

Enhanced Alkaloid Production from Cell Culture System of *Catharanthus roseus* in Combined Effect of Nutrient Salts, Sucrose and Plant Growth Regulators

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Abstract

Callus and biomass culture of *Catharanthus roseus* L. were established to check for the presence of total alkaloid and its subsequent yield. Various treatments like strength of nutrient salts, sucrose concentrations and combinations of plant growth regulators (PGR's) were applied to both MS and B5 in agar as well as suspension medium to test their effects on enhanced alkaloid content and its yield. There was no significant difference in any of the observable parameters of fresh wt, dry wt, alkaloid content, production, productivity and yield if the culture were treated similarly in both types of media formulations (MS or B5 salts). Physical state (agar solidified or the liquid suspension) of the medium had significant effect on all the parameters particular on fresh wt, alkaloid content and yields. Although, the fresh wt. and dry wt. of biomass in suspension culture was 2-3 times less than that of callus obtained from agar medium. However, the alkaloid content and yield was 2-3 times higher in suspension culture compared to agar medium in similar treatments. The highest alkaloid content observed was 5.67mg/g dwt in B5 suspension medium containing 3% sucrose and modified with 0.5mg/l 2,4-Dichlorophenoxy acetic acid (2,4-D) + 1 mg/l Kinetin (KIN) + 2mg/l α -naphthalene acetic acid (NAA). The combined effects of these factors on the enhanced production of total alkaloids were expected to contain higher yield of anticancer vinblastine and vincristine in the cell suspension culture system.

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Introduction

Plants are rich source of a number of secondary metabolites including the alkaloids with an estimated count of around 3000 different types till date [1]. The indole alkaloid, also known as monoterpene indole alkaloids (MIA) or terpenoid indole alkaloids (TIA) are derived from strictosidine which is obtained from tryptamine and a portion from iridoid secologanin [2, 3]. TIA pathway depends on indole and terpenoid precursors supplied by two convergent branches of the primary metabolites the shikimate and the isopentenyl diphosphate pathways. It involves more than twenty enzymes located in cytosol, (pro)vacuoles and chloroplasts/plastids [4]. These indole alkaloids such as antimalarial quinine from *Chincona officinalis*, the antineoplastic camptothecin from *Camptotheca acuminata*, the rat poison and tonic like strychnine from *Strychnos nux-vomica*, antihypertensive and tranquilizer reserpine from *Rauvolfia* sp., and the widely investigated antitumor agents vinblastine and vincristine from *Catharanthus roseus* are now being prescribed in modern therapeutics to treat the diseases.

Catharanthus roseus L. (Apocynaceae) also known as Madagascar periwinkle is one of the most extensively used and investigated medicinal plant [5]. This is a major source of the highly important anti-cancer bisindole alkaloids i.e. vinblastine and vincristine [6-9]. These alkaloids are responsible for control of white cells in blood (an indicator of leukemia) thus acting as an anticancer drug by preventing mitosis in the metaphase stage when they bind to the tubulin thus inhibiting the spindle fiber formation and the cell division [10]. Similarly antihypertensive alkaloids like ajmalicine and serpentine are found in the roots of these plants [11-12]. Apart from these activities this plant is also found to be anti-microbial where extracts of the plant is found to be effective against *S. typhi*, *S. aureus* and *B. subtilis* [13-14]. Other than these numerous similar pharmaceutical activities such as antidiabetic, melanomas of breast and lung, Hodgkin's and Non-Hodgkin's lymphoma are also described for this plant [15]. The water extract of this plant is also known to be effective against bleeding, fever and rheumatism [16]. The anti-oxidant activity (anti-2, 2, 1-diphenyl-1-picrylhydrazyl) of this plant was also recently documented [17-18]. Extensive work has been

carried out on the use of this plant against a wide range of diseases [17] [19-26]. Owing to very trace amount in *C. roseus*, has initiated widespread search for finding alternative solutions for cost effective and mass production of these drugs [7] [27-28].

Plant cell and tissue culture technique is widely used method to enhance the accumulation of such therapeutic secondary metabolites in a wide range of plants [29]. The cultured plant tissues have demonstrated much higher concentrations of alkaloids than the intact plants [30-32]. The biotechnological approaches to manipulate and regulate the production of these alkaloids of *C. roseus* have been overviewed recently [7].

In the present study we are applying factors such as physical state, concentrations and types of nutrient salts (MS and B5), the level of sucrose, and combinations of plant growth regulators with the aim to create stress conditions similar to that experienced by plant in the field as an inductive force for enhanced production of total alkaloids containing higher amount of anticancer vincristine and vinblastine.

Materials and Methods

Selection of Plants and Explants

The *Catharanthus roseus* L. were grown as seedlings in the GITAM campus as hedge plant. Axillary buds shoot tips, leaf segment and roots of six week old plants growing in the GITAM campus were collected for the culture establishment. These explants were used for callus induction following standard protocol [33]. Most of these explants were not successfully established due to heavy contamination by endophytic fungi (*Fusarium oxysporum*) and bacteria [34]. Since the leaf segments were only successful in culture establishment, further studies were confined with leaf segments as explants of choice.

Explants Preparation and Culture Establishment

The leaf explants were collected washed thoroughly under running tap water for 15 min. Surface disinfection was carried by treating with a mild antiseptic solution (1% Savlon, GSK India) and 8-10 drops of Tween-20 for 5-8 min by continuous shaking. Further, surface sterilization was carried under aseptic environment over a Laminar Air Flow hood by treating first with 70% ethanol for 60 seconds followed by

0.05% HgCl_2 for about 15 minutes with continuous stirring to ensure complete sterilization. Thereafter the explants were washed 3-4 times with autoclaved double distilled water to remove traces of HgCl_2 . The surface sterilized leaf explants were inoculated on the variously modified culture medium. The leaf explants were prepared either as circular disks with 1 cm diameter using a sterile cork borer or a squire segment of 1 cm^2 .

Culture Medium and Culture Conditions

Initially cultures were established following the standard protocols [33]. At first normal strength Murashige and Skoog (MS) [35] medium containing 3 % (w/v) sucrose and different combinations of 4-D, Kinetin, 2 and NAA was for culture establishment. Later normal strength B5 culture medium [36] was modified variously for optimization. The leaf explants were treated continuously for 4-weeks in the presence of above growth regulators and each experiment was repeated three times. The leaf explants were also established on B5 medium following similar combinations of growth regulators along with 3% sucrose.

To study the effect of nutrient strength on growth and multiplication of callus, biomass and alkaloid yield three different types of nutrient media (half strength, full strength and one and half strength) was prepared for both MS and B5 in agar and in suspension along with standard sucrose and PGR combination. Callus and biomass obtained from both the medium were further used for alkaloid extraction and quantification.

The pH of the culture media was adjusted to 5.8 ± 0.2 prior to autoclaving (121°C , 15 min) and solidified with 1% agar (if required). The cultures were maintained at $25 \pm 2^\circ\text{C}$ in an environmentally controlled air conditioned room. The culture racks were provided with a 16 h photoperiod under a photon flux density of 2,000-3,000 Lux, provided by fluorescent lamps.

