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Enhancing alkaloid production from cell culture system of *Catharanthus roseus* with different carbon sources

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Abstract
A carbon sources in the tissue culture medium is the absolute requirement for growth, development and metabolite production by plant cell culture system. Alkaloid are being high in carbon containing molecules, it is hypothesized that inclusion of additional carbohydrates along with the preferred sucrose as the carbon sources in the cell culture system of *Catharanthus roseus* might enhance its production. The present study is aimed at studying the effects of various sugars as additional carbon sources on biomass development and subsequent total alkaloid metabolism of *C. roseus*. Glucose, lactose and maltose were added separately at 1%, 3%, 5% concentrations along with 3% sucrose in both agar and suspension medium. It was observed that all three sugars significantly affect various observable parameters (fresh wt., dry wt., alkaloid content, production and the yield) in both agar solidified as well as in suspension medium. Although fresh wt and dry wt of cell biomass was 2-3 times less in the suspension medium but the total alkaloid production 2-3 times higher as compared to agar solidified medium. Addition of glucose at 3% concentration along with sucrose at 3% and standard PGR's combinations showed the maximally enhanced total alkaloid yield of (0.615% dry wt) as compared to the control (0.605% dry wt). Both lactose and maltose at 3% increased the fresh wt. and dry wt. but the total alkaloid production was lesser in comparison to the control cultures. Hence, 3% glucose added as additional carbon source along with sucrose and other standard PGR's combinations stimulated the total alkaloid production from the cell culture systems of *C. roseus* in suspension medium.

Keywords: *Catharanthus roseus*, carbon sources

Introduction
Plants are a rich repository of various medicinally important compounds with broad range of activity such as antimicrobial, antitumorogenic, antineoplastic, antiglycemic etc. [1]. Pharmaceutical industries depend at large on these medicinal properties of different plants for remedies and development of drugs against a wide range of diseases [2]. The active principles of the various drugs found in these medicinal plants are secondary metabolites [3, 4]. Plant secondary metabolites are categorized as alkaloids, tannins, phenolics, falvonoids, steroids etc. exhibiting one or the other medicinal property. Some of these secondary metabolites have been reported to have anticancer activity such as alkaloids of *Catharanthus roseus* [5]. The antimicrobial properties are rendered by the phenolic and aldehyde compounds [6].

The herb *Catharanthus roseus (Vinca rosea)* Linn. (Apocynaceae) popularly known as Madagascar periwinkle is possessing immense medicinal properties [7] attributed to its active compounds which are mainly of two types, tannins and alkaloids. The plant produces more than 130 different varieties of monoterpenoid indole alkaloids (TIA's) [8] in varying quantities in different organs. Many of these alkaloids have demonstrated medicinal properties like anticancer [9, 10]. The leaves and stems are reservoirs for the highly anticipated anti-cancer drugs vincristine and vinblastine while roots contain antihypersensitive ajmalicine and serpentine alklaoids [11]. The leaves also produce numerous pharmacologically important metabolites which have proved its efficacy in animal model against hyperglycemia and hypertension [12].

The cultivated and wild *C. roseus* plant have shown very trace quantities of these secondary metabolites (mainly the anti-tumorogenic alkaloids) making them a highly costly drug due to extensive and costly extraction and purification process. There is an urgent need for widespread research to find alternative technology for cost effective and mass production of these drugs [13, 14, 15]. The cell suspension system of plant cell and tissue culture technique is one of the alternative to enhance the accumulation of such therapeutic compounds in shake flask or bioreactor [16]. The levels of accumulated alkaloids in the cultured tissues are found at much higher concentrations than in the intact plants [17, 18, 19]. Different biotechnological approaches to manipulate and regulate the production of these alkaloids of *C. roseus* have been overviewed recently [13].

