International Journal of Secondary Metabolite
2019, Vol. 6, No. 2, 137-153

https://dx.doi.org/10.21448/ijsm. 559679

Published at http://ijate.net/index.php/ijsm http://dergipark.gov.tr/ijsm **Research Article**

Effect of Nitrogen, Phosphorus and Medium pH to Enhance Alkaloid Production from *Catharanthus roseus* Cell Suspension Culture

Malay Ranjan Mishra[1], **Rajesh Kumar Srivastava**[1], **Nasim Akhtar** [*,1]

[1] Department of Biotechnology, GITAM Institute of Technology, Gandhi Institute of Technology and Management, GITAM Deemed to be University, Rushikonda, Visakhapatnam (A.P.), India

Abstract: Several elevated levels of nitrogen and phosphate at varying pH of the medium which impart a major influence on callus and biomass development and subsequent production of alkaloids was investigated using suspension culture system of *Catharanthus roseus* in the present study. The B5 medium was buffered at pH 4.51, 5.82 and 7.32 by addition of different levels of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ representing the enhanced and varied supply of total nitrogen $(NH_4^+ + NO_3^-)$ and phosphate compared to MS medium (as control) for cell biomass production and alkaloid yield. The pH of the medium have shown significant effects with maximum biomass fresh wt., dry wt. and total alkaloid yield at 5.82 medium pH with elevated phosphate levels and total nitrogen concentration of 3710.10 mg/L compared to control MS medium with 2850 mg/L total nitrogen. At 3667.33 and 3752.48 mg/L of total nitrogen with enhanced phosphate supply showed reduced biomass fresh wt., dry wt. and total alkaloid yield at lower (4.51) and higher (7.32) medium pH respectively. Inclusion of 200 mg/L of tryptophan or phenylalanine as reduced nitrogen source in B5 medium buffered at 5.82 ± 0.2 pH showed enhanced biomass and alkaloid production. Hence, addition of nitrogen, phosphate, tryptophan, phenylalanine as nutrient in suspension culture stimulate their uptake to enhance cell biomass and total alkaloids production but as a function of pH of the medium.

ARTICLE HISTORY

Received: February 05, 2019
Revised: March 11, 2019
Accepted: April 30, 2019

KEYWORDS

Alkaloid,
Catharanthus roseus,
medium pH,
Nitrogen,
Phenylalanine,
Phosphorus,
Tryptophan

1. INTRODUCTION

The plant cell, tissue and organs cultured in vitro need to be supplied with complete nutrients in the medium [1]. Nitrogen and phosphate comprises the most important essential elements required for all plants growth and developmental process by feeding to the major metabolic pathways [2-6]. The nitrogen is available as nitrate and ammonia in the soil which are absorbed by plants through roots [7-9]. The nitrate gets transported through xylem reaches

*CONTACT: Nasim AKHTAR ✉ nasimakhtar111@gmail.com Department of Biotechnology, GITAM Institute of Technology, Gandhi Institute of Technology and Management, GITAM Deemed to be University, Rushikonda, Visakhapatnam (A.P.), India

ISSN-e: 2148-6905 /© IJSM 2019

to the parenchymatous cells of root and shoot wherein it is stored in vacuoles and does not cause any toxicity to the plants but need to be converted to the active form ammonia for metabolisms leading to growth, development and stress tolerance [10]. The uptake of nitrate is dependent on various factors like plant species, root pH, temperature etc. [11]. The energy required for ammonium assimilation is low in comparison to nitrate. To avoid the toxicity in roots of the plants ammonia is immediately converted to organic biomolecules [12]. In suspension culture of *C. roseus* the effect of UV-B and nitrate was compared individually as well as in combinations for cell biomass growth and alkaloid accumulation [13]. The increased supply of nitrogen inhibited the repair mechanism of the cell for UV-B radiation damages as under decreased N supply, plant becomes sensitive to UV radiation [14].

Nitrate supplied as nutrients was found as an important factor in alkaloid production by *C. roseus* plants under salinity stress [15]. On the other hand, changes in many growth parameters such as reduction of biomass, leaf epidermis damages, change in leaf colour and leaf fall due to reduced resistance was implicated with excess N deposition in plants [16]. It has also been reported that secondary metabolite production is affected differently by nitrate and ammonia, where nitrate promote secondary metabolite synthesis but ammonium ions inhibit it [17-18].

