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ABIOTIC STRESSES OF SALINITY AND WATER TO ENHANCE ALKALOIDS PRODUCTION IN CELL SUSPENSION CULTURE OF *CATHARANTHUS ROSEUS*

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ABSTRACT
The present study aimed to investigate effect of abiotic stresses of salinity induced by NaCl and water stress by PEG (6000) on *Catharanthus roseus* biomass and alkaloids production using B5 suspension culture medium. Different levels of saline stress was created by addition of 0, 25, 50, 75, 100, 125, 150, 175, 200 mM NaCl along with standard inductive treatments with 0.5 mg/l of 2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA in B5 suspension medium containing 3% sucrose. The water stress was created by addition of 3, 6, 9, 12, 15 and 18% PEG (6000) in B5 suspension medium to study their effect on biomass and alkaloid production following standard inductive treatments. The fresh and dry wt of the biomass tolerated the salinity stress upto 100mM NaCl and showed enhanced levels of alkaloids compared to control cultures. B5 suspension medium with 150mM NaCl showed best alkaloid yield than the control cultures while higher levels of salinity in the medium significantly decreased all the observable parameters of biomass fresh and dry wt. as well as alkaloid content, production, productivity and yield. There was significant increase in all the observable parameter by addition of PEG (6000) up to 15% concentrations. In B5 suspension culture maximum alkaloid content (6.562 mg/g dry wt) was observed in 150 mM NaCl containing medium and 6.383 mg/g dry wt. in 15% PEG (6000) added medium. The present study indicated that the alkaloid yield was enhanced significantly in B5 suspension medium by creating abiotic stresses of salinity and water with NaCl and PEG (6000).

KEYWORDS: Abiotic stress, Alkaloid, *Catharanthus roseus*, B5 medium, NaCl, PEG.

INTRODUCTION

Plants are frequently exposed to large number of environmental stresses such as high/low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity (Amirjani, 2010a, 2011a, 2011b, 2012a, 2012b, 2013, 2015; Mahajan and Tuteja, 2005). Being sessile in nature, the growth and development in plants are harmfully affected by both biotic and abiotic stresses (Andrade *et al*., 2013) which forced plant to adopt and evolve continually. These factors also play major role in determining the distribution of plant species across different types of environments (Zhu, 2002). These abiotic stresses are associated with low water availability to plant cells (drought), cellular dehydration by formation of extracellular ice (freezing), altered ion content and water uptake (salinity), decreased uptake of essential nutrients from the soil or the build-up of toxic ions (ionic stress), restricted photosynthesis (excess light) or increased hardness of the soil that restricts root and shoot growth (Akhtar *et al*., 2016; Verslues *et al*., 2006).

Many physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates and nutrient metabolism are affected during water deficit reducing the plant growth (Akhtar *et al*., 2016; Farooq *et al*., 2009; Jaleel *et al*., 2008a,b; Rahbarian *et al*., 2011). Abiotic stress such as drought and salt leads to the over production of highly toxic reactive oxygen species (ROS) in plants. Production of ROS results in oxidative stress causing damages to proteins, lipids, carbohydrates and DNA (Shalaby *et al*., 2010; Tang *et al*., 2009). Plants resist low water potential and related stresses naturally by modifying water uptake and loss to avoid low water potential, accumulating solutes and cell walls modification to overcome dehydration due to low water availability and synthesize protective proteins as mechanisms to tolerate reduced water content by preventing or repairing cell damages (Akhtar *et al*., 2016; Verslues *et al*., 2006). Nature has provided plants highly efficient antioxidant defense system for detoxification, mitigation and repair the damage initiated by ROS. These specific protective mechanisms include either the non-enzymatic constituents, like ascorbic acid (AsA), tocopherols, anthocyanin, flavonoids, carotenoids and glutathione (GSH) or the enzymatic components, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) nitrate reductase (NR), glutamine synthase (GS), and NADH-dependent glutamate dehydrogenase (NADH-GDH) (Akhtar *et al*., 2016; Amirjani, 2015; Fatima *et al*., 2015; Lee and Lee, 2000). Prolonged or severe stress condition has negative impact on growth and survival of the plant even though they will produce alkaloids and other secondary metabolites as their defense mechanism (Selmar, 2008). The abiotic stresses of both salinity and water not only adversely affected the plant growth development but also modulates the levels of secondary metabolites (Fatima *et al*., 2015; Flowers, 2004;

Radhakrishnan & Kumari, 2013; Sairam & Tyagi, 2004; Zhang *et al*., 2012).