As per the Pub Chem Compound Database the vinca alkaloids include vinblastine, vincristine and vinorelbine which are important antineoplastic agents used in many chemotherapeutic regimens for a wide variety of cancers as a safe drug without any apparent acute liver injury. The molecular formula of vinblastine ($C_{46}H_{58}N_4O_9$; MW 810.989 g/mol) and vincristine ($C_{46}H_{56}N_4O_{10}$; MW 824.972 g/mol) have very high C: N ratio. The nutrient medium is designed to

meet all the essential requirements for the growth and development of cultured plants cells in tandem to natural plants. Sucrose in the medium acts as the osmotic stabilizer as well as the preferred source of carbon by the cultured plant cell. The production of various secondary metabolites through hairy roots (such as hyoscyamine, isoflavones, sennosides A and B, pyranocoumarins, gymnemic acid, ginsenoside) is found to be affected by initial concentrations of carbon sources in the nutrient media [20, 21, 22, 23, 24, 25, 26]. It is hypothesized that enhancing the level of carbon source in the medium might divert the additional carbohydrates to maintain the high C: N ratio towards the synthesis of alkaloid.

In the present investigation, we have studied the effects of various carbon sources like glucose, lactose and maltose along with sucrose and other nutrient factors with the aim to create an osmotic potential in the culture medium for inducing a stress that is experience by plant in the field and to divert them for enhanced production of total alkaloids containing higher amount of anticancer vincristine and vinblastine.

Materials and methods

Selection of plants and explants

Explants like axillary buds, shoot tips, leaf segment and roots were collected from *Catharanthus roseus* L. plants (six week old seedlings) growing in the GITAM campus. Most of these explants were not successfully established due to heavy contamination by endophytic fungi (*Fusarium oxysporum*) and bacteria [27]. The leaf segments were successfully established for callus induction and selected for further studies.

Explants preparation and culture establishment

The leaf from field grown plants were collected washed thoroughly under running tap water for 15 min. Disinfection of leaf segments was performed by treating with 1% Savlon and 8-10 drops of Tween-20 for five min. The leaf segments were surface sterilized under sterile environment (LAF) first with 70% ethanol for 60 seconds followed by treatment with 0.05% $HgCl_2$ for about 15 minutes by continuous stirring. Thereafter the explants were washed 3-4 times with autoclaved double distilled water to remove traces of $HgCl_2$. The sterilized leaf segments were trimmed to explants size and inoculated on the variously modified culture medium. The leaf explants were prepared either as circular disks with 1cm diameter using a sterile cork borer or a squire segment of 1 cm^2.

Culture medium and culture conditions

Initially normal strength Murashige and Skoog (MS) [28] medium was prepared containing 3 % (w/v) sucrose and different combinations of 2,4-D, Kinetin, and NAA for culture establishment. The leaf explants were treated continuously for four weeks in the presence of above growth regulators and each experiment was repeated three times. To study the effect of enhanced carbon sources on growth and multiplication of callus, biomass and total alkaloid yield the medium was added with additional three different (1%, 3% and 5%) concentrations separately for glucose, lactose and maltose both in agar and in suspension conditions along with standard sucrose and PGR combination. 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. The cultures were maintained at 25 ± 2 °C maintained in an environmentally controlled air conditioned room and culture racks were provided with a 16/8 h photoperiodic cycle 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 on standard MS medium [29] 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 each at a concentrations of 0, 0.5, 1.0, 2.0 and 4.0 mg/l in a full factorial design.

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 cell biomass was harvested after 14 - days (2 – weeks) by filtration on a pre-weighed filter paper (Whatman No.1). 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.

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 the study in agar as well as in suspension normal strength of both MS and B5 medium. 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.

Sample size

A 100ml volume of MS agar medium was modified for each treatment and dispensed equally in 2 culture boxes. The culture box was inoculated with 4 - leaf segments ($1cm^2$) for callus induction or 4 – spots of 100 -150 mg of callus (4-week old) for sub-culture and multiplications. Callus cultures were maintained by sub-culturing every four weeks cycle.

Suspension culture was established by inoculating friable callus at the end of second subculture. Each treatment in suspension culture was modified similarly 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.

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 for 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 ± standard deviations.