The importance of phosphate in secondary metabolite production was demonstrated as depletion of its level showing decreased biomass and 20-hydroxyecdysone (20-HE) production in hairy root culture of *Ajuga* [19]. On the other hand, *C. roseus* batch culture added with different phosphate concentrations showed increased cell growth and alkaloids with higher intracellular phosphate level [20].

Catharanthus roseus a perennial medicinal herb of family Apocynaceae is the store house of more than 130 secondary metabolites. The most important are the alkaloids (vincristine and vinblastine) with anti-cancer activity, anti-hypertension (ajmalicine and serpentine) [21]. Majority of alkaloids possess nitrogen containing heterocyclic reaction centers for these activities. But the problem with these alkaloids is very low concentrations and gets stored in leaf vacuoles. As the genes involved in alkaloid synthesis are expressed in tissue specific manner might be the cause of its low yield [22-24].

Biosynthesis of alkaloids by *C. roseus* cell gets initiated by joining of amino acid and a monoterpenoid i.e. tryptophan and geraniol respectively. The whole process is expressed by the involvement of two regulatory genes controlling about 30 biosynthetic steps catalyzed by 30 enzymes to produce 35 intermediates compartmentalized among 7 intra- and inter- cellular components [25]. This emphasizes the importance of tryptophan in TIA biosynthesis. Addition of tryptophan, secologanin, phenylalanine etc as precursor have been applied in cell suspension and hairy root culture of *C.* roseus to enhance the alkaloid production [26-27]. The effect of tryptophan on metabolic flux of indole alkaloids in *Catharanthus* revealed tryptophan feeding at 17 days of culture mimicked auxin effect on the cultures and increased the flux density of the indole alkaloids [27].

Precursor feeding as elicitation is an important strategy for increasing secondary metabolite productions in plants [28]. Plants like *Crocus sativus* also showed enhanced ajmalicine and strictosidine production upon treatment with exogenous tryptamine and loganic acid [29]. The induced production of enzyme phenylalanine ammonia lyase (PAL) and tryptophan decarboxylase (TDC) in cell suspension culture of *C. roseus* are involved in the production of phenylalanine and tryptophan respectively [30]. The 2 amino acids are the branched point product of common intermediate chorismate of the pathway and serve as the precursors for the synthesis of phenolics and indole alkaloids. The shikimate and terpenoid pathway catalyzed the production of tryptamine and secologanin which are combined to produce strictosidine, the precursor for monomeric alkaloid ajamalicine, catharanthine and

vindoline. The coupling reaction between catharanthine and vindoline catalyzed by anhydrovinblastine synthase (AVLBS) to produce vinblastine from a-3',4'- anhydrovinblastine whose mechanism of conversion is still unclear [30]. Hence, the regulation of alkaloid production by *C. roseus* in response to nitrate and other cultural and environmental factors is not fully understood and need to be explored in detail.

There is pressing need for an alternative strategy for natural sources to produce enhanced level of alkaloids and other desirable substances. Therefore attempts are made in the present investigation to investigate the role of nitrogen along with phosphate for biomass growth leading to higher alkaloid production in suspension medium with varying pH. The experiments are designed to maintain different pH of the medium by buffering action with higher levels of nitrogen and phosphate as well as supplemented with reduced nitrogen (tryptophan and phenylalanine) sources to enhance total alkaloid synthe sis.

2. MATERIALS and METHODS

2.1. Selection of Plants and Preparation Explants

Catharanthus roseus plants grown as seedlings in the GITAM campus nursery were used for explant supply. Successful establishment of callus was induced only from leaf segments as other explants were not successful. Induction of callus was achieved by culturing 1 cm^2 as described earlier [31-33].