Induction and Multiplication of Callus and Biomass

Induction of callus from leaf explants was initiated as per the standard protocol [33] with slight modifications. To test the effect of growth regulators treatments on callus induction different combinations permutations of 2, 4-D, Kinetin, NAA were used.

To study the combined effect of sucrose and various

PGR combinations on growth and multiplication of callus and biomass and alkaloid yield three concentrations of sucrose (1%, 3%, 5%) were used in this study in normal strength of agar as well as in suspension for both MS and B5 formulations. Three different combinations of PGR's used were **(A)** 0.1 mg/l of 2, 4-D + 0.5 mg/l of KIN + 1.0 mg/l of NAA **(B)** 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA and **(C)** 1.0 mg/l of 2,4-D + 2.0 mg/l of KIN + 4.0 mg/l of NAA. Callus and biomass obtained from both the medium were further used for alkaloid extraction and quantifications.

The biomass doubling time was calculated by harvesting the suspension culture from 3 conical flasks after every 2-day interval filtered on a pre-weighed filter paper. The suspension cultures were maintained by continuous shaking at 150 RPM in a shaker incubator for 12-14 days. The suspension cultures were established and maintained for 2 cycles and sub-cultured every 12-14 days for the cell biomass harvesting and alkaloid yield. The cell biomass was harvested after 14 - days (2- weeks) by filtration on a pre-weighed filter paper (Whatman No.1).

Sample Size

For callus culture 100ml of medium was modified for each treatment and dispensed equally in 2 culture boxes. Each culture box was inoculated with 4 - leaf segments for callus induction or 4 - spots of 100 -150 mg of callus for sub-culture and multiplications. Hence, induction and proliferation of callus is represented by a sample size of 8 per 100ml culture medium per treatment. Callus cultures were maintained by sub-culturing every 4-weeks cycle.

The friable callus at the end of second subculture was used to establish suspension culture. In suspension culture each treatment was modified for 100ml of medium and dispensed 25ml each in to 4 - conical flasks of 150ml capacity. Each 25ml of suspension medium was inoculated with 100 - 150 mg of friable callus (4 - weeks old) from agar medium or 5ml of 2 - weeks old suspension culture for biomass production. Hence, sub-culture, proliferation and biomass production in suspension culture was represented by a sample size of 4 per 100ml medium per treatment.

The growth and development was measured by pooling callus from 2 culture boxes for 100ml medium, expressed in g/l and considered as first replicate. Similarly, cell biomass from 100ml of suspension medium was pooled, expressed in g/l unit and considered as first replicate. These experiments on each treatment were repeated thrice and the results represented as mean \pm standard deviations.

Growth Measurement

Callus Growth

The callus grown on agar medium was harvested after 4-weeks of sub-culture. The cell biomass from suspension culture was harvested every 2- weeks of culture/sub-culture. Callus grown in each of the culture tube or culture boxes were weighed separately.

The laboratory filter paper cut to the size of inner Petri-plate pair and dried in oven at 60°C for 2hrs to make the papers completely moisture free, cooled down to room temperature before weighing. To calculate callus fresh weight, the initial weight of dried filter paper was taken followed by weight of filter paper along with callus and represented as:

Callus Fresh Wt = (Weight of filter paper and the callus – Initial weight of dried filter paper)

To calculate dry weight, the callus along with the filter was dried by initially at room temperature for 2 - 4 days. The callus along with filter paper was further incubated at 40°C in dry heat oven for 3 – 4 hours to remove the traces of moisture before weighing for dry weight. Precaution was maintained to avoid moisture exposure of petri-pales with dried callus and the filter paper. The dried callus along with the filter paper was weighed and represented as:

Callus Dry Wt = (Weight of dried callus along with filter paper – Initial weight of dried filter paper)

Cell Biomass Growth

The cell biomass growth was calculated by harvesting the suspension culture from all 4 conical flasks of a treatment after every 2-days interval onto a pre-weighed filter paper. Filter papers (Whatman No.1) were dried in oven at 60°C for 2hrs to make the papers completely moisture free, cooled down to room temperature before weighing. One of the dried filter paper discs was used to filter simple distilled water

followed by keeping over three layer of blotting paper towel for 1 hr with 2-3 changes to absorb moisture. The weight of moisture free filter paper was taken.

The cell biomass were harvested by pooled filtration from 100ml medium for each treatment using the pre-weighed filter paper and kept in the petri-plates over 3 layers of blotting papers towel for absorbing of moisture by changing 2-3 times with fresh towels over a period of 1 hr. To calculate cell biomass fresh weight, the weight of moisture free filter paper along with cell biomass was taken and represented as:

Biomass Fresh Wt = (Weight of moisture free filter paper and the cell biomass – Initial weight of moisture free filter paper)

The cell biomass was allowed to dry at room temperature for 2-3 days by shifting on a fresh blotting papers towel every day. Finally cell biomass was dried in oven at 40°C for 2-3 hr to remove the remaining moisture before weighing for dry weight. Precaution was maintained to avoid moisture exposure of petri-pales with cell biomass. The dried cell biomass was calculated as:

Biomass Dry Wt = (Weight of the dried biomass with filter paper – Weight of the dried filter paper)

Extraction of Total Alkaloids

About 20mg of dried callus or cell biomass was ground with 10ml of methanol in a mortar pestle, and the whole mixture kept overnight in 100ml conical flasks in a rotary shaker at 25 – 50 rpm for proper mixing of the solvent. The total mixture was then centrifuged at 3000 RPM for 10 minutes and about 9.0 ml of supernatant collected. The supernatant was re-centrifuged 2-3 times until a clear supernatant was obtained. Finally 7-8ml of supernatant was collected in 25ml beakers and kept for drying at room temperature. After complete evaporation of solvent (overnight) the left over content was re-dissolved in 2ml methanol (by mixing for 1hr covered with Petri-plates) and stored in 2.0ml micro-centrifuge vials for further investigations.

Alkaloid Quantifications

Preparation of Standard curve for total Alkaloid Quantification

The calibration curve was prepared with Bismuth nitrate pentahydrate ($\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$) stock solution.

Bismuth nitrate stock solution was made by dissolving 10mg Bismuth nitrate and diluted with DDW (10mg of $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ + 5ml of Conc. HNO_3 + 95ml of DDW = 100ml of $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$). Series dilutions of the stock solution were made by pipetting out 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 mL stock solution into separate 10mL standard flasks and diluted to volume with double distilled water.

A 1mL amount of this solution was taken, and 5 mL thiourea solution (3%) was added to it. The absorbance value of the light yellow solution was measured at 435 (430) nm against the blank containing nitric acid and thiourea. The OD value against each of the concentration is presented in figure 1.