Catharanthus roseus (Apocynaceae), is widely cultivated and naturalized around Southeastern and Eastern Madagascar and other parts of the world. It is also called as Madagascar periwinkle, vinca, Cape periwinkle, rose periwinkle, and rosy periwinkle in different regions. The genus consists of two species categorized as '*Rosea*' for the pink flowered plant and '*Alba*' for the white flowered plant (Jaleel *et al*., 2007a-f). The plant is the store house of more than 130 different types of secondary metabolites. Amongst them the most important are alkaloids vincristine and vinblastine known to possess anticancer properties (Alam *et al*., 2017; Verpoorte *et al*., 1997). The plant is very well applied against a wide range of diseases like diabetes, hypertension, and cancer (Aslam *et al*., 2010; Naeem *et al*., 2017b) in Egypt, China, India, Greece and other places. A salt tolerant line has been selected for increased alkaloid production in *C. roseus* (Vázquez-Flota & Loyola-Vargas, 1994). Application of growth regulators has been reported to mitigate the adverse effects of salinity and other stresses (Jaleel *et al*., 2007a-f; Kaur *et al*., 2017; Mishra *et al*., 2018 a,b). Increased alkaloid content of *C. roseus* seedlings has been reported by NaCl stressed plants (Fatima *et al*., 2015; Wang *et al*., 2008) and by addition of sea water (Wang and Liu, 2010). Liu *et al.* (2014) showed that addition of $CaCl_2$ to NaCl stressed *C. roseus* plants has a significant role in partial alleviation of salinity stress and promoted the secondary metabolism by increased enzyme (TDC and POD) activities and alkaloid contents. Sodium buildup in *C. roseus* leaves has been affected by saline treatment with over 4- and 3-fold increases for Na^+ ions after 14days of low and high salt treated plants respectively. Most of the soluble sugar (glucose, sucrose), free amino acids (FAA) (proline, threonine) and total FAA as well as nitrate reductase (NR), NADH-dependent glutamate dehydrogenase (NADH-GDH) of both roots and shoots, glutamine synthase (GS) of roots and production of vinblastine, were significantly accumulated in high nitrogen fed salt treated plants in the first week and low nitrogen fed salt treatment in the second weeks respectively. The role of internal ammonium and free amino acids during salt stress of *C. roseus* plant was studied by Zhonghua *et al.* (2011). These compounds may acts as compatible solute to adjust osmotic stress and retain ion homeostasis in salt stressed plants (Osman *et al*., 2007; Zhonghua *et al.*, 2011). Despite the relative great number of reports on the medicinal aspects (Filippini *et al*., 2003), growth regulator effects (Idrees *et al*., 2011; Kalidass *et al*., 2010), salinity and water-stress (Jaleel *et al*., 2007a-f) studies on *C. roseus* plants, there are only a few attempts to explain the physiological basis of salt and secondary metabolite accumulation (Jaleel *et al*., 2007a, b; 2008a,b).

The field grown *C. roseus* plant produce very trace amount of these alkaloids, hence, alternative solutions for a cost effective and mass production of the drugs has been initiated applying various abiotic and biotic stresses (Iskandar and Prayogo, 2016; Jaleel *et al.*, 2007a-f; 2008; Naeem *et al*., 2017b; Vázquez-Flota and Loyola-Vargas, 1994; Verpoorte *et al*., 1991; Zhao and Verpoorte, 2007). In the present study we aim to enhance alkaloids production per unit culture system of *C. roseus* applying abiotic stresses of salinity and water deficit condition in B5 suspension medium.