2.2. Culture Media and Treatments

The normal strength of Murashige and Skoog's (MS) [1] and Gamborgh's (B5) culture medium [34] containing 3% (w/v) sucrose and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA was used as the standard treatments for the induction of callus and cell proliferations in suspension cultures. Callus induction from leaf explants and multiplication was achieved by continuous incubation for four weeks in the presence of this standard treatment on agar medium. The cell suspension culture was established in normal strength B5 culture salts with above standard treatment for control and modified variously for different treatments. About 100 ml of suspension medium was modified for each treatment and dispensed 25 ml in 4 conical flasks of 150 ml capacity. Each 25 ml suspension medium was inoculated either with 100 -150 mg friable callus (4-weeks old) from agar medium or with 5 ml of 2-weeks old suspension cultures.

The pH of the MS medium was adjusted to 5.8 ± 0.2. To study the effect of pH the B5 medium was buffered at 4.51 ± 0.2, 5.82 ± 0.2 and 7.32 ± 0.2 by the addition of specific volumes of molar concentrations of i) ammonium di-hydrogen orthophosphate ($NH_4H_2PO_4$) and ii) di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) along with standard sucrose and PGR combination. The B5 control and medium buffered at pH 5.82 ± 0.2 was also supplemented with 0 – 200 mg/L of reduced nitrogen sources either as tryptophan or as phenylalanine as precursor feeding in alkaloid biosynthetic pathway.

All these media were sterilized by autoclaving (15 lbs, 121°C, 15 min). The suspension cultures were maintained under continuous shaking at 150rpm over an orbital shaker incubator at 25 ± 2 °C. The callus cultures were maintained in an environmentally controlled air conditioned room. Each culture shelves were fitted with fluorescent lamps providing 2,000-3,000 Lux photon flux density under 16/8 hrs photoperiodic cycle.

2.3. Growth Measurement

2.3.1. Callus growth

Induction and proliferation of callus from leaf explants was achieved as described by [31-33]. 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. The procedure for measurement of fresh and dry weight of callus and cell biomass was described earlier [31-33] and represented as:

Callus Fresh Wt = {Weight of filter paper and the callus – Initial weight of dried filter paper}

Callus Dry Wt = {Weight of dried callus along with filter paper – Initial weight of dried filter paper}

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

Biomass Dry Wt = {Weight of the dried Biomass with Filter Paper – Weight of the dried Filter Paper}

2.4. Extraction and Quantifications of Total Alkaloids

Alkaloids were extracted using 20mg dried callus or cell biomass following the procedure detailed in by [31-33]. The total alkaloid was estimated following the modification of protocol [35; 36] along with Bismuth nitrate pentahydrate **(**$Bi(NO_3)_3.\ 5H_2O$**)** calibration curve. The alkaloid content was calculated as described earlier [31-33] and presented as:

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

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

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

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

2.5. Statistical Analysis

The mean of three replicates of experiments, standard deviation, analysis of variance (ANOVA) of results were performed by using SPSS 15 package for Window [37] in the present study.

3. RESULTS

3.1. Effect of Medium pH

The B5 medium is devoid of NH_4NO_3 and contain only KNO_3 as sources of nitrogen at a concentration lesser that present in MS medium. The results of the present study in B5 medium buffered at pH 4.51, 5.82 and 7.32 by addition of specific amount of molar solutions of **(A)** diammonium hydrogen phosphate **$(NH_4)_2HPO_4$** and **(B)** ammonium dihydrogen orthophosphate **($NH_4H_2PO_4$)** for the enhanced supply of total nitrogen (NH_4^++NO_3^-) and phosphate compared to MS medium on alkaloid production is presented in Table 1.

Table 1. Effects of nitrogen and phosphate source (combination of diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate) on modulation of medium pH for production of cellular biomass and yield of alkaloid in B5 suspension culture.

Sl. No.	Volume of 1M (A)	Volume of 1M (B)	Total nitrogen conc.	pH	Biomass Fresh wt	Biomass Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
	(ml)	(ml)	(mg/L)		g/L	g/L	mg/g dwt	mg/l	mg/L/d	% dwt
1	0	0	MS Medium (2850)	5.80 ± 0.2	16.806 ± 1.076	1.668 ± 0.162	5.575 ± 0.270	9.276 ± 0.689	0.662 ± 0.049	0.558 ± 0.027
2	10	90	1167.33 (3667.33)	4.51 ± 0.2	12.541 ± 0.098	1.463 ± 0.069	4.717 ± 0.044	6.900 ± 0.388	0.493 ± 0.028	0.472 ± 0.005
3	35	65	1210.10 (3710.10)	5.82 ± 0.2	19.166 ± 0.145	2.096 ± 0.090	5.844 ± 0.154	12.239 ± 0.204	0.874 ± 0.015	0.584 ± 0.016
4	60	40	1252.48 (3752.48)	7.32 ± 0.2	11.407 ± 0.093	1.317 ± 0.063	4.483 ± 0.087	5.905 ± 0.295	0.422 ± 0.021	0.448 ± 0.009
	F(3, 11)				79.140	23.115	36.757	90.961	91.490	36.838
	P<0.05				0.000	0.000	0.000	0.000	0.000	0.000