Quantitative Estimation of Total Alkaloids

The total alkaloid was estimated following the modification of protocol [33, 37]. A 1 mL of methanolic extract was taken and the pH brought down at 2–2.5

(pH paper) with dilute HCl. A 400 μL amount of Dragendorff's (DR) reagent (prepared by mixing equal volume of i. solution of 0.8g bismuth nitrate pentahydrate in 40 ml distilled water and 10 ml glacial acetic acid, and ii. solution of 8.0g potassium iodide in 20 ml distilled water used as stock solution) was added to it, and the precipitate formed was centrifuged for 10mins at 5000rpm. The centrifugate was checked for complete precipitation by adding additional 400 μL of DR solution. The centrifugate was discarded completely and the precipitate was further washed twice with methanol. The filtrate was discarded and the residue was then treated with 400 μL disodium sulfide (1% in DDW) solution. The resulting brownish black precipitate was then centrifuged for 10mins at 5000rpm. Completion of reaction was checked by adding 2 drops of disodium sulfide and centrifuged again. The residue was dissolved in 400 μL concentrated nitric acid by gentle warming. This solution was added with 1.6 ml of DDW to get a

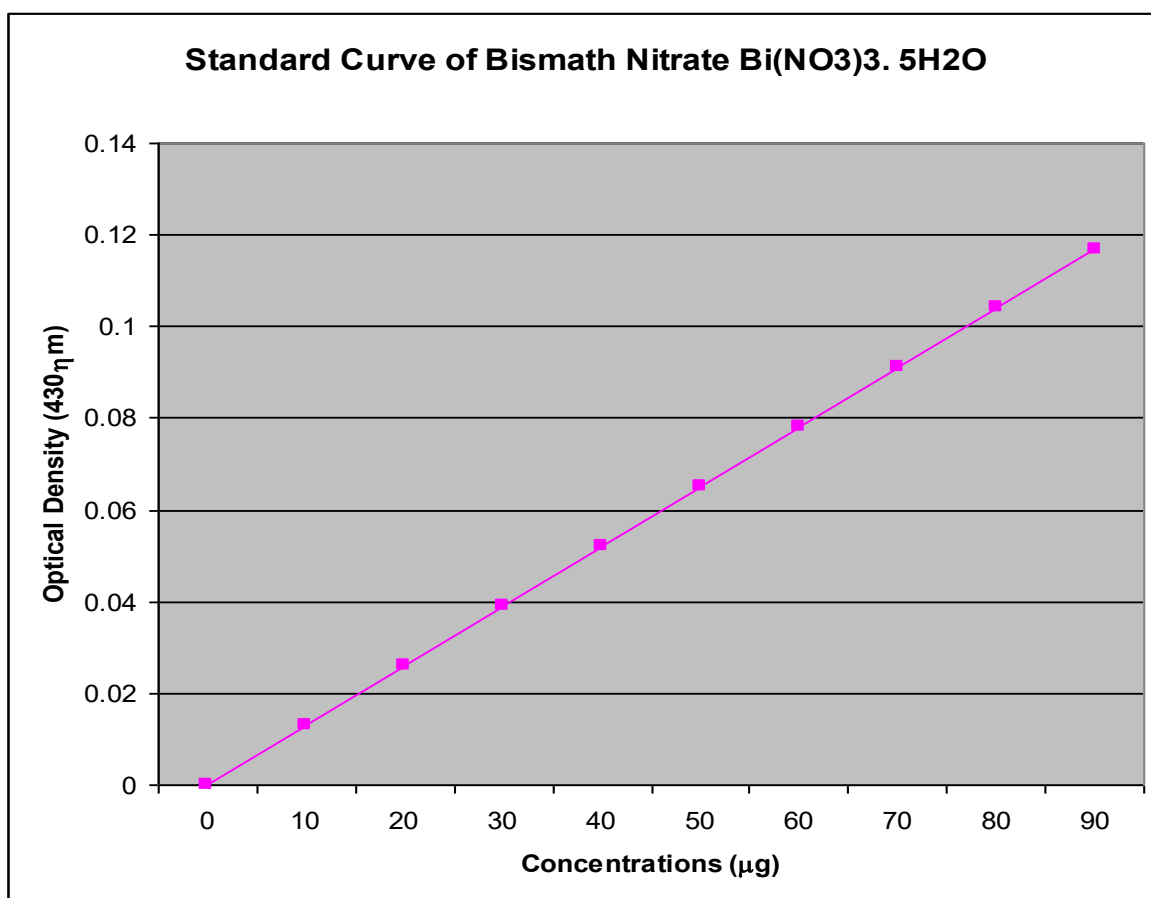


Figure 1. The calibration curve of Bismuth nitrate pentahydrate ($\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$) for the quantification of total alkaloids.

final mixture of 2ml. A 1 mL volume of this mixture was mixed with 5 mL thiourea solution (3% in DDW). The absorbance was measured at 435 (430) nm against the blank containing nitric acid and thiourea. The amount of bismuth present in the solution was calculated by multiplying the absorbance values with the factor taking suitable dilution factor into consideration. The factor was calculated from standard graph shown in figure 1 as a constant value for different concentrations.

Factor = Concentration / absorbance at 430nm

or

Concentration = Factor x Absorbance at 430nm

The alkaloid content was represented as mg/g dry weight of callus or the cell biomass and calculated as under:

Alkaloid content (mg/g dwt): {(Concentrations ((μ g) / 10mg Dry weight} x 1000

Alkaloid Production

It is defined as the alkaloid content per litre of the culture establishment. It is calculated as:

Alkaloid Production (mg/l) = Dry wt (g/l) x Alkaloid content (mg/g)

Alkaloid Productivity

The rate of production of alkaloid per litre per day is the productivity of system (treatment). It is calculated as

Alkaloid Productivity (mg/l/day) = {Alkaloid Production (mg/l) / no. of days the product is harvested}

Alkaloid Yield

The yield of the alkaloid per treatment is represented as the percentage of alkaloid per gram dry weight of the callus or the cell biomass produced. It is calculated as

Alkaloid Yield (% dwt) = {Alkaloid Content (mg/g) / 1000} x 100

Statistical Analysis

The mean of three replicates of experiments, standard deviation, monovariate and multivariate analysis of variance (ANOVA and MANOVA) of results were performed by using SPSS 15 package for Window (SPSS Inc., USA) in the present study.

