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PHYSICOCHEMICAL STUDIES OF DIFFERENT CALLI RAISED FROM LEAF EXPLANTS OF *CATHARANTHUS ROSEUS*

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Abstract

Cancer is the abnormal growth of our body cells that can lead to death. Chemotherapy is used for the treatment of cancer, but due to high death rate and serious side effects of this therapy, the search for alternative method for treatment is still in progress. *Catharanthus roseus*; a renowned medicinal plant contains many powerful antitumor drugs such as vindoline, vincristine, vinblastine, serpentine, ajmalicine etc. for the treatment of different types of cancer. Biotechnological approaches specially plant tissue culture could be used for production of bioactive plant metabolites. In the present study the calli were developed on MS medium supplemented with 2, 4-D, NAA and Kn alone or in combination by using leaf and stem as explants from *Catharanthus roseus* plant. Efficient callus induction and growth was found on MS medium supplemented with 2mg/l NAA cultured in dark. The callus so obtained was also transferred on MS medium supplemented with different salts concentration (25mM, 50nM, 75mM and 100mM) keeping NAA concentration constant. In general the callus sub-cultured on MS medium supplemented with 2mg/l NAA was best in term of total crude alkaloid content when compared to different calli and plants parts.

Key words: Alkaloids, Plant growth regulator, Callus induction, Plant metabolites

Introduction

Plants have been used for treating various human and animal diseases since time immemorial. They maintain health and vitality of individuals, and also cure diseases including cancer without causing toxicity. According to the report of WHO in the late 90's, a very high percentage of the world's population rely on plant based therapies to cover the needs of the primary health care (Dikshit *et al.*, 2004). More than 50% of all modern drugs in clinical use are of natural product origin (Roja *et al.*, 2000 and Haung *et al.*, 1992). A recent survey shows that more than 60% of cancer patients use vitamins or herbs as therapy (Sivalokanathan *et al.,* 2005). Many plants like *Colchicum autumnale, Taxus cuspidate, Vitex rotundifolia, Panax gingseng* etc. possess antitumor property. However, it is difficult to obtain sufficient amount of drugs due to reduction in plant availability. Destructive and non-sustainable collective methods coupled with low regeneration and habitat destruction has also posed serious threat to survival of wild medicinal plants. By these relentless and indiscriminate practices one out of ten plants is either already extinct or its extinction is imminent (Nair *et al.,* 1991).

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Abbreviations: MS (Murashige and Skoog), 2, 4-D (2, 4-dichlorophenoxy acetic acid), NAA (Naphthalene Acetic Acid), Kn (Kinetin), OC (Organic carbon)

Catharanthus roseus (family- Apocynaceae) is one of the important medicinal plants and is the only source for the powerful antitumor drugs vinblastine and vincristine. Some other pharmaceutical compounds from this plant, *e.g.* ajmalicine and serpentine are also of economical important. However, the antitumor alkaloids in this plant are produced in trace amounts (0.0003% dry weight). The high prices of such anticancer products have led to a widespread research interest over the past 25 years in the development of alternative source for the production of these compounds (Verpoorte *et al.,* 1991). Researchers are also focusing their attention to enhance the yield of alkaloids by various ways (chemically, enzymatically, synthetically or by cell culture method).

Biotechnological approaches, specifically, plant tissue cultures could be used for production of bioactive plant

metabolites (Rao and Ravishankar, 2002). Research in the field of plant tissue culture has made possible the production of many pharmaceutical substances. Advances in the area of cell culture for the production of medicinal compounds has resulted in production of a wide variety of pharmaceuticals as alkaloids, terpenoids, steroids, saponins, phenolics, and flavanoids (secondary metabolites) (Vanisree *et al.,* 2004). Moreover, maximum production of these secondary metabolites through *in vitro* culture requires knowledge of many different factors such as- biochemical pathway, relationship between cell differentiation and product formation, nutrient formulation, hormone and other environmental factors like stress (Jaleel *et al.*, 2007e), pH, light and temperature. The present study was carried in order to find out the suitable combinations of hormones for induction as well as growth of callus by using leaf and stem as explants from *Catharanthus roseus* and also to investigate the effect of salts on biochemical parameters including the enhancement of total crude alkaloid contents, if any.

Materials and Methods

The present study was carried on a well-known medicinal plant- *Catharanthus roseus* Linn. G. Donn (Apocynaceae). The plant was collected from herbal garden of Shobhit University Campus, Modipuram, Meerut. It is erect or decumbent, deciduous under shrub up to 1.0 m tall, usually with white latex and an unpleasant smell; roots up to 70 cm long; stems narrowly winged, green or red, shortly hairy, often woody at base. Leaves are opposite, decussate, simple, entire, herbaceous and hairy to glabrous on both sides. Leaf blade is elliptical to obovate or narrowly obovate, glossy green above and pale green below.