The Gamborgh's (B5) suspension medium [34] was supplemented with 3% sucrose (w/v) and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA. For increased pH, total nitrogen and phosphorus, respective volume of 1M stock solution each of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ were mixed as shown in the table to get specific pH and the total amount of nitrogen and phosphorus in the medium. The suspension culture was established with 100-150 mg of callus (4-weeks) or by cell biomass (2- weeks) inoculated @ 20% per culture vessel with 25 ml medium. The growth of cell biomass in suspension culture was recorded after 2-week of subculture.

The B5 medium maintained at lower pH (4.51 ± 0.2) prevented the solidification of agar even at 2.5% concentration in the medium. While at higher pH (7.32 ± 0.2) precipitation of nutrient salts was common both in agar and suspension conditions of B5 medium. This led to reduced biomass and alkaloid production at lower and higher pH even in the presence of elevated levels of nitrogen and phosphate even in the suspension medium. The B5 medium maintained at pH of 5.82 ± 0.2 by supplementing with elevated levels of nitrogen and phosphate was almost indispensable for enhanced production of biomass (fresh and dry weight) and alkaloids (content, production, productivity and the yield) compared to control cultures in MS suspension medium. All the observable parameters such as fresh and dry weight of biomass as well as content, production, productivity and the yield of alkaloid was significantly reduced at both higher or lower pH even though media contained elevated levels of both nitrogen and phosphorus. The possible reason for this reduction in response to the media pH had been reported in various studies discussed in the following section.

3.2. Effect of Nitrogen and Phosphate

The increased supply of nitrogen and phosphate source in B5 medium showed a significant effect on biomass (fresh and dry wt.) and alkaloid (content, production, productivity and yield) as compared to the MS medium (Table 1). Maximum biomass production (fresh wt., dry wt.) and yield of total alkaloid was obtained with elevated phosphate levels and total nitrogen concentration of 3710.10 mg/L compared to control MS medium with 2850 mg/L of total nitrogen (Table 1) both maintained at 5.8 ± 0.2 pH. The B5 medium with 3667.33 mg/L of total nitrogen buffered at lower pH (4.51 ± 0.2) or with 3752.48 mg/L of total nitrogen adjusted at higher pH (7.32 ± 0.2) containing elevated levels of phosphate showed significant reduction in all the observable parameters compared to the control MS medium with normal levels of nitrogen and phosphate. These reductions might be attributed to the precipitation of nutrient salts as a function of medium pH. Hence, the elevated levels of nitrogen and phosphate showed significant effects on all observable parameter as a function of pH of the medium.

3.3. Effect of Nitrogen Phenylalanine and Tryptophan

The effects of tryptophan or phenylalanine added in the medium as reduced sources of nitrogen showed significant ($P<0.05$) response on all the observable parameters (Table 2 & 3). Both the reduced sources of nitrogen followed similar response trend while tryptophan showed slightly better response compared to phenylalanine. There was continuous increase in all the observable parameters with the addition of either the tryptophan or phenylalanine. Hence, addition of 200 mg/L of either tryptophan or phenylalanine in the B5 medium was the threshold for maximum alkaloid yield as well as other parameters.

Table 2. Effects of tryptophan and nitrogen source (combination of diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate) on biomass production alkaloid yield.