Growth measurement

Callus growth

The callus was harvested from agar medium after 4-weeks of sub-culture. The cell biomass was harvested every 2- weeks of culture/sub-culture in suspension medium. 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 was 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 before weighing for dry weight. Precaution was maintained to avoid moisture exposure of petri-pales with cell biomass. Finally the dry 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

A 20mg of dried callus or cell biomass from cell suspension cultures 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 (12hrs) 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 quantzifications

Quantitative estimation of total alkaloids

The total alkaloid was estimated following the modification of protocol [29, 30]. A 1 mL of methanolic extract was taken and the pH brought down at 2–2.5 (pH paper) with dilute HCl. A 400µL 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 400µL 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 400µL 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 400µL concentrated nitric acid by gentle warming. This solution was added with 1.6 ml of DDW to get a 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 the bismuth nitrate pentahydrate **(**$Bi(NO_3)_3.5H_2O$**)** standard graph shown as a constant value for different concentrations.

Factor = concentration/absorbance or Concentration = Factor * Absorbance

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

Alkaloid content (mg/g dwt): {(Absorbance at 430nm * Factors)/10} * Dry weight (in gm)

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

Alkaloid Production (Callus) (mg/l) = Alkaloid content (mg/g) * 20
Alkaloid Production (Biomass) (mg/l) = Alkaloid content (mg/g) * 40

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

Alkaloid Productivity (Callus) (mg/l/day) = Alkaloid production (mg/l) / nos of days the product is harvested.
Alkaloid Productivity (Biomass) (mg/l/day) = Alkaloid production (mg/l) / nos 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} * 100

Statistical analysis

The mean of three replicates of experiments, standard deviation and monovariate analysis of variance (ANOVA) of results were performed by using SPSS 10 package for Window (SPSS Inc., USA) in the present study.

Results and discussion

Effect of glucose as additional carbon source on total alkaloids

The analysis of results for induction and growth of callus and production of alkaloids by addition of glucose as extra carbon source in different concentrations in MS agar medium along with standard combinations of sucrose and PGR's is presented in table 1. Addition of different concentrations of glucose along with the standard (3%) sucrose in MS agar medium showed a significant ($P \leq 0.05$) effect on the callus and alkaloid production. It has been observed that 3% glucose along with 3% sucrose containing MS agar medium showed maximally enhanced response of all the observable parameters of callus fresh wt, dry wt and total alkaloid production compared to the control treatments. Addition of 1% glucose along with the standard (3%) sucrose in MS agar medium showed slightly reduced response of all the observable parameters compared to control cultures without it. While at 5% glucose along with the standard sucrose (3%) containing agar media all observable parameters were enhanced compared to control but in significantly less efficiency than those added with 3% glucose. Hence, only slight improvement in the alkaloid production was observed from callus produced in MS agar solidified medium with 3% glucose along with standard sucrose (3%) and PGR's combination (Table 1 footnote) compared to control without glucose.

Table 1: Effect of Glucose as Carbon Source on Callus and Alkaloid Production in MS Agar Medium.

Sl No.	Glucose 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
1	0	48.400 ± 0.917	4.986 ± 0.124	2.882 ± 0.105	14.375 ± 0.792	0.514 ± 0.029	0.288± 0.011
2	1	49.867 ± 1.419	5.095 ± 0.110	2.524 ± 0.075	12.860 ± 0.553	0.459 ± 0.020	0.253 ± 0.008
3	3	58.800 ± 1.400	4.986 ± 0.124	2.984 ± 0.067	14.877 ± 0.545	0.531 ± 0.019	0.298 ± 0.007
4	5	57.267 ± 2.301	4.526 ± 0.080	2.727± 0.209	12.336 ± 0.897	0.441 ± 0.032	0.273 ±0.021
	F(3, 8)	30.633	15.627	7.413	8.595	8.594	7.178
	P≤0.05	0.000	0.001	0.011	.007	0.007	0.012

(MS agar solidified (1%) medium containing 3% sucrose (w/v) were supplemented with different concentrations of glucose as additional carbohydrates 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 calluses (4 – weeks old) were inoculated per culture vessel and the growth was recorded after 4- weeks of sub-culture).

The carbon has been a limiting factor for TIA biosynthesis in cell culture system as it is proved that at low glucose concentration the production of ajmalicine is found to be higher [31]. It was found that the presence of various carbon source in the culture medium have varied response towards alkaloid production from *C. roseus* [5]. It was also indicated that glucose as the best carbon source after sucrose for callus development and alkaloid production in *Solanum nigrum* [32].