Preparation of culture media

Murashige and Skoog's (MS) medium (1962) was used as basal medium throughout present tissue culture studies. The medium was supplemented with 3%w/v sucrose and 100mg/l meso-inositol. The stock solutions of growth regulators (2, 4–D, NAA and kinetin) were prepared in 20 ml double distilled water. The auxins were dissolved first in few drops of absolute alcohol; made final volumes 20 ml by addition of double distilled water and stored at low temperature. For the preparation of semi-solid medium 0.8% (w/v) agar was added as gelling agent into the medium. The pH of all media was adjusted to 5.6-5.8 by using 0.1N HCI or 0.1N NaOH before dispensing a known volume of medium into culture vessels. The amount of medium dispensed into the various culture vessels was 20 ml/tube (15 and 25ml), 30ml/100ml flask for cultures. The culture vessels were plugged with non-absorbent cotton and wrapped with paper or aluminum foil. The prepared media was carefully sterilized at 121°C temperature and 1.05 kg/cm^2 (15psi) pressure for 20 minutes in a vertical autoclave.

Surface sterilization of explants

Leaves and stem were used as a source of explants. Leaves were first disinfected with the help of 70% alcohol, rinse in Tween-20 and washed with distilled water. Afterwards, they were treated with 0.1% $HgCl_2$ for 3-5 minutes followed by rinsing 3-4 times with sterile double distilled water. The leaves were excised into small pieces (1.0 mm approx.) and inoculated on agar medium containing growth regulators for establishing callus culture.

Inoculation

The working table of laminar airflow chamber was cleaned with 70% alcohol. Before carrying out operations the cabinet as well as media vessels, sterilized petri plates, scalpel, forceps, inoculation loop, spatula etc. were irradiated in U.V. light for 30 minutes. These implements were sterilized by flaming with 90% alcohol inside laminar airflow chamber before use, while not in use these were kept immersed in alcohol. All inoculations were done in laminar airflow chamber.

Incubation of cultures

The cultures were incubated in culture room and provided with light of 40 μmols.m^{-2}s^{-1} intensity with a photoperiod of 16/8 h light/dark cycles at 25±2°C temperature maintained by automated photoperiod controlled device and air conditioner or room heater.

For culture initiation, MS medium supplemented with different amount of growth regulators (2, 4–D, NAA and kinetin) were used. The callus induced from explants was maintained on same medium for minimum of 2-3 passages and used for further studies. The undifferentiated stock of calli was regularly transferred after every 3-4 week in their exponential phase of growth on the fresh media.

Physicochemical analysis: The obtained calli were analyzed for various parameters such as-

- Moisture percentage = $\frac{Fresh\ weight - Dry\ weight \times 100}{Fresh\ weight}$
- Growth Index = $\frac{Weight \times 100}{Volume}$

Biochemical analysis: The biochemical analysis was performed as per the following methods-

- Estimation of total nitrogen (Snell and Snell, 1967)
- Determination of total proteins (Bradford, 1976)

Table 1: *In vitro* morphological response of *Catharanthus roseus* leaf explants on different media.

S.N.	MS Medium + 3% sucrose + GR (mg/l)			Day of Response	Condition of culture	Growth of culture	Description of callus	
	2,4-D	Kn	NAA				Colour	Texture
1	2	0	0	21	Dark	+++	Off white	Compact
					Light	+	Off white	Compact
2	4	0	0	21	Dark	++	Off white	Compact
					Light	+	brown	Compact
3	1	0.25	0	40	Dark	+	Only swelling and curling observed	
					Light	+		
4	0.5	1	0	21	Dark	No response		
5	1.5	0.5	0	21	Dark	+	Greenish	Compact
6	0.45	0.25	0	36	Dark	+	Only swelling and curling observed	
7	3	1	0	21	Dark	+	Off white	Compact
					Light	+	Off white	Compact
8	0	0	0.5	21	Dark	No response		
9	0	0	1	30	Light	+	Off white	frabile
10	0	2	2	90	Dark	+	Only swelling and curling observed	
11	0	0.5	0.25	30	Dark	No response		
12	0	0	2	21	Dark	++++	white	frabile
					Light	++	white	frabile
13	0	0	4	60	Dark	+	Only swelling and curling observed	
14	0	0	3	21	Dark	No response		
15	0	0	5	60	Dark	+	Only swelling and curling observed	
16	0.9	0.5	0	21	Dark	+	Only swelling and curling observed	
					Light	+		
17	1	2	0	21	Dark	No response		
18	0	1	0.5	21	Light	No response		
19	0.5	0.5	0	40	Dark	+	Only swelling and curling observed	
					Light	++	Off white	Compact

+ Poor growth, ++ Normal growth, +++ Good growth, ++++ Very good growth

Table 2: Amount of protein, nitrogen, %OC, total phenolics and total crude alkaloids in different plant parts of *Catharanthus roseus* plant.