Result and Discussion

Callus Induction, Growth and Development

Both axillary buds and shoot tip explants were found difficult for successful establishment of cultures due to high contamination (100%) by endophytic fungus *Fusarium oxysporum* [34]. Induction of callus from leaf explants was initiated as per the protocol [33] with different combinations permutations of 2, 4-D, Kinetin, NAA each at a concentrations of 0.0, 0.5, 1.0, 2.0 and 4.0 mg/l as randomized design in normal strength MS agar medium with 3% sucrose. The leaf segments used for culture establishment have shown 20-30% contaminations (mostly bacterial) during 3-4 weeks of culture initiation. About 88-100% of the leaf segments (1 cm²) have shown the callus formation within a week of culture initiation. The callus mass continued to grow slowly and ceased growing beyond 4- weeks of culture initiation. There was very fast growth of callus when sub-cultured on to fresh medium. The result of callus development from leaf explants and growth in the first and second sub-culture is presented in figure 2. Induction and development of callus from leaf explants as well as their growth in the first and second sub-culture was found best in a combination of 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA in 3% (w/v) sucrose containing MS normal strength agar solidified medium. . Callus sub-cultured for more than 2 cycles (4-weekd each) were prone to bacterial contaminations (20-30%) and became more friable with diverse cyto-differentiation and irregular morphology. While those maintained for longer than 4 weeks onto the same culture media develop into more compact, solid calli. Therefore, multiplication of callus was performed only for two subcultures cycle to maintain the uniformity in morphological characteristics and the alkaloid content. As a consequence fresh callus induction was performed from leaf explants for every 12-16 weeks. Both MS and B5 medium supplemented with various growth regulators (NAA, Kinetin, BAP, 2, 4-D etc.) and other growth supplements were reported to induce callus and biomass containing these alkaloids is enhanced [29, 38-41].

Growth Rate of Cell Biomass

The growth rate in suspension culture was calculated by harvesting cell biomass after every 2-day interval over a period of 1 month. The fresh weight of

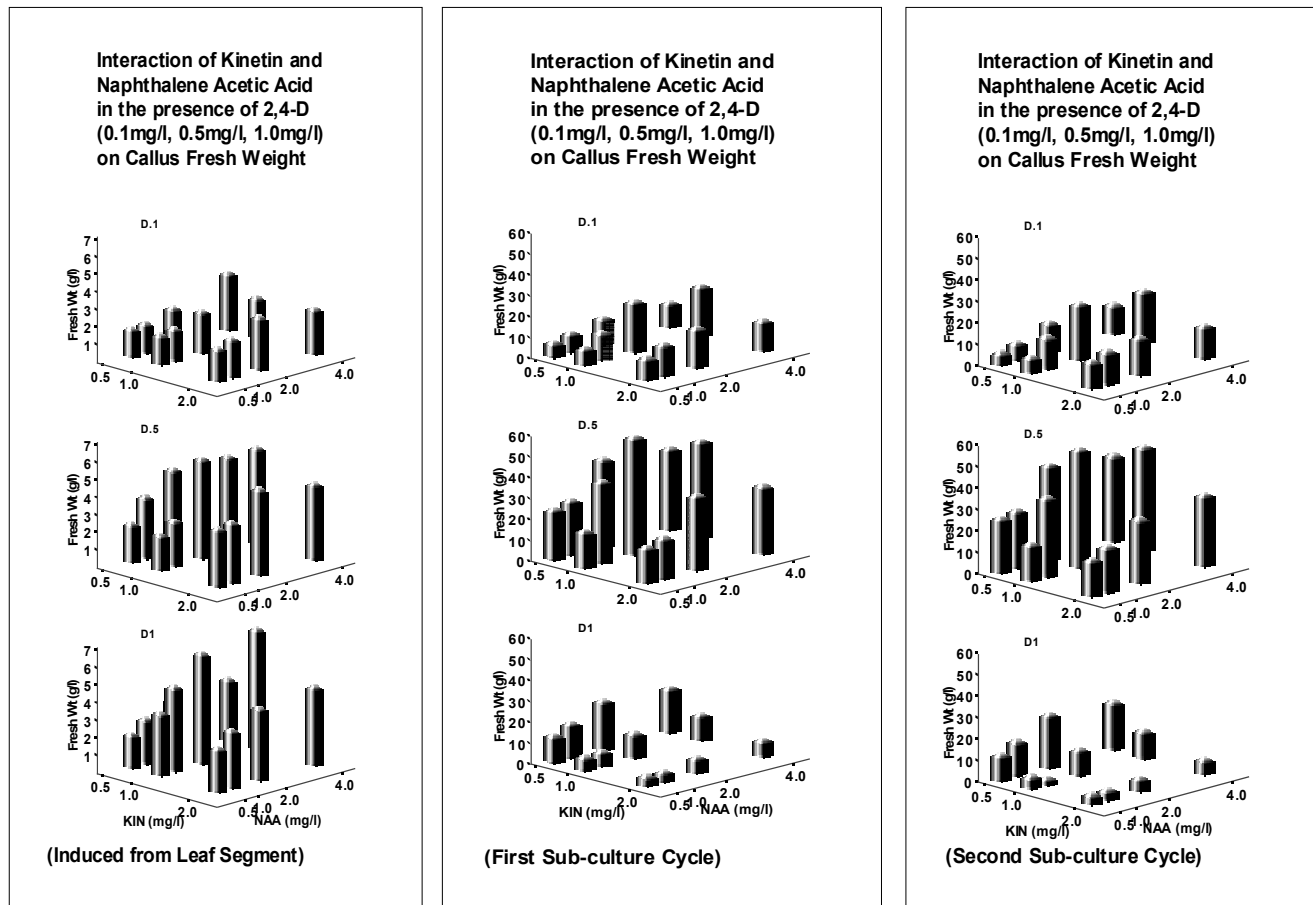


Figure 2. Interactive effects of 2, 4-D, Kinetin, NAA on induction and multiplication of callus growth and development*.

* (Normal strength MS agar (1%) solidified medium was added with 3% sucrose (w/v). This medium was modified with different combinations permutations of 2,4-D (0.1, 0.5 and 1.0mg/l) + KIN (0.5, 1.0 and 2.0 mg/l) + NAA (1.0, 2.0 and 4.0 mg/l). About 100-150 mg of leaf derived callus was inoculated per culture vessel and growth was recorded after 4- weeks of sub-culture).

biomass growth calculated for 4-flasks was represented as mg/100ml suspension culture medium as shown in figure 3. The cell biomass in suspension culture followed a sigmoid pattern with 2-3 days of lag period followed by exponential growth during 3-9 days of culture initiation. The response plateau formed during 10-14 days of culture beyond which there was as a stationary without any change in growth of cell biomass between 14-28 days of culture.

Effects of Strength and Physical State of Medium

All the observable response of fresh wt., dry wt., alkaloid content, production, productivity and the yield showed different pattern in two different physical state (agar and suspension) of the medium irrespective of MS or the B5 nutrient formulation (Table 1-4). The B5 nutrient formulation in agar solidified condition showed higher response of all the parameter compared to the MS agar medium irrespective of their strength (half, normal or one and half) (Table 1 and 3). Strength of medium (half, normal or one and half) in both the

nutrient (B5 or MS) formulations under agar solidified conditions exerted significant effect with the maximum response of all the observable parameters in full strength medium and the least response in half strength medium (Table 1 and 3). Enhancing the nutrient levels to one and half strength did not improve any of the observable response.