The effect of different concentrations of glucose as extra carbon in MS suspension medium along with standard combinations of sucrose and PGR's on biomass and alkaloid production is presented in table 2. The monovariate analysis variance of these results showed a significant effect on all the observable parameters of biomass and alkaloid. In general, the cell biomass (fresh wt and dry wt) in MS suspension medium were significantly less (2-3 times) while the alkaloid content, production, productivity and the yields were significantly more (2-3 times) compared to agar medium modified with similar combination of glucose, sucrose and PGR's combinations (Table 2 footnote).

Table 2: Effect of Glucose as Carbon Source on Callus and Alkaloid Production in MS Suspension Medium.

Sl No.	Glucose 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
1	0	16.587 ± 0.638	1.564 ± 0.119	6.050 ± 0.311	9.436 ± 0.246	0.674 ± 0.018	0.605 ± 0.031
2	1	18.267 ± 0.140	1.838 ± 0.137	4.665 ± 0.118	8.585 ± 0.852	0.613 ± 0.061	0.467 ± 0.012
3	3	20.213 ± 0.306	2.423 ± 0.085	6.152 ± 0.077	14.910 ± 0.656	1.065 ± 0.047	0.615 ± 0.008
4	5	19.867 ± 0.442	2.309 ± 0.087	5.870 ± 0.117	13.555 ± 0.686	0.968 ± 0.049	0.587 ± 0.012
	F(3, 8)	46.390	40.761	43.828	67.709	67.698	44.470
	P<0.05	0.000	0.000	0.000	.000	0.000	0.000

MS suspension medium containing 3% sucrose (w/v) were supplemented with different concentrations of glucose as additional carbohydrates 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 calluses or 5ml of 2- week old suspensions were inoculated per culture vessel and the biomass was recorded after 2- weeks of sub-culture).

Addition of glucose in the presence of sucrose and PGR's in MS suspension medium was found stimulatory on all the observable parameters irrespective of its concentrations. Maximum stimulatory response was observed with 3%

glucose along with 3% sucrose and the standard PGR's combinations (Table 2 footnote). Both lower (1%) and higher (5%) glucose concentration in the medium containing 3% sucrose irrespective of PGR's combinations (Table 2 footnote) showed enhanced responses of all observable parameters.

The findings of these experiments clearly indicated that suspension medium was better than the agar solidified medium for the production vinca alkaloids. In both MS agar as well as suspension medium sucrose had been the preferred source of carbon whose potential of alkaloid production were enhanced significantly by inclusion of additional glucose in the medium containing the standard PGR's combinations (Table 2 footnote).

In tissue culture and secondary metabolite production medium sucrose plays dual role as carbon sources and as osmotic regulators [33]. There is an inverse relationship between concentrations of carbon source and the osmotic potential of the medium. The increase in carbon concentration leads to an initial increase, followed by a reduction in the values of the examined parameters. This reduction can be attributed to the excessive osmotic contribution or by toxicity of the carbohydrate[34][35] when using fructose or glucose (3%, w/v) (instead of sucrose) in the culture medium, the growth rate of hairy roots of *Plumbago zeylanica* L. is significantly slower. It is also indicated that sucrose and glucose may influence directly (such as signaling molecules) or indirectly via variety of metabolic pathways [36]. The influence of various carbon sources like sucrose and glucose on the growth and production of primary and secondary metabolites of *Arnica montana* hairy root cultures is found to be favorable for production of flavonoids which is not commonly present in the roots of the plant [37].

Effect of lactose as additional carbon source on total alkaloids

The effect of addition of different concentrations of lactose as extra carbon source in MS agar and suspension medium along with standard combinations of sucrose and PGR's was analyzed by ANOVA and presented in table 3 and 4. Analysis of these results indicated a significant ($P \leq 0.05$) effect of lactose on all the observable parameters in both agar solidified as well as in suspension medium.