Plant Part	Protein (mg/gfwt)	Nitrogen (mg/gdwt)	% Organic carbon	Phenolics meq. Cinnamic acid/gfwt	Total crude alkaloids (gdwt)
Leaf	12.267±3.156	0.426±0.005	0.396±0.004	23.73±6.86	0.0317
Stem	11.249±3.481	0.425±0.020	0.336±0.002	52.69±17.74	0.0849
Root	1.697±0.1955	0.481±0.002	0.37±0.004	18.08±1.17	0.0096

- Estimation of total phenolic content (Bray and Thorpe, 1954)
- Estimation of organic carbon (Datta *et al.*, 1962)
- Method of extraction of tropane alkaloids (Gupta *et al.*, 1996).

Result and Discussion

The leaf and stem from *Catharanthus roseus* plant were used as explants to induce the callus, when they were inoculated on MS medium supplemented with different growth regulators (NAA, 2, 4-D and Kn). The growth regulators which were tested for callus induction were used alone or in combination. After inoculation of explants for callus induction the culture vessel are cultured in dark and light in order to find out the response of explants under such conditions (effects of light on callus induction and growth). In general, good callus induction and growth occurred in dark in comparison to light in all tested media. Similar results were obtained by Park *et al.,* (2003), when they used modified Gamborg's medium supplemented with 2 mg/l 2, 4-D. On both, MS medium supplemented with 2mg/l NAA and MS medium supplemented with 2mg/l 2, 4-D callus induction took place after 21 days. Moreover, as compared to MS medium supplemented with 4mg/l2, 4-D, callus cultured on MS medium supplemented with 2mg/l2, 4-D showed good growth. The callus so obtained was off-white and compact (Plate-1). Among various concentration of NAA tested good callus induction was observed on MS medium

Table 3: Physico-chemical attributes of different calli raised and subcultured on MS medium supplemented with growth regulators and with different salt concentration.

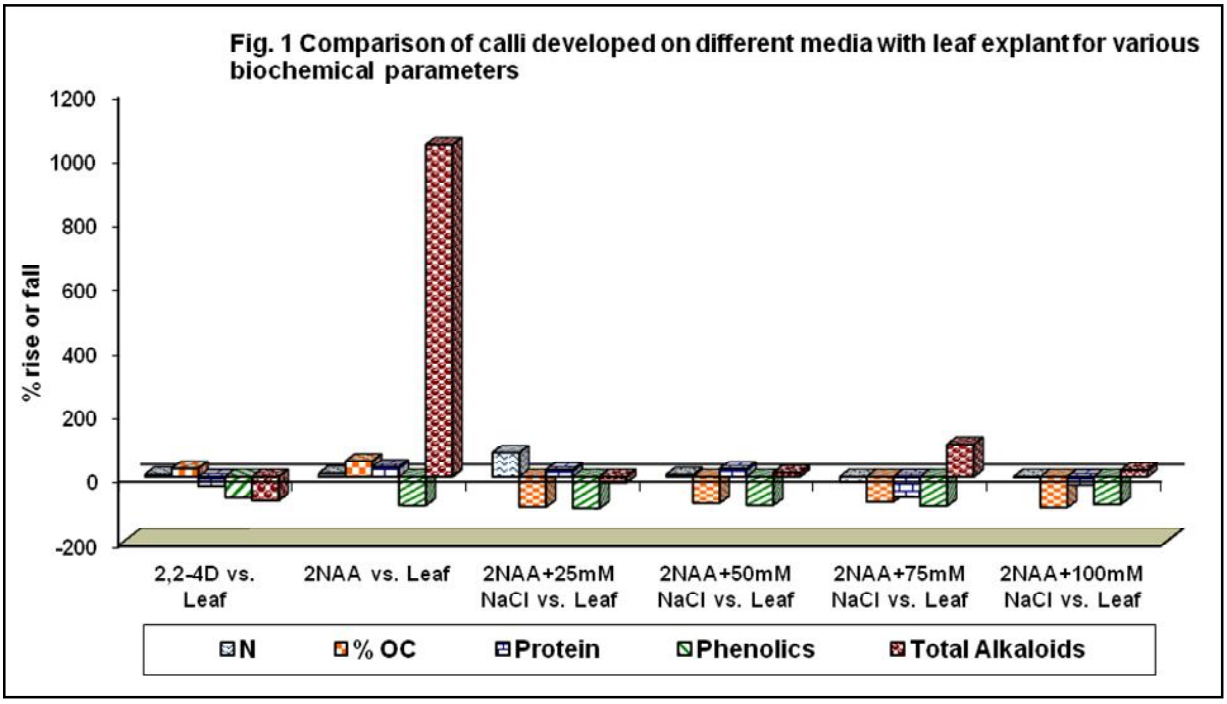
Media tested MS+3% sucrose+	Callus transferred on MS+3% sucrose+	Physico-chemical parameters						
		Protein (mg/gfwt)	Nitrogen (mg/gdwt)	% Organic carbon	Phenolics meq. Cinnamic acid/gfwt	Total crude alkaloids (gdwt)	Moisture %	Growth index
2-2,4-D	2-2,4-D	8.7±0.100	0.453±0.018	0.495±0.014	8.65±0.751	0.0086	56.99	105.26
2NAA	2NAA	16±0.926	0.467±0.055	0.588±0.007	2.71±0.249	0.3605	54.9	105.26
2NAA	2NAA+25mMNaCl	14.82±0.359	0.747±0.048	0.026±0.001	0.569±0.134	0.0258	38.57	100
2NAA	2NAA+50 mMNaCl	15.23±0.572	0.451±0.060	0.077±0.014	2.844±0.55	0.0356	39.4	96.5
2NAA	2NAA+75 mMNaCl	4.65±0.534	0.353±0.082	0.091±0.004	2.351±0.723	0.064	40.5	95.7
2NAA	2NAA+100 mMNaCl	9.12±0.945	0.418±0.079	0.02±0.001	3.632±0.411	0.038	42.7	85