MATERIALS AND METHODS

Selection of Plants and Preparation Explants:

The leaves were collected from *Catharanthus roseus* plants grown as seedlings in the GITAM campus nursery as hedge plant. Callus cultures were established with $1cm^2$ leaf segments as other explants were not successful (Mishra *et al*., 2018a, b). The leaf were washed thoroughly under running tap water for 15 min, treated in 1% Savlon along with 8-10 drops of Tween-20 for five min. Surface sterilization was carried out under sterile environment (LAF) by treating with 70% ethanol for 60 seconds followed by 0.05% $HgCl_2$ for about 15minutes with continuous stirring to ensure complete sterilization. The explants were washed 3-4 times with autoclaved double distilled water to remove traces of $HgCl_2$. The surface sterilized leaf explants were trimmed to size and 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 Treatments

Induction and proliferation of callus was performed on normal strength Murashige and Skoog (MS) (Murashige and Skoog,1962) medium containing 3% (w/v) sucrose and different combinations of 2,4-D, Kinetin, and NAA. The leaf explants were treated continuously for four weeks in the presence of above growth regulators and each experiment was repeated three times. For establishment of cell suspension normal strength of both MS and B5 culture medium (Gamborgh *et al*., 1968) was used.

To study the effect of salinity on growth and multiplication of callus, biomass and alkaloid yield both MS agar and B5 suspension medium were added with 0, 25, 50, 75, 100, 125, 150, 175 and 200 mM NaCl along with standard sucrose and PGR combination. Similarly, both MS agar and B5 suspension medium were added with 0, 1, 3, 6, 9 and 12% PEG (6000) along with standard sucrose and PGR combination to study the effect of water stress on growth and multiplication of callus, biomass and alkaloid yield. Callus and biomass obtained from both the medium were used for alkaloid extraction and quantification.

The pH of the culture media was adjusted to 5.8 ±0.2 before autoclaving (121°C, 15 min). The cultures were incubated at 25 ±2°C 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.

Growth Measurement

Callus growth

Induction and proliferation of callus from leaf explants was achieved as described by Mishra *et al.* (2018a, b). 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 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 - 3days. The callus along with filter paper was further incubated at 40°C in dry heat oven for 2-3 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 from suspension culture were pooled by filtration of 100ml medium for each treatment using the pre-weighed dried 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 1hr. 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.

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

Extraction of Total Alkaloids:

A 20mg 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 –50rpm for proper mixing of the solvent. The total mixture was then centrifuged at 3000 RPM for 10 minutes and about 9.0ml 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

The total alkaloid was estimated following the modification of protocol (Kalidasss *et al*., 2010, Sreevidya and Mehrotra, 2003) along with Bismuth nitrate pentahydrate **(**$Bi(NO_3)_3.\ 5H_2O$**)** calibration curve. A 1mL 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 40ml distilled water and 10ml 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 entrifugate 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 1mL 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 estimated were represented as mg/g dry weight of callus or the cell biomass and calculated as under:

Alkaloid content (mg/g dwt): {(Concentrations (µg) / 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 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, analysis of variance (ANOVA) of results was performed by using SPSS 15 package for Window (SPSS Inc., USA) in the present study.

RESULTS AND DISCUSSION

Effect of salinity on biomass and alkaloid production

Successful callus establishment was achieved (Mishra *et al*., 2018a, b) only with leaf segments as other explants contaminated (100%) with *Fusarium oxysporum* an endophytic fungus of *C. roseus* (Kumar *et al*., 2013). The condition for the production of callus and biomass were optimized using various combinations of 2,4-D, kinetin and NAA along with different concentrations of sucrose and other carbohydrates using variable strength of B5 and MS medium formulation under agar solidified and suspension conditions (Kaur *et al*., 2017; Mishra *et al*., 2018a,b; Naeem *et al*., 2017a).

TABLE 1: Effect NaCl on Growth and Multiplication of Biomass and Alkaloid Production in B5 Suspension Medium*.

Sl. No.	NaCl Concentrations	Biomass Fresh wt	Biomass Dry wt	Alkaloid Content	Alkaloid Production	Alkaloid Productivity	Alkaloid Yield
	mM	g/l	g/l	mg/g dwt	mg/l	mg/l/d	% dwt
1	0	18.267 ± 0.442	1.945± 0.024	5.229± 0.277	10.171± 0.538	0.727± 0.039	0.523± 0.028
2	25	18.320± 0.342	1.868± 0.026	5.332± 0.248	9.956± 0.407	0.711± 0.029	0.533± 0.025
3	50	18.187± 0.061	1.923± 0.073	5.460± 0.335	10.491± 0.651	0.750± 0.047	0.546± 0.033
4	75	18.493± 0.092	2.010± 0.067	5.588± 0.160	11.227± 0.105	0.802± 0.007	0.559± 0.016
5	100	18.373± 0.450	2.024± 0.065	6.075± 0.204	12.288± 0.329	0.878± 0.024	0.608± 0.021
6	125	17.267± 0.281	1.980± 0.095	6.357± 0.270	12.601± 1.101	0.900± 0.079	0.636± 0.027
7	150	16.773± 0.467	1.976± 0.225	6.562± 0.270	12.982± 1.759	0.927± 0.126	0.656± 0.027
8	175	16.227± 0.489	1.735± 0.122	5.281± 0.270	9.144± 0.246	0.653± 0.017	0.528± 0.027
9	200	13.227± 0.761	1.444± 0.068	4.998± 0.277	7.214± 0.431	0.515± 0.031	0.500± 0.028
	F(8, 18)	47.921	9.807	13.174	16.465	16.465	13.256
	P<0.05	0.000	0.000	0.000	0.000	0.000	0.000