Table 3: Effect of Lactose as carbon source on Callus and Alkaloid Production in MS Agar Medium

Sl No	Lactose 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
1	0	49.133± 0.503	5.366 ± 0.369	2.719 ± 0.118	14.572 ± 0.713	0.520 ± 0.026	0.272 ± 0.012
2	1	48.600 ± 1.058	4.962 ± 0.118	2.535 ± 0.148	12.585 ± 0.940	0.449 ± 0.033	0.253 ± 0.015
3	3	51.267 ± 0.902	5.347 ± 0.188	2.901 ± 0.100	15.507 ± 0.565	0.554 ± 0.020	0.290 ± 0.010
4	5	49.467 ± 0.808	4.317 ± 0.295	2.567 ± 0.175	11.050 ± 0.287	0.395 ± 0.010	0.257 ± 0.018
	F(3, 8)	5.651	10.618	4.401	26.772	26.767	4.482
	P<0.05	0.022	0.004	0.042	0.000	0.000	0.040

(MS agar solidified (1%) medium prepared with 3% sucrose (w/v) were supplemented with different concentrations of lactose as additional carbohydrates 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 calluses (4 – weeks old) were inoculated per culture vessel and the growth was recorded after 4- weeks of sub-culture).

Addition of lactose along with standard combinations of sucrose and PGR's (Table 3 footnote) in agar medium showed comparable callus fresh weight in all the three concentrations compared to control. All the parameters were observed to their maximum response in 3% lactose in MS agar medium as additional carbon source. The other two concentrations (1% and 5%) were less effective in enhancing the response of all the observable parameter of callus (fresh and dry weight) and total alkaloids (content, production, productivity and yield) compared to the control cultures. The potential of lactose was found to be less stimulatory compared to the glucose supplemented agar medium irrespective of concentrations. However, lactose in the agar medium showed response of all the observable parameter almost comparable to that exerted by maltose added agar medium.

A comparison of Table 3 and 4 indicated that in general biomass production (fresh and dry weight) was 2-3 times less in suspension compared to agar medium. While the other parameters like alkaloid content, production, productivity and yield were almost 2-3 times higher in suspension culture compared to that of agar solidified medium at all the three concentrations.

Table 4: Effect of Lactose as carbon source on Callus and Alkaloid Production in MS Suspension Medium.

Sl No.	Lactose 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
1	0	16.667 ±0.424	1.708± 0.023	5.947 ± 0.311	10.157 ± 0.595	0.725 ± 0.043	0.595± 0.031
2	1	17.440 0.144	1.738 ± 0.085	4.434 ± 0.089	7.710 ± 0.491	0.551 ± 0.035	0.443 ± 0.009
3	3	18.173 ± 0.281	1.805 ± 0.143	5.921 ± 0.154	10.700 ± 1.112	0.764 ± 0.079	0.592 ± 0.016
4	5	17.053 ± 0.180	1.534 ± 0.047	4.948 ± 0.044	7.589 ± 0.283	0.542 ± 0.020	0.495 ± 0.005
	F(3, 8)	15.895	5.253	51.636	16.489	16.401	50.613
	P<0.05	0.001	0.027	0.000	0.001	0.001	0.000

(MS suspension medium prepared with 3% sucrose (w/v) were supplemented with different concentrations of lactose as additional carbohydrates 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 calluses or 5ml of 2- week old suspensions were inoculated per culture vessel and the biomass was recorded after 2- weeks of sub-culture).

In MS suspension medium addition of lactose along with standard combinations of sucrose and PGR's (Table 4

footnotes) significantly affected all the observable parameter in a manner similar to that observed in the presence of other two carbon sources (glucose and maltose). Although, addition of lactose showed stimulatory effect on biomass fresh and dry weight but it did not improve any of the total alkaloid parameter (content, production, productivity and yield) as compared to the control cultures. Lactose at 3% concentration showed slightly higher production of biomass fresh and dry weight but the total alkaloid parameters were slightly less compared to control cultures glucose containing media. However, in 1% and 5% lactose containing suspension medium all the observable parameters (except for alkaloid production and productivity) were slightly higher than that of control and the maltose containing medium. In 5% lactose supplemented suspension medium showed better response compared to the 1% lactose containing medium.