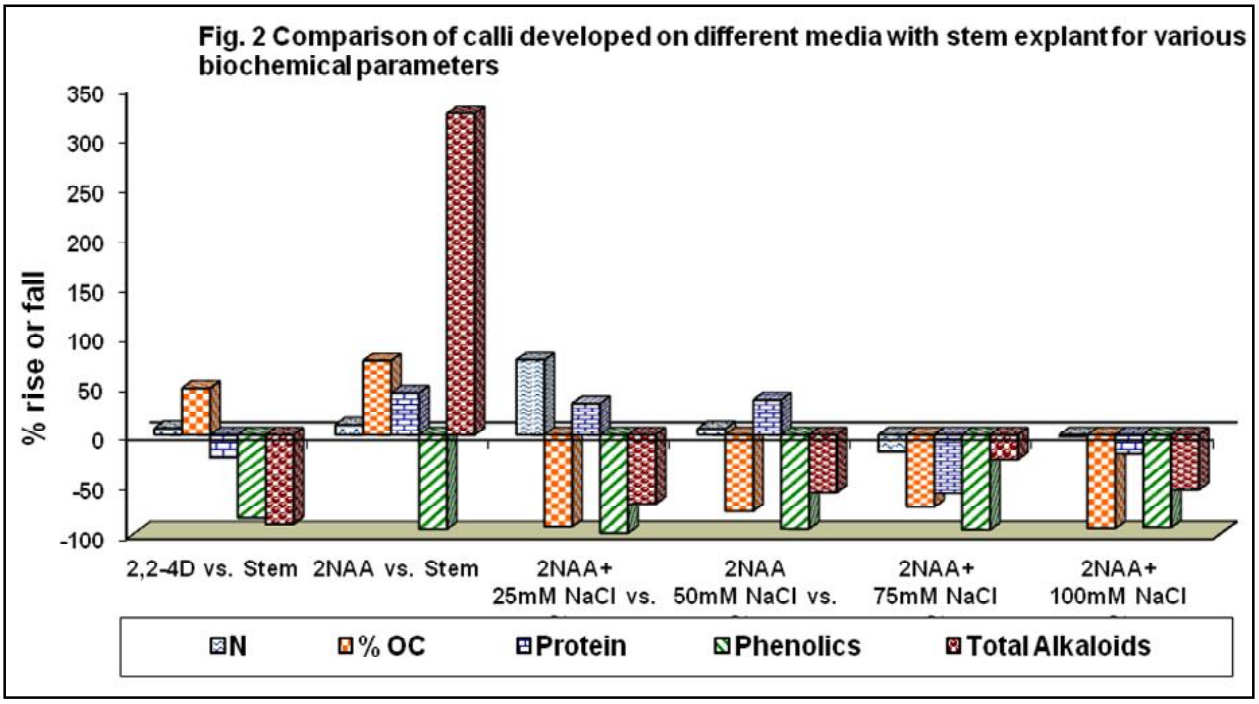
supplemented with 2mg/l NAA in comparison to the medium supplemented with 0.5mg/l, 1mg/l NAA (low NAA) and MS medium supplemented with 3mg/l, 4mg/l, 5mg/l (high NAA). The callus was white and frabile as compared to the medium supplemented with 2mg/l 2, 4-D. Therefore, the medium supplemented with 2mg/l NAA was found to be best medium and used as control callus (Plate-2). The callus obtained on MS medium supplemented with 2mg/l NAA was also subculture on media supplemented with various salt concentration keeping the NAA concentration constant (2mg/l NAA). MS medium supplemented with 2mg/l 2, 4-D in combination with Kn could not show promising results, although swelling in inoculated explants was observed. Likewise, NAA in combination with Kn was not found good (Table 1). Although, Saifullah and Khan (2011) reported the callus induction from young shoot tip of *Catharanthus roseus* by the use of MS medium supplemented with 1.5 mg/l 2, 4-D and 0.5 mg/l Kn incubated in complete dark.