Sl. No.	Tryptophan Concentration	Biomass Fresh wt	Biomass Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
	mg/L	g/L	g/L	mg/g dwt	mg/L	mg/L/d	% dwt
1	0.0	17.987 ± 0.122	2.089 ± 0.174	5.972 ± 0.160	12.492 ± 1.311	0.892 ± 0.093	0.597 ± 0.016
2	50	18.493 ± 0.101	2.293 ± 0.080	6.665 ± 0.160	15.287 ± 0.895	1.092 ± 0.064	0.666 ± 0.016
3	100	18.947 ± 0.122	2.450 ± 0.120	7.306 ± 0.203	17.913 ± 1.363	1.279 ± 0.097	0.731 ± 0.021
4	150	19.120 ± 0.120	2.672 ± 0.083	7.947 ± 0.118	21.240 ± 0.847	1.517 ± 0.061	0.794 ± 0.012
5	200	19.387 ± 0.061	2.766 ± 0.120	8.844 ± 0.231	24.478 ± 1.700	1.748 ± 0.121	0.884 ± 0.023
F(4, 10)		78.876	15.726	116.539	42.169	42.266	116.374
P<0.05		0.000	0.000	0.000	0.000	0.000	0.000

The Gamborgh's (B5) suspension medium[34] was supplemented with 3% sucrose (w/v) and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA. Additionally, the media was fortified with 35 ml and 65 ml of 1M stock solution each of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ respectively to get enhanced amount of nitrogen, phosphorus and medium pH at 5.82. This medium was further supplemented with 0 – 200 mg/L tryptophan to study the combined effects of nitrogen source. The suspension culture was established with 100-150 mg of callus (4-weeks) or by cell biomass (2- weeks) inoculated @ 20% per culture vessel with 25 ml medium. The growth of cell biomass in suspension culture was recorded after 2-week of subculture.

Table 3. Effects of phenylalanine and nitrogen source (combination of diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate) on biomass production alkaloid yield.

Sl. No.	Phenylalanine Concentration	Biomass Fresh wt	Biomass Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
	mg/L	g/L	g/L	mg/g dwt	mg/L	mg/L/d	% dwt
1	0.0	17.600 ± 0.080	1.942 ± 0.122	5.742 ± 0.247	11.174 ± 1.165	0.798 ± 0.083	0.574 ± 0.025
2	50	18.333 ± 0.122	2.106 ± 0.101	6.409 ± 0.160	13.502 ± 0.891	0.964 ± 0.064	0.641 ± 0.016
3	100	18.800 ± 0.120	2.317 ± 0.101	7.049 ± 0.248	16.346 ± 1.263	1.167 ± 0.090	0.705 ± 0.025
4	150	18.880 ± 0.120	2.499 ± 0.080	7.690 ± 0.204	19.229 ± 1.104	1.374 ± 0.079	0.769 ± 0.020
5	200	19.280 ± 0.160	2.593 ± 0.101	8.664 ± 0.248	22.478 ± 1.492	1.606 ± 0.107	0.866 ± 0.025
F(4, 10)		44.971	21.064	76.585	42.022	41.911	77.094
P<0.05		0.000	0.000	0.000	0.000	0.000	0.000

The Gamborgh's (B5) suspension medium [34] was supplemented with 3% sucrose (w/v) and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA. Additionally, the media was fortified with 35 ml and 65 ml of 1M stock solution each of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ respectively to get enhanced amount of nitrogen, phosphorurus and medium pH at 5.82. This medium was further supplemented with 0 – 200 mg/L phenylalanine to study the combined effects of nitrogen source. The suspension culture was established with 100-150 mg of callus (4-weeks) or by cell biomass (2-weeks) inoculated @ 20% per culture vessel with 25 ml medium. The growth of cell biomass in suspension culture was recorded after 2-week of subculture.