Hence, lactose as additional carbon source in the suspension medium was found to be ineffective for the growth of callus, biomass and production of total alkaloids. Even though the biomass (fresh and dry weight) production was higher in lactose containing medium but it was significantly less effective in alkaloid production at all the concentrations in comparison to control cultures.

Studies on Japanese pear showed that lactose along with maltose and mannitol was found inferior to other carbon sources in terms of shoot proliferation and increase of fresh mass of the plant [38]. Shoot survival was negatively affected in presence of lactose. Lactose as a source of carbon in plant cell culture was not suitable for biomass yield and growth as the rates vary greatly between the different carbon sources and there is a variation in response between different cell cultures to individual carbon sources [39]. Lactose proved to be the least effective source of carbon for growth and proliferation of fresh mass of *Chrysanthemum* in both dark and light conditions [40].

Effect of maltose as additional carbon source on total alkaloids

The effect of different concentrations of maltose as an extra carbon source in MS agar and suspension medium along with standard combinations of sucrose and PGR's was analyzed by ANOVA and presented in table 5 and 6. These analyses revealed that addition of maltose in MS medium (both agar and suspension) exerted a significant ($P \leq 0.05$) effect on all the observable parameter.Addition of maltose along with standard combinations of sucrose and PGR's (Table 5 footnote) showed enhanced callus fresh weight in all the three concentrations compared to control. However, when compared to the other two additional carbon sources (glucose and lactose) inclusion of maltose was found almost equally stimulatory for callus and total alkaloid following similar response trend. At 3% maltose in MS agar medium all the parameters of callus (fresh and dry weight) and total alkaloids (content, production, productivity and yield) were enhanced compared to the control cultures. Addition of lower (1%) and higher (5%) concentration of maltose in agar medium did not show improvement in any of the observable parameters except for fresh weight as compared to the control cultures.

Table 5: Effect of Maltose as carbon source on Callus and Alkaloid Production in MS Agar Medium.

Sl No.	Maltose 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
1	0	43.200 ± 1.400	5.080 ± 0.096	2.882 ± 0.105	14.635 ± 0.271	0.522 ± 0.010	0.288 ± 0.011
2	1	47.933 ± 0.462	4.990 ± 0.132	2.494 ± 0.104	12.453 ± 0.820	0.445 ± 0.029	0.249 ± 0.011
3	3	49.867 ± 0.757	5.427 ± 0.075	2.901 ± 0.080	15.750 ± 0.593	0.563 ±0.021	0.290 ± 0.008
4	5	49.333 ± 0.643	4.707 ± 0.165	2.485 ± 0.150	11.713 ± 1.109	0.418 ± 0.040	0.249 ± 0.015
	F(3, 8)	34.952	17.748	12.825	18.158	18.106	12.724
	P<0.05	0.000	0.001	0.002	0.001	0.001	0.002

(MS agar solidified (1%) medium containing 3% sucrose (w/v) were supplemented with different concentrations of maltose as additional carbohydrates 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 calluses (4 – weeks old) were inoculated per culture vessel and growth was recorded after 4- weeks of sub-culture).

As indicated in table 6 that addition of maltose in MS suspension medium significantly affected all the observable parameter in a manner similar to that observed with the addition of other two carbon sources (glucose and lactose). In general biomass production (fresh and dry weight) was 2-3 times less in suspension compared to agar medium. The other parameters like alkaloid content, production, productivity and yield were almost 2-3 times higher in suspension culture compared to that of agar solidified medium.

Table 6: Effect of Maltose as carbon source on Biomass and Alkaloid Production in MS Suspension Medium

Sl No.	Maltose 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
1	0	16.867 ± 0.122	1.785 ± 0.370	5.844 ± 0.504	10.430 ± 0.870	0.745 ± 0.621	0.585 ± 0.050
2	1	17.680 ± 0.280	1.713 ± 0.007	4.845 ± 0.077	8.301 ± 0.123	0.593 ± 0.089	0.484 ± 0.008
3	3	19.080 ± 0.661	1.949 ± 0.643	5.765 ± 0.081	11.242 ± 0.527	0.803 ± 0.038	0.576 ± 0.008
4	5	18.133 ± 0.289	1.837 ± 0.005	5.537 ± 0.077	10.717 ± 0.117	0.726 ± 0.008	0.554± 0.008
	F(3, 8)	16.659	21.218	9.080	17.452	17.480	9.157
	P<0.05	0.001	0.000	0.006	0.001	0.001	0.006

(MS suspension medium containing 3% sucrose (w/v) were supplemented with different concentrations of maltose as additional carbohydrates 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 calluses or 5ml of 2- week old suspensions were

inoculated per culture vessel and the biomass was recorded after 2- weeks of sub-culture).