Biochemical status of different plant parts and calli of *Catharanthus roseus*:

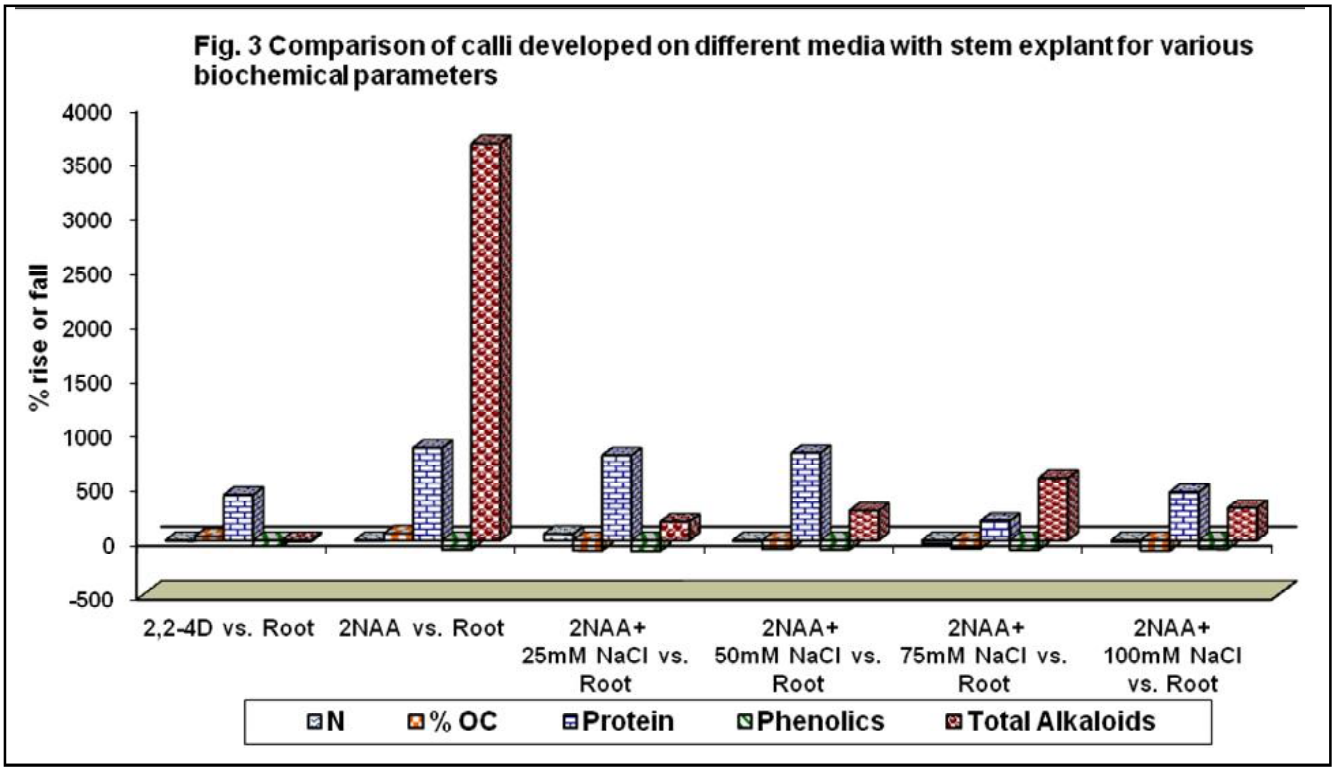
The biochemical estimation of different parts such as leaf, stem and root of *Catharanthus roseus* plant has been carried out. The results indicate that maximum % OC and protein were found in leaf. Phenolics and total alkaloid contents were found maximum in stem and minimum in roots. In addition, maximum nitrogen and minimum protein were found in roots (Table 2).