*(B5 suspension medium prepared 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. Salinity stress was created by addition of 0-200mM of NaCl into suspension medium. About 100-150 mg friable calluses or 5ml of 2- week old suspensions were inoculated per culture vessel. The biomass was recorded after 2- weeks of sub-culture. Data represent the mean values of 3 replicates of experiments ± standard deviations).

TABLE 2: Effect PEG (6000) on Biomass and Alkaloid Production in B5 Suspension Medium*.

Sl. No.	PEG Concentrations	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	0.0	18.280 ± 0.461	1.946 ± 0.021	5.203 ± 0.118	10.129 ± 0.323	0.723 ± 0.024	0.521 ± 0.012
2	3.0	18.067 ± 0.500	2.067 ± 0.025	5.306 ± 0.077	10.970 ± 0.289	0.784 ± 0.021	0.531 ± 0.008
3	6.0	18.520 ± 0.288	2.045 ± 0.139	5.691 ± 0.077	11.632 ± 0.684	0.831 ± 0.049	0.569 ± 0.008
4	9.0	17.560 ± 0.629	1.985 ± 0.131	5.998 ± 0.077	11.913 ± 0.934	0.851 ± 0.067	0.600 ± 0.008
5	12.0	18.227 ± 0.522	1.946 ± 0.091	6.383 ± 0.154	12.408 ± 0.358	0.886 ± 0.026	0.638 ± 0.016
6	15.0	12.907 ± 0.978	1.415 ± 0.092	5.640 ± 0.160	7.971 ± 0.373	0.570 ± 0.027	0.564 ± 0.016
7	18.0	9.947 ± 0.782	0.889 ± 0.037	4.717 ± 0.118	4.191 ± 0.149	0.299 ± 0.011	0.471 ±0.012
	F(4, 10)	86.953	73.953	66.248	97.979	97.026	65.898
	P<0.05	0.000	0.000	0.000	0.000	0.000	0.000

*(B5 suspension medium prepared 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. Water stress was created by addition of 0 - 18% of PEG (6000) into suspension medium. About 100-150 mg friable calluses or 5ml of 2- week old suspensions were inoculated per culture vessel. The biomass was recorded after 2- weeks of sub-culture. Data represent th e mean values of 3 replicates of experiments ± standard deviations).

The analysis of variance for salinity stress using various concentrations of NaCl in B5 suspension medium along with the standard combinations of sucrose and PGR's on biomass and alkaloids production was performed and presented in table 1. Different concentrations of NaCl significantly affected the all the observable parameters of biomass fresh and dry weight as well as alkaloid content, production, productivity and the yield. There was fluctuating response up to 75mM NaCl followed by significant decrease in fresh of cell biomass in higher concentrations. The fresh and dry weight of biomass was fluctuating but increased with addition of upto 100mM NaCl followed by decrease in higher concentrations as compared to the control. Alkaloid content, production, productivity and the yield was significantly enhanced by addition of NaCl in the B5 medium with maximum response in the presence of 150mM concentration. All the parameters were significantly reduced in NaCl concentrations higher than 150mM compared to the control culture (Table 1).

The role of NaCl as a stress for increasing the content of alkaloids in *C. roseus* L. was studied by Smith *et al.* (1987). They proved that at lower salinity stress in MS medium *C. roseus* suspension culture showed high accumulation of catharanthine. Zhao *et al*. (2000a) showed the positive impact of rare earth and heavy metals in inducing indole alkaloid production in *C. roseus* in MS suspension culture. The osmotic shock rendered by sodium alginate (28 mg/l), polyvinyl pyrrolidone (24mg/l) stimulated more catahranthine production in *C. roseus* in MS suspension culture compared to NaCl or KCl added medium (Zhao *et al*., 2000b).