Maltose at 3% concentration showed slightly higher production of biomass fresh and dry weight but the total alkaloid were slightly less compared to control cultures as well as to that of other two carbon sources (glucose and lactose). However, in 1% and 5% maltose containing suspension medium all the observable parameters (except for alkaloid content and productivity) were slightly higher than that of control and the lactose containing additional carbon source. A 5% maltose supplemented suspension medium showed better response compared to the 5% lactose containing medium.

In conclusion all these three additional carbon sources (glucose, lactose and maltose) affected the process significantly, while the glucose served as the best supplement to enhance the productivity. Amount of total alkaloid was best observed in suspension than the agar solidified medium. Glucose at 3% concentration stimulated the maximum yield of total alkaloid compared to other two carbon sources. Lactose was found to be better than the maltose at 3% concentration. While at 5% concentration maltose was better for process stimulation compared to the lactose added medium. A 1% maltose containing suspension medium showed enhanced response compared to control cultures but found to be less stimulatory than its higher concentrations.

Studies on the effects of additional carbon sources have indicated that a concentration of 3% maltose induces the highest percentage of hairy root cultures in leaves of *Camellia sinensis* followed by dextrose, sucrose and fructose although maltose seems not to be significant enough for alkaloid production[41]. Maltose is proved to be the second most efficient carbon source for growth and development of *Arnica montana* hairy roots [37]. However, in many in vitro responses maltose has not been indicated as preferred carbon source or as the supplements to the medium. Maltose supplemented medium is not beneficial for somatic embryogenesis in *Linum usitatissium* [42]. It was also reported that shoots of 'Hosui' Japanese pear failed to develop roots when the media is supplemented with maltose. Maltose was found to be an inferior choice as carbon source against other carbon sources for shoot elongation, proliferation and survivability [38]. Similarly it was shown in their investigation on micro propagation of common ninebark (*Physocarpus opulifolius* maltose as being ineffective for shoot and root proliferation. Rather at higher concentrations (30–50 gdm^{-3}) maltose has inhibitory effect on the number of shoots per proliferated explants as well as on the shoot length [43]. Maltose was one of the least preferred source for shoot fresh mass in chrysanthemum thin cell layers. A low level of end reduplication or polysomaty was obtained in callus cultures derived from TCLs grown on mannose (light), maltose (light and dark) and raffinose (light and dark) supplemented media [40].

Conclusion

The plant cell cultured in suspension medium experiences a constant and uniform osmotic stress by simple inclusion of additional carbon sources as compared to agar solidified medium. Increasing the concentration of carbon sources by inclusion of glucose, lactose or maltose in the standard agar medium although stimulated the production of higher fresh wt and dry wt but the alkaloid production was significantly more in suspension culture. The extra carbon sources in the form of lactose, maltose and glucose showed significant enhancement of total alkaloid production from callus grown on agar medium and the cell biomass in suspension of *Catharanthus roseus* compared to control cultures. Among the three carbohydrates, glucose proved to be the best sugar that can be used in tandem with sucrose. Though at 1% and 5% concentrations in both agar and suspension medium the fresh wt and dry wt of cultures were enhanced in comparison to the control but a 3% concentration of any of these sugars along with sucrose showed the best results across all observable parameters including the total alkaloid content. Lactose and maltose though showed significant effect on all the observable parameters but both were found to be less stimulatory than glucose towards growth of callus, biomass and production of total alkaloids. A more extensive study need to be conducted on the inclusion of other carbohydrate in order to verify the need for mobilization of carbon sources towards secondary metabolite production by cultured cell of *C. roseus* under stressed condition.

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