4. DISCUSSION

The growth of biomass and accumulation of products in cultured plant cells were dramatically altered by manipulating various media and environmental factors [38]. In the previous report enhanced production of biomass and alkaloid accumulation was optimized for the composition and strength of nutrient media, carbon source and plant growth regulators [31-33]. The normal or single strength B5 medium supplanted with 3% sucrose (w/v) was found as the best choice for the optimal biomass accumulation and production of secondary metabolite in cell suspension culture of *L. macranthoids* [39] and *C. roseus* [31-33]. Li et al [39] observed similar trends in biomass and chlorogenic acid production using 6-BA (2.0 mg/L) and NAA (0.5 mg/L) in B5 suspension culture of *Lonicera macranthoids*. In the present study production of biomass (fresh wt. and dry wt.) and alkaloid (content, production, productivity and yield) was investigated in response to increased supply of nitrogen (inorganic and organic) and phosphorus in suspension culture using B5 medium buffered at varying pH. The plant physiological process such as mineral nutrition, signaling, cell elongation, growth, development, environmental stress adaptation has been related to the pH of external media [40]. The pH of plant tissue culture media is mostly adjusted between 5 and 6 prior to the autoclaving which is dropped by 0.6 to 1.3 units after sterilization [41]. The cytosolic and vacuollar pH was increased by 3.0 and 1.3 units with a change in media pH from 4.5 to 6.3 in *Chenopodium rubrum* cell suspension culture [42] while the specific buffering potential was decreased continuously by 60% [40]. Production of biomass and withanolide A in *Withania somnifera* cell culture was not affected by media pH set at high or low. On the other hand *Daucus carota* cell culture excreted 90% of anthocyanin at 5.5 media pH than at 4.5 [43]. The release of secondary metabolites in to medium was due to cell membrane permeability associated with shift of media pH at low or high [44]. Reducing the media pH even for shorter time was found beneficial for up to 50% release of betalains from *Beta vulgaris* transformed root culture system [45]. The flavonolignan content in *Silybium marianum* hairy root culture was maximum at 5.0 medium pH and decreased with the increase of pH to 5.7, 6, and 7 [46]. The growth and ginsenoside production in hairy root culture of ginseng was maximum when the initial pH of the medium adjusted between 6.0 to 6.5 prior to autoclaving and significantly reduced at initial pH 4.0 or lower or above 7.0 [47]. Li et al [39] studied the effect of pH on the production biomass and chlorogenic acid in cell suspension cultures of *Lonicera macranthoids*. The highest biomass of 6.52 g/L and 6.76 g/L was achieved at 5.5 and 6.0 media pH respectively, showing maximum chlorogenic acid content. They found that production of biomass and secretion of chlorogenic acid was unaffected at higher or lower pH of the media, however, intracellular accumulation of acid was continued in medium at pH 7.0. Similar results were also observed in *E. ulmoides* cell suspension culture with maximum accumulation of chlorogenic acid at medium pH of 5.3 and continued production of chlorogenic acid up till the medium pH adjusted to 7.0 [48].

The inorganic and organic nutrients of culture media are easily manipulated for the enhanced and modified production of different chemical components of plant cells. In an earlier report Nowacki et al. [49] reported that the phosphate and nitrogen source provided by both ammonium di-hydrogen orthophosphate ($NH_4H_2PO_4$) and di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) helps in increasing the alkaloid production by converting the excess amino acids produced to secondary metabolites. When different levels of fructose, galactose, glucose and sucrose carbon source were compared, the medium containing 3% sucrose showed maximum growth of cell biomass and the alkaloid yield [50]. In *L. aestivum* shoot culture, increased production of galanthamine was optimized applying different concentrations of sucrose, NO_3^-, NH_4^+, PO_4 ions [51]. Culture medium supplied with enhanced nitrogen levels and the ratio of NH_4^+/NO_3^- (molar concentrations) showed to stimulate alkaloid synthesis. But the uptake of

nitrate from medium and assimilation of ammonia by plant cell decreased the medium pH [52]. The uptake of nutrients and buffering components (NH_4^+, NO_3^-, PO_4^{3+}) from the medium during initial growth phase or the acids (lactate, malate, succinate) secretion at the later stages in suspension culture caused change in hydrogen ion concentration affecting the secondary metabolite production [40; 53]. An optimum of 90 mM concentration of total nitrogen in 4:1 ratio of NH_4^+/NO_3^- in hairy roots culture of *Anisodus acutangulus* showed highest yield of tropane alkaloid from the maximum biomass produced [50]. The hairy roots induced from leaf explants culture of *A. acutangulus* was significantly affected by variations in media pH. At pH 6.5 the hairy roots biomass increased two fold as compared to media with pH 4.5. Whereas, at pH 4.5 tropane alkaloid was produced with maximum yield [50]. Influence of external pH on alkaloid production and excretion by *C. roseus* resting cell suspensions reported [54]. In root cultures of *Brugmansia candida* production of scopolamine as well as hyoscyamine was increased at medium pH 5.5 adjusted with acetic acid inducing the release of these alkaloids [55]. While these alkaloids were reduced significantly at pH 3.5 and 4.5, but a medium pH of 4.5 and above showed significant release of scopolamine and hyoscyamine. A change in cell membrane permeability stimulated by acetic or citric acid might be the cause for the release of these two alkaloids [55].