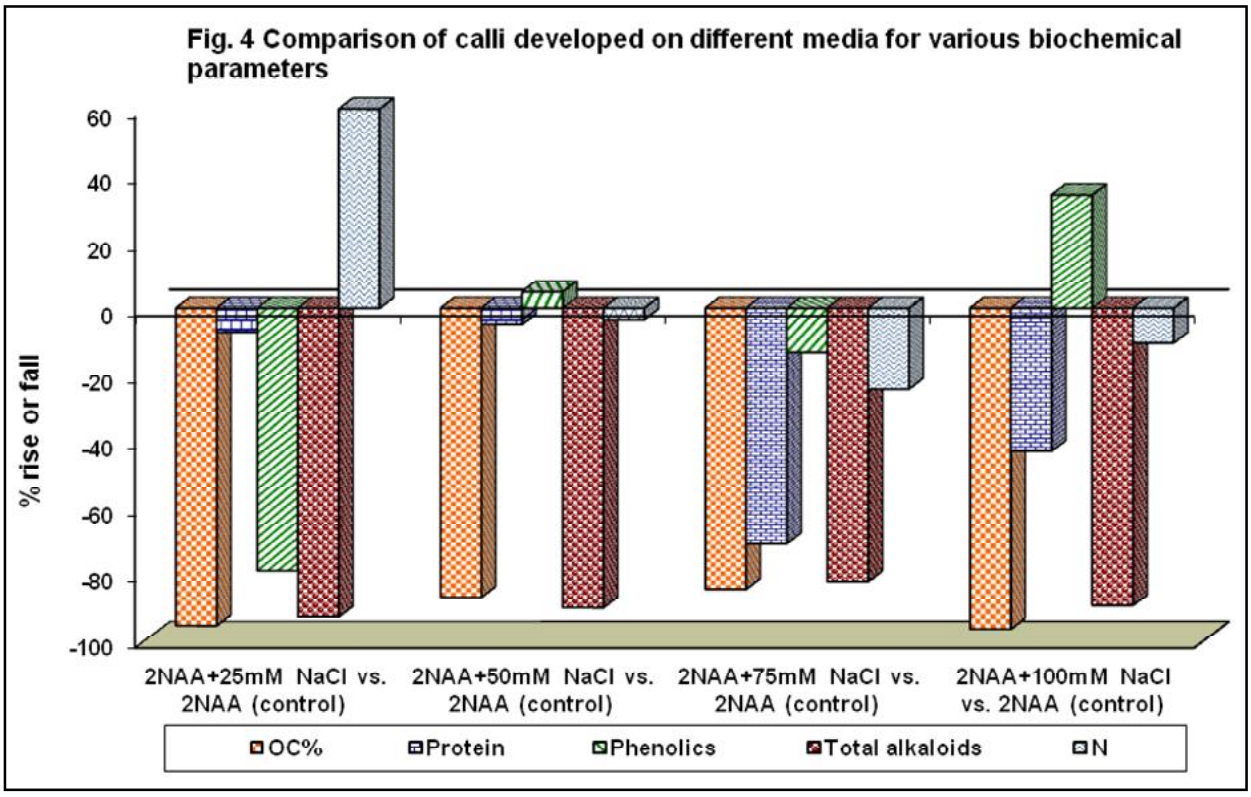
The callus raised on MS medium supplemented with 2mg/l NAA (control) was transferred on MS medium supplemented with 2mg/l NAA and different salt concentration (25mM, 50mM, 75mM and 100mM NaCl) in order to find out the effect of salt concentration on various physicochemical parameters such as growth





index, moisture %, nitrogen, %OC, protein, phenolics and total crude alkaloids. Growth index clearly depicts a reduction in the growth of calli with supplementaion of salts in the medium. Similarly, reduction in the leaf growth of *Catharanthus* plant was observed due to toxic effect of NaCl (Jaleel *et al*., 2008). %OC, protein and total crude alkaloid contents were found maximum in the callus treated as control i.e. raised on MS medium supplemented with 2mg/l NAA. Minimum nitrogen and proteins were found in the callus transferred on MS medium supplemented with 2mg/l NAA and 75mM NaCl. The callus raised and subcultured on MS medium supplemented with 2mg/l 2, 4-D exhibited maximum phenolics whereas minimum total crude alkaloids which was similar to Morris (1986a) observations. Liman *et al*., (1998) also found reduced activity of peroxidase with adition of 2, 4-D in the culture medium. Since, peroxidase perform a singnificant role in alkaloid production its activity might





get reduced in presence of 2, 4-D in the present study, resulting in less alkaloid production.

Interestingly, the total crude alakloid contents were found maximum in callus raised and subcultured on MS medium supplemented with 2mg/l NAA (control) as compared to the calli subcultured on MS medium supplemented with 2mg/l NAA and different salt concentrations. Among, the calli subcultured on different salt concentration the maximum total alkaloid contents were found on MS medium supplemented with 2mg/l NAA and 75mM NaCl (Table 3).

Comparision of callus with different plant parts

The production of secondary metabolites in the callus culture is controlled by factors such as plant material, medium components, pH and temperature. Plant growth regulators like auxins and cytokinins have shown the remarkable affects on growth, differentiation and metabolism of cultured cells (Zenk *et al*., 1977). The role of plant growth regulators in alkaloid production in *Catharanthus roseus* has been studied and it was found that the response varies with genetic make up of used explants, types and quality of phytohormones (Ganpati *et al.*, 1990 and Smith *et al.*, 1987a). In present investigation, amongst various combinations of growth regulators MS medium supplemented with 2mg/l NAA was found to be the best in terms of callus induction and growth. Although, Kalidass *et al.,* (2010) reported more callus growth on MS medium supplemented with 2mg/l 2, 4-D as compared to NAA. The callus raised on MS medium suplemented with 2mg/l NAA was subcultured at regular intervals of 45 days. The callus was treated as control and transferred to MS medium suplemented with 2mg/l NAA and different salt concentrations (25mM, 50mM, 75mM and 100mM NaCl). The callus growth on various medium supplemented with salts was recorded time to time. Such calli were harvested harvested after 15 days and analysed.