Effect of water stress on biomass and alkaloid production:

The analysis of variance for different concentrations of PEG (6000) in B5 suspension medium along with standard combinations of sucrose and PGR's was performed and presented in table 2. Addition of PEG (6000) in B5 suspension medium mimicking more negative water potential, significantly affected the biomass and alkaloid production along with the other nutrient and sucrose. All the parameters including the fresh and dry weight of cell biomass was fluctuating but showed significantly enhanced response up to 12% of PEG (6000) in the medium as compared to the control. The lower concentration (3-6%) of PEG (6000) were supportive to enhance biomass fresh and dry weight but alkaloid content, production, productivity and yield was less compared to 12% PEG (6000) concentration. The higher concentration (15-18%) of PEG (6000) was detrimental on the entire process of biomass and alkaloid production. Hence, a 12% PEG (6000) in the medium along with standard sucrose (3%) and PGR's combination (Table 2 footnote) showed maximum alkaloid content, production productivity and yield along with the fresh and dry weight of biomass as compared to control.

In the present study on *C. roseus,* the increased biomass might be due to the accumulation of osmolyte. Enhanced antioxidative potential, indole alkaloid accumulation, proline metabolism under water deficit and salinity in *C. roseus* has been reported recently (Ahmed *et al*., 2007; Idress *et al*., 2017; Jaleel *et al*., 2007a-f,; Osman *et al*., 2007). Similarly the alkaloid production was enhanced due to water stress experienced by cell suspension culture tolerating up to 12% of PEG (6000) in the present study. The higher levels of PEG (6000) in the medium reduced biomass as well as alkaloid production significantly. Iskandar and Prayogo (2016) have reported the production of vinblastine and vincristine callus culture treated with 12% PEG (4000). Although plants response to adverse environments resulting in elevated accumulation of alkaloid, compatible solutes, enzyme systems to overcome reactive oxygen species has been achieved in many studies but the detailed mechanism still remains unclear (Zhao *et al*., 2005).

Aslam *et al.* (2008) for the first time showed the effect of PEG on *C. roseus* embryos. They found that PEG at 1.87g/l affect embryo proliferation while other higher concentrations were toxic and inhibited the growth. Ahmed *et al*. (2007) found that increase in osmotic pressure in *Oryza sativa* induced either with PEG or NaCl at lower levels lead to accumulation of proline in the cytosol as an osmolyte hence, subsequently increased the dry wt. of the biomass.

Al-bahrany (2002) found drastic reduction of callus fresh wt. with a progressive reduction in callus water content of *Oryza sativa* with increasing PEG concentration in MS suspension. Biswas *et al.* (2002) also showed that PEG 6000 at higher levels had a negative correlation towards rice seed germination in various varieties. Osmotic regulation can enable the maintenance of cell turgor for survival or to assist plant growth under severe drought conditions (Shao *et al*., 2008). The reduction in plant height was associated with a decline in the cell enlargement and more leaf senescence (Bhatt and Rao, 2005). The content of alkaloids in *C. roseus* has been found influenced by individual factor, such as stage of plant growth, drought and other stress (Misra and Gupta, 2006; Osman *et al*., 2007). Under drought stress the height, the chlorophyll content, fresh and dry weight of plants has been reduced. Amirjani (2013) has concluded that all the physiological, biochemical and growth parameters were significantly reduced under the applied drought stress on *C. roseus*. For example total protein decreased to 77% and total chlorophyll decreased by 27%. Whereas, total alkaloid content significantly increased to maximum 187% compared to the control. Vincristine and vinblastine content of the seedlings grown under drought increased to 175% and 171% compared to the control, respectively.

Enhanced production of indole alkaloids provided protection against microbial infection, herbivores consumption, and many abiotic environmental stresses (Cordell, 2013). Oxidative injuries and metabolism disturbance in plants under conditions of high-energy irradiation with UVB expressed with largely released reactive oxygen species (ROS) (Apel and Hirt, 2004). Zhu et al. (2015) found that the contents of the indole alkaloids strictosidine, vindoline, catharanthine, and ajmalicine in *C. roseus* leaves were increased under the binary stress of UV-B irradiation and dark incubation. Both UV-B irradiation and dark incubation were essential stresses for alkaloid induction (Zhang *et al*., 2014).