Both tryptophan and phenylalanine at all the levels in B5 medium showed highly significant increase in all the parameters in comparison to the standard control conditions. The highest growth was observed at 200mg/l concentration of both the precursors across all parameters. A combination of the precursors along with the diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate showed even higher response across all parameters. In conformity to the results of present study Whitmer et al. [56] reported that supplying tryptamine or tryptophan along with the iridoid precursors to transgenic cell lines S1 of *C. roseus* resulted in even further increase of alkaloid accumulation. Addition of a moderate concentration of reduced nitrogen source as L-glutamine followed by L-asparagine in medium also enhances the in vitro process of somatic embryo proliferation and maturation in *C. roseus* [57]. There was 2 fold increase in production of glucotropaeolin from *Tropaeolum majus* L. when phenylalanine and cystein fed as precursor amino acids in hairy root cultures [58]. Moreover, *Cistanche deserticola* cell culture fed with L- phenylalanine as precursor showed 75% higher phenylethanoid glycosides than the media without it [59]. On other hand, Namdeo et al. [60] reported that the key to successful protocol using precursor feeding to plant cell culture system lies in identification of cheapest by product of the other process which can be converted to desired secondary metabolites by selected plant cell line. Where as in *L. macranthoids* cell growth was inhibited by addition of phenylalanine at concentrations higher than 50 mg/L contrary to the content of chlorogenic acid which was gradually increased as compared to the control. The B5 medium added with 200 mg/L phenylalanine showed highest amount (18.0 mg/g DW) of chlorogenic acid production [39]. The total alkaloid of the *Fritillaria cirrhosa* cultures was significantly improved by adding different concentration of phenylalanine [61]. Similarly, soybean cell suspension cultures showed increased content of daidzein when medium was supplemented with phenylalanine for 48 h [62].

Nutrients and environment conditions such as potassium nutrition [63], nitrogen nutrition [12], and salinity [15] showed enhanced accumulation of alkaloids in *C. roseus*. Plants exposed to excess UV-B radiation and other physical or chemical factors [64-66] produce serious oxidative stress response to tolerate them. Similarly, *C. roseus* cell suspension cultures supplied with adequate nitrogen source showed enhanced alkaloid production under UV-B treatment [67]. The yield and accumulation of UV absorbing compounds in liverwort was found changing dynamically in response to UV-B and photosynthetic radiations to revert the inhibition due to UV-B radiation [68]. Malik et al. [69] also showed that an optimum pH of 5.5-6.5 enhanced *in vitro* shikonins production from various species like *Lithospermum, Arnebia, Alkanna,*

Anchusa, Echium and *Onosma*. Nitrogen is an important constituent of alkaloids and required for their synthesis while, phosphorus influence greatly the alkaloid synthesis in *C. roseus* L. [70]. Hassan et al. [71] showed enhanced alkaloid and other growth characteristics of *C. roseus* under the field application of nitrogen and potassium fertilizers. The highest yield of total alkaloid including the vincristine and vinblastine was obtained with maximum nitrogen but with lowest concentrations of potassium. Abdolzadeh et al. [72] found that feeding *C. roseus* plants with 11 mM of total nitrogen (nitrate+ammonia) significantly enhanced the vincristine, vinblastine and the total alkalods production with increased cellular pool of nitrogen, amino acids and proteins. Nitrogen deficiency showed reduction in total chlorophyll content [73; 74]. While highest plant biomass in *C. roseus* was reported at 200 mg N dm^{-3} in the substrate [70] and the highest yield of alkaloids was obtained at 300 mg N dm^{-3} [75].