The callus on MS medium suplemented with 2mg/l NAA, MS medium suplemented with 2mg/l 2, 4-D and those transferred on different salt concentrations were compared with different plant parts such as leaf, stem and roots. As compared to leaf maximum rise (75.35%) in nitrogen was found in callus subcultured on MS medium suplemented with 2mg/l NAA and 25mM NaCl, whereas with high salt concentration it declined. The callus grown on MS medium suplemented with 2mg/l NAA also exhibited rise in nitrogen when compared to leaf. Moreover, as compared to leaf, %OC was increased by 48.48% and 25% in the calli raised on MS medium suplemented with 2mg/l NAA and MS medium suplemented with 2mg/l 2, 4-D, respectively. In addition, it declined in all the calli subcultured on various salt concentrations. In the callus subcultured on MS medium suplemented with 2mg/l NAA and 100mM NaCl maximum decline (-94.94%) was found as compared to leaf. Maximum rise in protein and total crude alkaloids (30.5% and 1037.20, respectively) was recorded in the

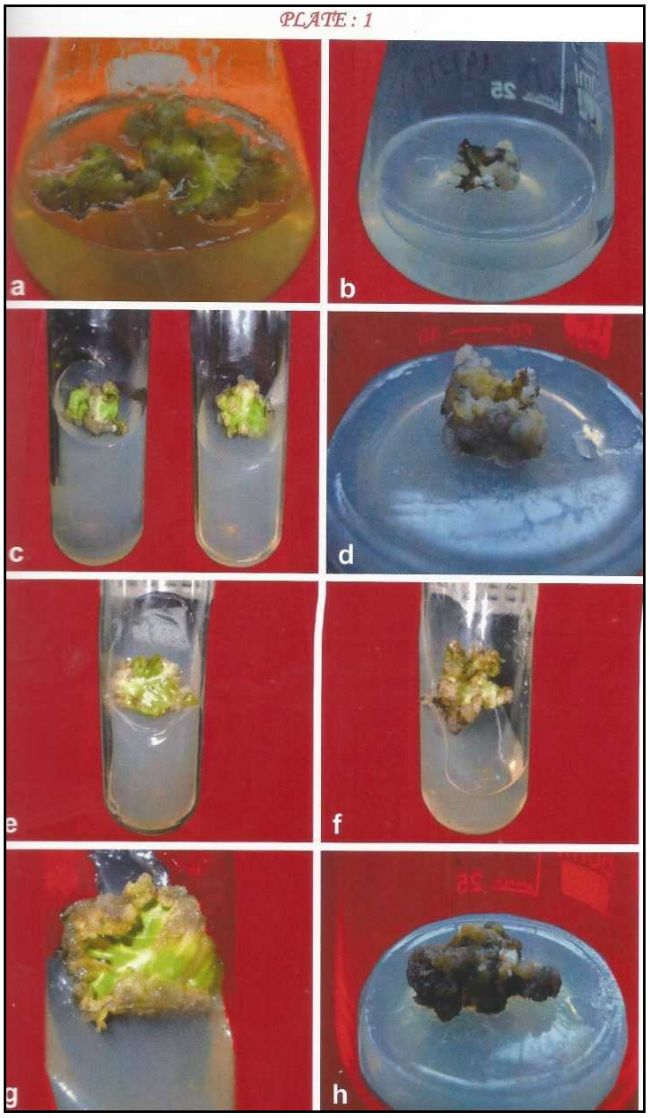


Plate-1: Callus raised on MS medium supplemented with different concentration of 2,4-D by using leaf explants. a) Callus induction from leaf explants on MS+2mg/l 2,4-D in light. b) Callus grown on MS+2mg/l 2,4-D in light after 30 days. c) Callus induction from leaf explants on MS+2mg/l 2,4-D in dark. d) Callus grown on MS+2mg/l 2,4-D in dark after 21 days. e) Callus induction from leaf explants on MS+4mg/l 2,4-D in light. f) callus grown on MS+4mg/l 2,4-D in light 21 days. g) Callus induction from leaf explants on MS+4mg/l 2,4-D in dark. h) Callus grown on MS+4mg/l 2,4-D in dark after 21 days.

callus raised on MS medium suplemented with 2mg/l NAA. In the callus subcultured on MS medium suplemented with 2mg/l NAA and high salt concentration increase in total crude alkaloids was recorded as compared to leaf explant. In general, the phenolics declined in all the calli as compared to leaf (Fig.1).

