm/l on 50th days of incubation, when the callus was cultured in MS + 2.0 mg/l 2,4-D + 1.0 mg/l KN + 300 mg of tryptophan. This was followed by the biomass of hypocotyl callus which was 11 6.2 gm/l and 104.2 gram for leaf explant when the above medium was supplemented with 200 mg/l of tryptophan. In the control, in the same medium the biomass for hypocotyl was 56.6 gm/l and for leaf explant 52.4 gm/l after 50th days of incubation. It may be noted from the table that there was increase in biomass in comparison to the control even when the medium was supplemental with 50 mg/l of tryptophan along with other hormones. It may further be noted that along with the increasing concentration of tryptophan from 50 mg/l to 300 mg/l, there was gradual increase in the callus biomass that was from 88.4 gm/l to 116.2 gm/l for the callus raised from hypocotyl explant and from 85.6 gm/l to 110.8 gm/l for the calli raised from the leaf explants.

Elicitation of the calli through methyl jasmonate increased alkaloid production many folds in *Catharanthus roseus* cell suspension culture were reported by Gaines(2004).Similarly Choudhary and Gupta (1999) observed impact of dikigulac on biomass and alkaloid production in the *Catharanthus roseus* under in vitro conditions. Schlatmann *et al*., (1995), studied impact of glucose concentration on callus biomass and ajmalicine production in case of *Catharanthus roseus.* Renu *et al.,(*2011), reported *in vitro* micropropagation and callus induction among different explants of *Catharanthus roseus*.Ashutosh *et al.,(*2012), published increase amount of alkaloid in callus culture of *Catharanthus roseus*. Therefore, the enhancement in callus biomass after supplementation with tryptophan is in accordance with the findings of the above workers.

Table: 1 Different explants viz., the leaf and hypocotyl were inoculated in MS+ various concentration and compositions of the plant growth regulators

MS+Growth regulators (mg/l)		Explants	Percent response	Days required	Texture	Growth rate
2,4-D	0.5	Leaf	28	18.4±0.36	WL	+
		Hypocotyl	32	16.6±0.42	WL	+
	1.0	Leaf	31	18.4±0.36	WL	+
		Hypocotyl	38	16.6±0.42	WL	+
	1.5	Leaf	42	16.4±0.44	WC	++
		Hypocotyl	50	14.8±0.62	WC	++
	2.0	Leaf	44	16.2±0.44	WC	++
		Hypocotyl	56	14.4±0.38	WC	++
	2.5	Leaf	26	18.6±0.28	WL	+
		Hypocotyl	34	17.4±0.36	WL	+
2,4-D+KN	0.5+0.5	Leaf	44	18.2±0.28	WL	++
		Hypocotyl	58	16.4±0.38	WGL	++

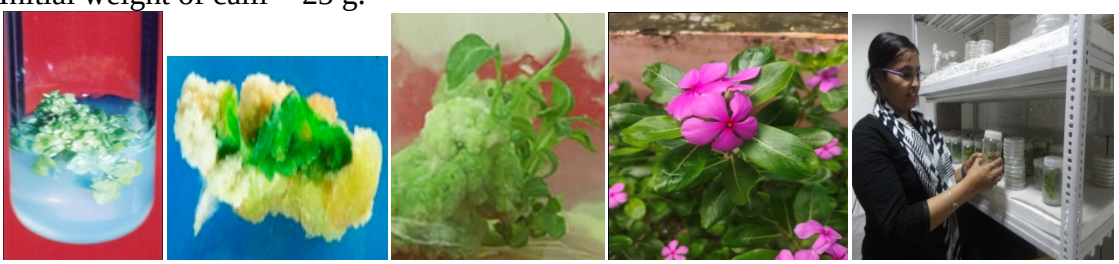
	1.0+0.5	Leaf Hypocotyl	48 66	17.8±0.46 16.2±0.26	WL WC	++ ++
	1.5+0.5	Leaf Hypocotyl	78 84	15.4±0.28 14.4±0.36	WC WYC	++ +++
	2.0+0.5	Leaf Hypocotyl	80 88	15.2±0.18 14.5±0.26	WL GYC	+++ ++++
	0.5+1.0	Leaf Hypocotyl	42 56	18.2±0.20 16.2±0.26	WL WL	++ ++
	1.0+1.0	Leaf Hypocotyl	46 60	18.2±0.28 16.2±0.26	WC WC	++ ++
	1.5+1.0	Leaf Hypocotyl	82 90	14.6±0.40 13.8±0.60	WC WC	+++ +++
	2.0+1.0	Leaf Hypocotyl	87 93	13.4±0.40 12.6±0.42	WC WCN	++++ +++++

+ = Average, ++ = good, +++ = better, ++++ = best , +++++ = excellent
WL= White Loose, WC= White Compact, WYC= White yellow compact, WCN= White compact nodular
GYC= Green yellow compact

Table- 2 MS liquid culture medium was prepared and supplemented with 1.5mg/l 2,4-D +different concentrations of tryptophan separately

Concentration of Tryptophan(mg/l)	Explant	Weight after 50 days/l	Difference
50	Leaf Hypocotyl	85.6 88.4	60.6 63.4
100	Leaf Hypocotyl	90.6 94.4	65.6 69.4
150	Leaf Hypocotyl	100.6 108.4	75.6 83.4
200	Leaf Hypocotyl	104.2 112.6	79.2 87.6
300	Leaf Hypocotyl	110.8 116.2	85.8 91.2
Control	Leaf Hypocotyl	52.4 56.6	27.4 31.6

Initial weight of calli = 25 g.



Conclusion

Based on the above findings, it maybe concluded that for better and rapid callus induction in *Catharanthus roseus* ,hypocotyl explant is the best explant in comparison to leaves and internodal explants. It may be due to the fact that the hypocotyl cells have greater potential of division in comparison to the cells of leaf and the internodal segments. The growth hormones as was observed in the present work

have synergistic impact on callogenesis in comparison to their use as alone. Therefore, 2,4-D, 2.0 mg/l + 1.0 mg/l KN have better synergistic impact on callogenesis in case of *Catharanthus roseus.*

Similarly addition of tryptophan 300 mg/l initiated maximum biomass of the calli cultured in it. Such biomass may be utilised for the extraction of the most important secondary metabolites of *Catharanthus roseus* i.e., vincristine and vinblastine. In this way at one hand *Catharanthus roseus* may be protected in its natural habitat and on the other hand there shall be constant production of the aforesaid secondary metabolites of immense medicinal value.

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