Further, An et al. [76] and Zhang et al. [77] have pointed out that nitrate reductase-dependent nitric oxide signaling mediated the flavonoid accumulation in UV-B-induced plant leaves fed with nitrate. Adequate supply of nitrogen source showed significantly increased contents of H_2O_2 and MDA compared to control *C. roseus* plants indicating an enhanced tolerance to oxidative stress caused by UV-B radiation treatment [13]. The increased biosynthesis of different compounds with nitrogen, and alkaloids by extra nitrate supply might be involved in fighting the plants to various environmental and radiation stresses [13]. This hypothesis was further supported by the findings of unique catharanthine transporter as UV-B-induced signaling events under suspension cultures [67] and the involvement of ATP-binding cassette transporter with changing environmental conditions in *C. roseus* leaf surface cells [78].

Monnerat et al. [79] found that *C. roseus* plants supplied with nitrogen fertilizer in combinations with mycorrhizal fungi *C. etunicatum, G. margarita* and *R. intraradices,* showed increased production of ajmalicine. Hashemabadi et al. [80] reported enhanced level of leaf vindoline (1.94 mg/g DW) and root alkaloid (1.11 mg/g DW) and other plant characteristics by application of 40 mg kg^{-1} soil nitrogen fertilizer along with *Azotobacter* and fungal compost. A positively significant effect of plant growth promoting rhizobacteria (PGPR) on the alkaloid content of *C. roseus* was reported recently [81-84]. Similarly, application of PGPR on the induction of secondary metabolite synthesis, particularly of alkaloids had been presented earlier [85].

Hence, all these studies demonstrated that both biotic and abiotic stresses have either inductive, stimulatory or enhancing effects on the production of alkaloids and other secondary metabolites under the field conditions as well as in cell culture system of *C. roseus* and many other plants by enhanced organic or inorganic nitrogen and phosphorus supply in a pH dependent manner similar to our present study.

5. CONCLUSION

The callus and cell suspension culture technique has proven as a feasible alternative for enhancing the production of alkaloids and other secondary metabolite. Since, *C. roseus* are categorized as high nitrogen demanding plants which tolerate wide range of soil nutrients, salinity and pH. Plants supplied with balanced level of essential nutrients have shown increased crop growth, biomass and yield of the various secondary metabolites. Applying similar strategies in cell suspension culture system would have positive effects on biomass and alkaloid accumulation. We found that B5 medium supplemented with enhanced levels of nitrogen and phosphorus as a combination of **(A)** diammonium hydrogen phosphate **$(NH_4)_2HPO_4$** and **(B)** ammonium dihydrogen orthophosphate (**$NH_4H_2PO_4$**) significantly affected all the observable parameters such as fresh and dry weight of cell biomass as well as alkaloid content, production, productivity and the yield in a pH dependent manner. The medium pH of 5.82 showed the maximum response of biomass and alkaloid yield while the lower and higher pH of the medium

were slightly inhibitory compared to the control cultures established in MS medium. The enhanced alkaloid biosynthesis in response to increased total nitrogen (NH_4^++NO_3^-) and phosphate at an adequate pH of test culture medium might caused by uptake and transport of these nutrients increasing the intracellular pool of nitrogen containing compounds and the intermediates of the pathway as supported by various published reports reffered in the present study. Addition of tryptophan or phenylalanine as reduced nitrogen source in B5 medium buffered at 5.82 ± 0.2 pH further enhanced the biomass and alkaloid production. There might be involvement of more complex interactions mechanisms of pH, phosphate, organic and inorganic nitrogen as nutrient combined with other stresses to trigger the induction of alkaloid biosynthesis. There is need for further research to elucidate the mediation of molecular regulation and signaling mechanisms involved in the pathways leading to enhanced production of specific alkaloids.

Acknowledgement

The financial support provided by University Grant Commission, New Delhi for the major research project (F.: 42-207/2013 (SR) for the period 1.4.2013-31.3.2017) to the corresponding author is gratefully acknowledged.

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).

Conflicts of Interest

All the authors declared that there is no conflict of interest with regards to any part of the manuscript.

ORCID

Malay Ranjan Mishra https://orcid.org/0000-0003-1714-0061
Rajesh Kumar Srivastava https://orcid.org/0000-0001-5524-1045
Nasim Akhtar http://orcid.org/0000-0002-8867-985X

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