As compared to the stem, nitrogen was increased in all the calli except the callus subcultured on MS medium

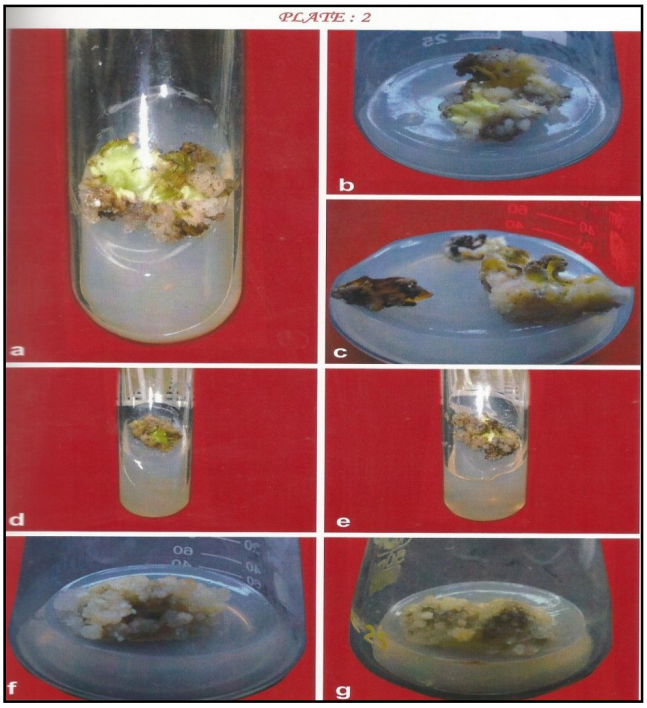


Plate-2: Callus raised on MS medium supplemented with different concentration of NAA, using leaf explants. a) Callus induction from leaf explants on MS+2mg/l NAA in light. b) Callus grown on MS+2mg/l NAA in light after 45 days. c) Callus induction from leaf explants on MS+2mg/NAA in dark. f) & g) Callus grown on MS+2mg/l NAA in dark after 30 days.

suplemented with 2mg/l NAA and 75 mM NaCl, where it declined significantly (-16.94%). In addition, maximum rise in nitrogen (75.76%) was recorded in the callus subcultured on MS medium suplemented with 2mg/l NAA and 25mM NaCl. Maximum rise in %OC, proteins and total alkaloids (75%, 42.34% and 324.61%, respectively) was recorded in the callus raised on MS medium suplemented with 2mg/l NAA as compared to stem. Interstingly, decline in total alkaloids was found in all the calli subcultured on various salt concentrations, being maximum -69.61% at low salt concentration as compared to stem. The callus subcultured on high salt concentration exhibited decline in proteins (Fig.2).

As compared to roots, nitrogen was declined in all the calli subcultured on various salt concentration being maximum (-26.16%) in the callus subcultured on MS medium suplemented with 2mg/l NAA and 75mM NaCl. Maximum rise in %OC, proteins and total alkaloids (58.91%, 846.74% and 3655.2%, respectively) was found in MS medium suplemented with 2mg/l NAA raised callus as compared to roots. In addition rise in proteins was also recorded in all the calli subcultured on MS medium suplemented with 2mg/l NAA and various salt concenrations. On the other hand, decline in phenolics

was found in all the calli subcultured on different media being maximum (-96.9%) in the callus subcultured on MS medium suplemented with 2mg/l NAA and 25mM NaCl as compared to roots. Total alkaloids were also increased in all the calli subcultured on MS medium suplemented with 2mg/l NAA and various salt concentrations (Fig.3).

The calli subcultured subcultured on MS medium suplemented with 2mg/l NAA and various salt concentrations were also compared to the control callus. In general, as compared to control callus decline in %OC, proteins and total crude alkaloids and increase in phenolics were recorded in all the calli. Reduction in nitrogen under NaCl treatment was also recorded by Rother *et al.,* (1983) and Jaleel *et al.,* (2003). In the present investigation, high protein content in the callus subcultured on NAA supplemented medium is perhaps due to synthesis of enzymes related to alkaloids biosynthesis leading to high amount of crude alkaloids. In the presence of low salts (25mM and 50mM NaCl) with NAA, synthesis of other proteins related to stress releiving might have occured. However, destruction of most of the proteins might have occured in the presence of high salts (75mM and 100mM NaCl). According to Levitt (1980), protein degardation in saline environment has been atributed to decreased protein synthesis, accelerated proteolysis, decreased avalibility of amino acids and denaturation of enzymes involved in protein synthesis. Moreover, through pot plant study Jaleel (2009) investigated an increase in alkaloids contents (ajmalicine) in two varieties- 'rosea' and 'alba', with NaCl treatment. However, in present study the quantity of ajmalicine is not determined in callus which might show an increase (Fig. 4).

Conclusion

The present study highlights that MS medium supplemented with 2mg/l 2, 4-D and MS medium supplemented with 2mg/l NAA were best for callus induction and growth. However, significant amount of total crude alkaloids was recorded in the calli subcultured on MS medium supplemented with 2mg/l NAA as compared to 2, 4-D supplemented medium and various plant parts (leaf, stem and roots). Although, the amount of total crude alkaloids in the calli subcultured on MS medium supplemented with 2mg/l NAA and different salt concentrations declined as compared to the calli subcultured on NAA (alone) supplemented medium, but it was still found to be higher than leaf and roots. It is clear from present study that the positive effect of NAA in terms of total crude alkaloids accumulation is neutralized by salts (NaCl).

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