In *Podophyllum peltatum* tissue cultures Kadkade [42-43] showed that full strength MS medium was best suited for callus and podophyllotoxins production. Similar results were also observed by Drewes & Staden [44] for solasodine production in *Solanum mauritianum*. Likewise Rosli et al. [45] showed that 9-methoxycanthin-6-one production was enhanced in *Eurycoma longifolia* callus cultures. The concentration of basal medium was also reported to contribute towards the variation of solasodine production in callus cultures of *Solanum aviculare* [46]. Similar findings were observed where along with auxins normal strength MS medium was found to be best for callus production of *C. roseus* [47].

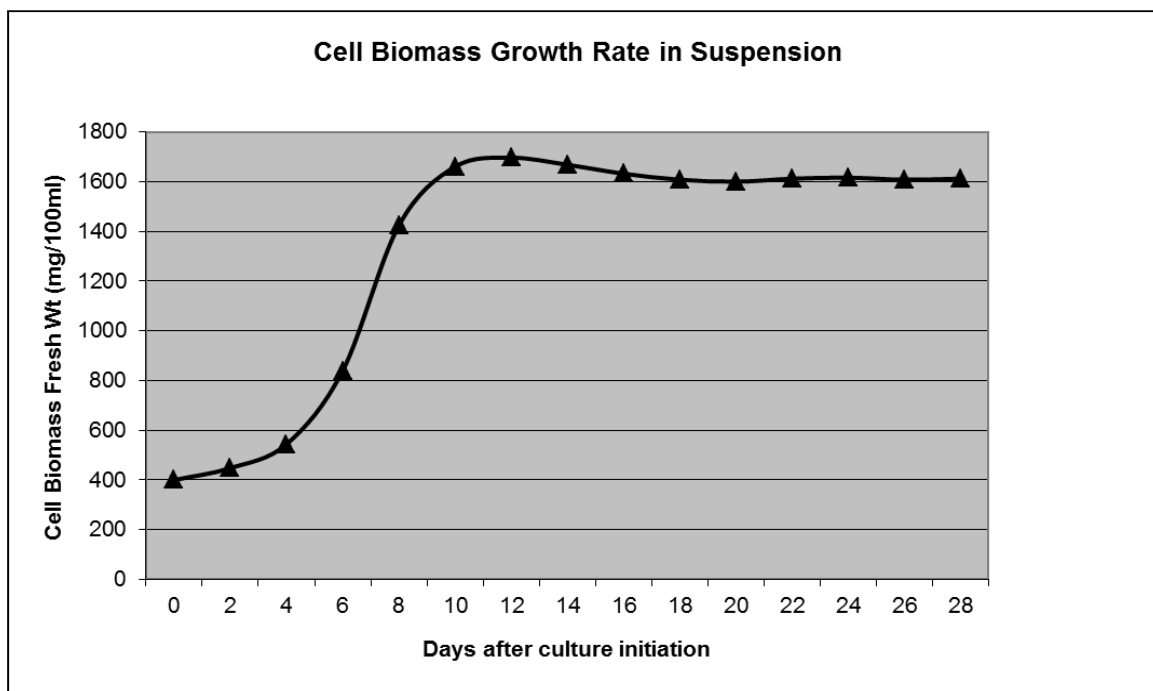


Figure 3. Growth rate of cell biomass in 2nd and 3rd subculture cycle*.

*(The normal strength MS suspension medium was added with 3% sucrose (w/v) and modified with 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA. About 100-125 mg of friable callus (4-weeks old) was inoculated per culture vessel containing 25ml of medium. The growth of biomass in suspension culture was recorded by harvesting cultures every 2- day intervals over a period of 1 month)

Table 1. Effect of Nutrient Strength on Growth and Multiplication of Callus and Alkaloid in MS Agar Medium*.

Sl. No.	Media Strength	Callus Fresh wt	Callus Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
		g/l	g/l	mg/g dwt	mg/l	mg/l/d	% dwt
1	0.50	30.400 ± 4.331	3.042 ± 0.373	1.785 ± 0.147	5.396 ± 0.293	0.193 ± 0.011	0.179 ± 0.015
2	1.00	50.000 ± 2.425	5.230 ± 0.120	2.411 ± 0.262	12.595 ± 1.227	0.450 ± 0.044	0.241 ± 0.026
3	1.50	49.400 ± 2.905	5.108 ± 0.273	2.453 ± 0.115	12.545 ± 1.195	0.448 ± 0.043	0.245 ± 0.012
F(2, 6)		33.805	59.755	12.128	51.146	50.590	12.133
P<0.05		0.001	0.000	0.008	.000	0.000	0.008

*(Agar (1%) solidified MS medium prepared in the above strength (concentrations) were added with 3% sucrose (w/v) and modified with 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA. About 100-150 mg callus was inoculated per culture vessel and the growth was recorded after 4- weeks of sub-culture).

Table 2. Effects of Nutrient Strength on Biomass and Alkaloid in MS Suspension Culture*.

Sl. No.	Media Strength	Biomass Fresh wt	Biomass Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
		g/l	g/l	mg/g dwt	mg/l	mg/l/d	% dwt
1	0.50	10.200 ± 0.144	1.153 ± 0.119	4.793 ± 0.311	5.546 ± 0.902	0.396 ± 0.064	0.479 ± 0.031
2	1.00	19.400 ± 0.262	2.070 ± 0.108	6.021 ± 0.351	12.451 ± 0.627	0.889 ± 0.045	0.602 ± 0.035
3	1.50	19.480 ± 0.560	2.010 ± 0.075	5.844 ± 0.231	11.761 ± 0.904	0.840 ± 0.065	0.584 ± 0.024
F(2, 6)		635.286	75.216	14.548	64.323	64.149	14.351
P<0.05		0.000	0.000	0.005	.000	0.000	0.005

*(MS suspension medium prepared in the above strength (concentrations) were added with 3% sucrose (w/v) and modified with 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA. About 100-150 mg of callus/ cell biomass was inoculated per culture vessel and the biomass growth was recorded after 2- weeks of sub-culture).

Table 3. Effect of Nutrient Strength on Growth and Multiplication of Callus and Alkaloid in B5 Agar Medium*.

Sl. No.	Media Strength	Callus Fresh wt	Callus Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
		g/l	g/l	mg/g dwt	mg/l	mg/l/d	% dwt
1	0.50	33.267 ± 2.053	3.242 ± 0.066	2.140 ± 0.185	6.930 ± 0.465	0.247 ± 0.016	0.214 ± 0.019
2	1.00	52.267 ± 2.641	5.450 ± 0.313	2.709 ± 0.057	14.769 ± 1.040	0.527 ± 0.037	0.271 ± 0.006
3	1.50	53.267 ± 1.501	5.521 ± 0.154	2.507 ± 0.097	13.838 ± 0.632	0.494 ± 0.023	0.251 ± 0.009
F(2,6)		85.045	120.057	16.017	97.465	99.465	15.934
P<0.05		0.000	0.000	0.004	.000	0.000	0.004

*(Agar (1%) solidified B5 medium prepared in the above strength (concentrations) were added with 3% sucrose (w/v) and modified with 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA. About 100-150 mg of callus was inoculated per culture vessel and the growth was recorded after 4- weeks of subculture.)

Table 4. Effects of Nutrient Strength on Biomass and Alkaloid in B5 Suspension Culture*.

Sl. No.	Media Strength	Biomass Fresh wt	Biomass Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
		g/l	g/l	mg/g dwt	mg/l	mg/l/d	% dwt
1	0.50	10.560 ± 0.655	1.223 ± 0.060	5.101 ± 0.424	6.225 ± 0.378	0.445 ± 0.027	0.510 ± 0.043
2	1.00	20.307 ± 0.361	2.129 ± 0.079	6.203 ± 0.178	13.214 ± 0.825	0.944 ± 0.058	0.621 ± 0.018
3	1.50	20.253 ± 0.599	2.165 ± 0.075	5.970 ± 0.325	12.938 ± 1.094	0.924 ± 0.078	0.597 ± 0.032
F(2, 6)		308.481	167.032	9.600	69.794	70.130	9.578
P<0.05		0.000	0.000	0.013	.000	0.000	0.014

*(B5 suspension medium prepared in the above strength (concentrations) were added with 3% sucrose (w/v) and modified with 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA. About 100-150 mg of callus/ cell biomass was inoculated per culture vessel and the biomass growth was recorded after 2- weeks of sub-culture).

Suspension Medium

The fresh and dry weight of the cell biomass was reduced 2-3 times in suspension condition when compared to agar solidified medium of both the nutrient (B5 and MS) formulation (Table 2 and 4). However, the alkaloid content, productivity and the yield were enhanced almost 2-3 time in suspension conditions as compared to agar solidified medium in their respective formulations and strength (Table 2 and 4). The total alkaloid production remained unchanged irrespective of the strength and the physical state of both (B5 and MS) the nutrient formulation.

Strength of the medium played a significant role with maximum response of all the observable parameters in normal strength and least response in half strength suspension culture (Table 2 and 4). Hence it was inferred that normal strength MS medium along with standard sucrose and PGR combinations is the best combination for biomass and alkaloid yield in suspension culture.

The culture production conditions of Egyptian henbane had shown that full strength MS medium as the best suited for callus and metabolites production [48]. Similar results were also observed [49-51] for alkaloid production in *Holarrhena antidysenterica* to study the effect of biochemical engineering on secondary metabolites derived from plant source by implementation of biochemical engineering production using cell suspension culture. MS suspension culture at normal strength proved to be the best nutrient source for biomass and alkaloid production [52] while studying the production of some important secondary metabolites from medicinal plants by plant tissue cultures.

These findings corroborated with the findings where cytokinin in B5 media was found to very helpful in increasing the total alkaloid content in *Catharanthus roseus* [53-54]. Hence, it was inferred that both normal strength MS and B5 suspension culture along with standard sucrose and PGR combinations as the best combination with better response of biomass and alkaloid yield in B5 suspension culture. Similar finding has proved that *Catharanthus roseus* biomass when transferred to B5 suspension medium showed extensive alkaloid production when used as immobilized

cells [55-56].

Effect of Sucrose and PGR's

MS Agar Medium

The result of different concentrations of sucrose (1%, 3% and 5%) along with three variable combinations of PGR's in MS agar medium is shown in table 5. Multivariate analysis of variance (MANOVA) was performed and presented in table 5a. Both sucrose and PGR's significantly affect all observable parameters when analyzed alone irrespective of other factor as well as interact significantly when analyzed in combined form (Table 5a). The lower concentrations of both sucrose and PGR's were found significantly less effective than their higher concentrations. Sucrose at 3% concentrations along with PGR's combinations B (Table 5 footnote) were sufficient for maximum responses of callus fresh wt, dry wt, alkaloid content, production, productivity and the yield in MS agar medium. A 5% sucrose containing MS agar medium in all the PGR's combinations were found to induce improved responses but slightly lesser than that shown by 3% sucrose interacting in a significant manner.

These results confirm that induction, growth, multiplications and alkaloid production from callus culture of *Catharanthus roseus* depends strongly on the concentrations of both sucrose as the carbon source and PGR's. The favorable effect of sucrose in this study can be explained by the fact that sucrose gets easily assimilated by the cells of *C. roseus*. Similar results were reported by Petersen et al. [57] where they showed that sucrose at 3% concentrations had improved significantly the induction, maintenance and plant regeneration from embryogenic callus of *Miscanthus x ogiformis* Honda 'Giganteus'. It was also observed that sucrose at 3% in basal MS medium was best for callus development for *Solanum nigrum* [58]. Earlier Kadkade [43] showed sucrose as the best carbon source for the production of podophyllotoxin in callus culture of *Prodophyllum peltatum* while glucose, fructose and galactose were less effective. Bondarev et al. [59] optimized accumulation of steviol glycoside from developing *Stevia rebaudiana* shoots cultivated in the roller bioreactor using nutrient medium containing 3% sucrose. Enhanced alkaloid content was from callus and cell biomass of *C. roseus* with maximum production in

Table 5. Effect of Sucrose and PGR's Concentrations on Growth and Multiplication of Callus and Alkaloid in MS Agar Medium*.

PGR Comb.	Sucrose Conc.	Callus Fresh wt	Callus Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
	%	g/l	g/l	mg/g dwt	mg/l	mg/l/d	% dwt
A	1	7.393 ± 0.303	1.887 ± 0.061	1.783 ± 0.178	3.371 ± 0.440	0.120 ± 0.016	0.178 ± 0.018
A	3	38.933 ± 2.403	4.230 ± 0.083	2.709 ± 0.057	11.455 ± 0.165	0.409 ± 0.006	0.271 ± 0.006
A	5	42.067 ± 1.206	4.301 ± 0.120	2.507 ± 0.097	10.778 ± 0.445	0.385 ± 0.016	0.251 ± 0.009
B	1	33.067 ± 2.194	3.375 ± 0.174	2.079 ± 0.174	6.999 ± 0.379	0.250 ± 0.014	0.208 ± 0.018
B	3	49.133 ± 2.845	5.215 ± 0.294	3.101 ± 0.077	16.181 ± 1.249	0.578 ± 0.044	0.310 ± 0.008
B	5	45.067 ± 3.101	4.297 ± 0.163	2.873 ± 0.077	12.352 ± 0.785	0.441 ± 0.028	0.287 ± 0.008
C	1	23.800 ± 1.778	2.359 ± 0.134	2.109 ± 0.132	4.980 ± 0.520	0.178 ± 0.019	0.211 ± 0.013
C	3	47.400 ± 1.114	4.863 ± 0.264	2.829 ± 0.065	13.746 ± 0.432	0.491 ± 0.016	0.283 ± 0.006
C	5	48.533 ± 0.702	4.374 ± 0.133	2.395 ± 0.157	10.475 ± 0.699	0.374 ± 0.025	0.240 ± 0.016

*(Normal strength MS agar solidified medium was prepared containing different sucrose (w/v) concentrations and modified with (A) 0.1 mg/l of 2,4-D + 0.5 mg/l of KIN + 1.0 mg/l of NAA (B) 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA and (C) 1.0 mg/l of 2,4-D + 2.0 mg/l of KIN + 4.0 mg/l of NAA. About 100-150 mg of callus was inoculated per culture vessel and the callus growth was recorded after 4 - weeks of sub-culture. Data represent the mean values of 3 replicates of experiments ± standard deviations).

Table 5a. Multivariate Analysis of Variance: Tests of Between-Subjects Effects of different Concentrations of Sucrose and PGR's on Callus and Alkaloid in MS Agar Medium.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Biomass Fresh wt	4645.515(a)	8	580.689	150.473	.000
	Biomass Dry wt	30.024(b)	8	3.753	123.527	.000
	Alkaloid Content	4.520(c)	8	.565	38.367	.000
	Alkaloid Production	416.568(d)	8	52.071	127.166	.000
	Alkaloid Productivity	.532(d)	8	.066	127.846	.000
	Alkaloid Yield	.045(c)	8	.006	38.416	.000
	Intercept	Biomass Fresh wt	37496.229	1	37496.229	9716.361
Biomass Dry wt		406.019	1	406.019	13363.633	.000
Alkaloid Content		167.029	1	167.029	11341.884	.000
Alkaloid Production		2720.338	1	2720.338	6643.545	.000
Alkaloid Productivity		3.468	1	3.468	6673.151	.000
Alkaloid Yield		1.670	1	1.670	11357.991	.000
PGR Comb.		Biomass Fresh wt	850.028	2	425.014	110.133
	Biomass Dry wt	3.053	2	1.526	50.236	.000
	Alkaloid Content	.580	2	.290	19.701	.000
	Alkaloid Production	50.534	2	25.267	61.707	.000
	Alkaloid Productivity	.064	2	.032	61.694	.000
	Alkaloid Yield	.006	2	.003	19.817	.000
	Sucrose Conc.	Biomass Fresh wt	3389.780	2	1694.890	439.195
Biomass Dry wt		25.046	2	12.523	412.173	.000
Alkaloid Content		3.708	2	1.854	125.898	.000
Alkaloid Production		357.128	2	178.564	436.084	.000
Alkaloid Productivity		.456	2	.228	438.790	.000
Alkaloid Yield		.037	2	.019	125.986	.000
PGR Comb. * Sucrose Conc.		Biomass Fresh wt	405.707	4	101.427	26.283
	Biomass Dry wt	1.926	4	.482	15.850	.000
	Alkaloid Content	.232	4	.058	3.934	.018
	Alkaloid Production	8.906	4	2.226	5.437	.005
	Alkaloid Productivity	.011	4	.003	5.450	.005
	Alkaloid Yield	.002	4	.001	3.931	.018
	Error	Biomass Fresh wt	69.463	18	3.859	
Biomass Dry wt		.547	18	.030		
Alkaloid Content		.265	18	.015		
Alkaloid Production		7.370	18	.409		
Alkaloid Productivity		.009	18	.001		
Alkaloid Yield		.003	18	.000		

Total	Biomass Fresh wt	42211.208	27			
	Biomass Dry wt	436.590	27			
	Alkaloid Content	171.815	27			
	Alkaloid Production	3144.276	27			
	Alkaloid Productivity	4.009	27	a R Squared = .985 (Adjusted R Squared = .979) b R Squared = .982 (Adjusted R Squared = .974) c R Squared = .945 (Adjusted R Squared = .920) d R Squared = .983 (Adjusted R Squared = .975)		
	Alkaloid Yield	1.718	27			
Corrected Total	Biomass Fresh wt	4714.978	26			
	Biomass Dry wt	30.571	26			
	Alkaloid Content	4.785	26			
	Alkaloid Production	423.938	26			
	Alkaloid Productivity	.541	26			
	Alkaloid Yield	.048	26			

Table 6. Effects of Sucrose and PGR's Concentrations on Biomass and Alkaloid in MS Suspension Medium*.

PGR Comb.	Sucrose Conc.	Biomass Fresh wt	Biomass Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
	%	g/l	g/l	mg/g dwt	mg/l	mg/l/d	% dwt
A	1	11.69 ± 0.69	1.29 ± 0.08	4.49 ± 0.27	5.78 ± 0.43	0.41 ± 0.03	0.45 ± 0.03
A	3	14.85 ± 0.65	1.73 ± 0.05	5.82 ± 0.04	10.05 ± 0.28	0.72 ± 0.02	0.58 ± 0.00
A	5	15.80 ± 0.48	1.61 ± 0.04	5.46 ± 0.08	8.77 ± 0.08	0.63 ± 0.01	0.55 ± 0.01
B	1	17.68 ± 0.14	2.01 ± 0.04	5.28 ± 0.19	10.61 ± 0.53	0.76 ± 0.04	0.53 ± 0.02
B	3	18.41 ± 0.32	2.12 ± 0.13	6.00 ± 0.28	12.68 ± 0.91	0.91 ± 0.07	0.60 ± 0.03
B	5	18.35 ± 0.12	2.02 ± 0.15	5.67 ± 0.31	11.47 ± 0.98	0.82 ± 0.07	0.57 ± 0.03
C	1	13.63 ± 0.62	1.16 ± 0.08	4.61 ± 0.20	5.37 ± 0.56	0.38 ± 0.04	0.46 ± 0.02
C	3	16.80 ± 0.74	1.90 ± 0.08	6.33 ± 0.32	12.01 ± 0.87	0.86 ± 0.06	0.63 ± 0.03
C	5	16.91 ± 0.80	2.00 ± 0.06	5.15 ± 0.20	10.32 ± 0.21	0.74 ± 0.02	0.52 ± 0.02

*(Normal strength MS suspension medium was prepared different sucrose (w/v) concentrations and modified with (A) 0.1 mg/l of 2,4-D + 0.5 mg/l of KIN + 1.0 mg/l of NAA (B) 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA and (C) 1.0 mg/l of 2,4-D + 2.0 mg/l of KIN + 4.0 mg/l of NAA. About 100-150 mg of callus/ cell biomass was inoculated per culture vessel and the biomass growth was recorded after 2- weeks of sub-culture. Data represent the mean values of 3 replicates of experiments ± standard deviations).

the presence of 3% glucose followed lactose and maltose each at 3% level respectively along with standard sucrose (3%) in the medium [60].

MS Suspension Medium

The effect of different combination permutations of sucrose and PGR's in MS suspension medium on biomass and alkaloid production is presented in table 6. Multivariate analysis of variance (MANOVA) was performed for these results and presented in table 6a. Both sucrose and PGR's in MS suspension medium interacted significantly affecting all the observable parameters irrespective of whether analyzed alone or in different combinations permutations (Table 6a). In MS suspension medium fresh wt and dry wt of the cell biomass were significantly (2-3 times) less compared to agar medium. Whereas alkaloid content, productivity and the yield were 2-3 times higher than that produced in MS agar medium. The alkaloid production (in mg/l) remained unaffected irrespective to the level of sucrose or the PGR combinations in suspension medium (Table 6). The lower concentrations of both sucrose and PGR's were found least responsive than the higher concentration. All the responses were at best in the presence of 3% sucrose along with PGR's combination B (Table 6 footnote). The 5% sucrose in MS suspension medium showed enhanced responses but slightly lesser than that observed in 3% sucrose irrespective of PGR's combinations but interacted significantly.

Amongst the two physical states of media, MS nutrient salts in suspension condition produced enhanced amount of alkaloid content, productivity and the yield despite the compromised fresh and dry wt of biomass as compared to agar solidified medium. Additionally, cell biomass culture cycle in suspension medium required just half the time (14-days) compared to callus multiplication cycle (28-days) in agar solidified medium (see footnotes of table 5 & 6). Hence, suspension medium were found more efficient for enhancing the alkaloid content, productivity and the yield compared to agar solidified medium.

Sucrose at 3% was beneficial in the production of ajamalicine and catharanthine from immobilized cells of *C. roseus* using a conditioned medium [61]. However, sucrose ranging from 2-6% increases the production of arbutin from suspension cultures of *C. roseus* by

glucosylation of exogenous hydroquinone [62]. Maximum accumulation of betacyanin in suspension culture of *Phytolacca americana* was enhanced by increasing the cell number in the presence of 88mM sucrose and by fresh weight in 175mM sucrose containing medium [63]. Additionally, sugar type and the osmotic potential of the culture medium affected the somatic embryogenesis in *Euonymus europaeus* [64]. *Fragaria ananassa* callus when grown in dark in MS medium in the presence of sucrose at 3% showed enhanced production of anthocyanin content [65].

Conclusion

In the present study we concluded that there exist a strong interaction of PGR's and sucrose concentrations for the induction, growth and multiplication of leaf derived callus in both MS agar and suspension medium. Further, the physical state of the medium (agar solidified and liquid suspension) played an important role on all the observable parameter of fresh wt and dry wt as well as alkaloid content, production, productivity and yield. The standard culture conditions induced considerable amount of variations in total alkaloid production from callus grown on agar medium and the cell biomass in suspension of *Catharanthus roseus*. Different factors such as concentrations of nutrient salts and sucrose used as carbon source along with the plant growth regulators tested in two media formulations (MS and B5 salts) significantly effected all the parameters of fresh wt, dry wt, alkaloid content, production, productivity and yield irrespective of whether analyzed alone or in combinations. The yield of total alkaloid was optimized for enhanced production in different combinations permutations of nutrient salts, sucrose and plant growth regulators. There was considerably enhanced amount (5.67 mg/g dwt) of total alkaloid yield in the present study similar to that observed in other reports [61-65]. The obtained results need to be exploited further for separation and purification of different indole alkaloids to test their pharmacological activities.

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Table 6a. Multivariate Analysis of Variance: Tests of Between-Subjects Effects of different Concentrations of Sucrose and PGR's on Biomass and Alkaloid in MS Suspension Medium.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Biomass Fresh wt	123.446(a)	8	15.431	48.654	.000
	Biomass Dry wt	2.828(b)	8	.354	47.076	.000
	Alkaloid Content	8.996(c)	8	1.124	21.229	.000
	Alkaloid Production	161.241(d)	8	20.155	52.594	.000
	Alkaloid Productivity	.823(e)	8	.103	52.218	.000
	Alkaloid Yield	.090(f)	8	.011	21.098	.000
	Intercept	Biomass Fresh wt	6923.525	1	6923.525	21830.060
Biomass Dry wt		83.544	1	83.544	11124.273	.000
Alkaloid Content		794.009	1	794.009	14989.631	.000
Alkaloid Production		2525.669	1	2525.669	6590.620	.000
Alkaloid Productivity		12.885	1	12.885	6538.928	.000
Alkaloid Yield		7.941	1	7.941	14892.169	.000
PGR Comb.		Biomass Fresh wt	73.873	2	36.937	116.462
	Biomass Dry wt	1.238	2	.619	82.391	.000
	Alkaloid Content	.739	2	.369	6.974	.006
	Alkaloid Production	54.327	2	27.164	70.883	.000
	Alkaloid Productivity	.277	2	.139	70.388	.000
	Alkaloid Yield	.007	2	.004	6.971	.006
	Sucrose Conc.	Biomass Fresh wt	38.589	2	19.295	60.836
Biomass Dry wt		1.011	2	.506	67.312	.000
Alkaloid Content		7.099	2	3.550	67.009	.000
Alkaloid Production		87.883	2	43.941	114.663	.000
Alkaloid Productivity		.449	2	.224	113.827	.000
Alkaloid Yield		.071	2	.035	66.515	.000
PGR Comb. * Sucrose Conc.		Biomass Fresh wt	10.984	4	2.746	8.658

	Biomass Dry wt	.580	4	.145	19.301	.000
	Alkaloid Content	1.158	4	.289	5.465	.005
	Alkaloid Production	19.030	4	4.758	12.415	.000
	Alkaloid Productivity	.097	4	.024	12.329	.000
	Alkaloid Yield	.012	4	.003	5.454	.005
Error	Biomass Fresh wt	5.709	18	.317		
	Biomass Dry wt	.135	18	.008		
	Alkaloid Content	.953	18	.053		
	Alkaloid Production	6.898	18	.383		
	Alkaloid Productivity	.035	18	.002		
	Alkaloid Yield	.010	18	.001		
Total	Biomass Fresh wt	7052.680	27	a R Squared = .956 (Adjusted R Squared = .936) b R Squared = .954 (Adjusted R Squared = .934) c R Squared = .904 (Adjusted R Squared = .862) d R Squared = .959 (Adjusted R Squared = .941)		
	Biomass Dry wt	86.507	27			
	Alkaloid Content	803.958	27			
	Alkaloid Production	2693.807	27			
	Alkaloid Productivity	13.744	27			
	Alkaloid Yield	8.041	27			
Corrected Total	Biomass Fresh wt	129.155	26	e R Squared = .959 (Adjusted R Squared = .940) f R Squared = .904 (Adjusted R Squared = .861)		
	Biomass Dry wt	2.964	26			
	Alkaloid Content	9.949	26			
	Alkaloid Production	168.139	26			
	Alkaloid Productivity	.859	26			

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Contribution of Authors

All the experiments were executed and performed by Mr. Malay Ranjan Mishra. The second author Dr. Rajesh K. Srivastava constantly reviewed the experiments and results of various experiments. The experiment design and planning for execution of entire study was conducted by the corresponding author Dr. Nasim Akhtar to achieve the objective of the major research project sanctioned to him by the funding agency University Grant Commission, New Delhi (F.: 42-207/2013 (SR) for the period 1.4.2013-31.3.2017). All the authors declared that there is no conflict with regards to any part of the manuscript.

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