Zhongua *et al.* (2011) found an inhibition of biomass but with increased resistance by enhanced production of soluble sugars, free amino acids such as proline, threonine, arginine, and aspartate, increased photosynthetic rates combined with reduced transpirational rate in the

treatment of *C. roseus* plants with salinity and fed with N_2 sources. Further, salt stress clearly inhibited GS and NR activity with enhanced NADH-GDH activity.

Many factors such as stage of plant growth, abiotic stresses, and nitrogen fertilization was found to influence the plant height, weight, leaf number, and leaf length but increased leaf weight in *C. roseus* (Misra and Gupta, 2006). Indole alkaloid production in *C. roseus* was increased by abiotic stresses, such as salinity (Vázquez-Flota and Loyola-Vargas, 1994), drought (Jaleel *et al*. 2007a, b, e) and wounding (Vázquez-Flota *et al*., 2004). Idrees *et al.* (2011) found that salt stress significantly reduced plant height, leaf-area index, shoot and root fresh weights, shoot and root dry weights. Activities of nitrate reductase, carbonic anhydrase and many photosynthetic parameters were gradually decreased with increasing NaCl concentrations. Ascorbic acid, total alkaloids and antioxidants enzymes superoxide dismutase, catalase and peroxidase also declined in NaCl-treated plants. They found that the content of proline, vincristine (14.0%) and vinblastine (14.6%) and electrolyte leakage in plants treated with NaCl was enhanced by foliar spray of salicylic acid to overcome the adverse effect of salinity. Salicylic acid also known to alter antioxidant and phenolics metabolism in *C. roseus* grown under salinity stress (Misra *et al.*, 2014). Li and Liu (2003) found that *C. acuminata* seedlings tolerated moderate NaCl levels (upto 75mM) in growth media while higher concentrations significantly reduced all morphological characteristics. Furthermore, high NaCl levels (above 75mM) increased camptothecin concentrations both in leaves and roots, whereas low NaCl levels (below 75 mM) had no effect on camptothecin concentrations. Treatment of cell cultures of *C. roseus* with GA_3 and Pseudomonas elicitors improved indole alkaloid production (Jaleel *et al*., 2007a,c).

Salt-tolerant plants exhibited various antioxidative enzyme systems for efficient removal of ROS (Liu *et al.*, 2014). In a sandy culture experiment Liu *et al.* (2014) reported that NaCl-stressed plants showed decrease in fresh and dry weight of one month old *C. roseus* plants but there was increase of malondialdehyde (MDA) content compared to control. They found that tryptophan decarboxylase (TDC) activity increased significantly under 50 $mmol^{-1}$ NaCl without $CaCl_2$ addition, 50 $mmol^{-1}$ NaCl with 7.5 $mmol^{-1}$ $CaCl_2$, and $CaCl_2$ treatment without NaCl addition. There was significant increase in peroxidase (POD) activity under NaCl stress compared to control plants. The content of vindoline, catharanthine, vincristine and vinblastine was increased under salt stress (50 $mmol^{-1}$ NaCl treatment with or without $CaCl_2$). Further, addition of $CaCl_2$ in NaCl-stressed plants showed increased biomass, TDC activity, vindoline, and catharanthine contents while, MDA and vincirstine contents was lowered compared to the plants without $CaCl_2$. Similarly, Fatima *et al.* (2015) observed maximum callus biomass reduction in 125mM NaCl amended MS medium. They found that antioxidant enzymes *i.e.* superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase and glutathione reductase activities were increased linearly with increasing NaCl level in medium showing 4.97 EU min^{-1} mg^{-1} SOD and 3.14 EU min^{-1} mg^{-1} CAT at 125mM concentrations. Enhanced content of vinblastine (14.17 µg/g dry wt) and vincristine (5.12 µg/g dry wt) was observed on 25 mM NaCl amended medium. Although, alkaloid production in periwinkle was enhanced along with the accumulation of compatible solutes, free amino acid and antioxidant enzymes as the adaptive mechanisms to protect plant against the oxidative damages caused under the abiotic stresses of salinity and water deficit conditions in suspension medium but the detailed mechanisms yet to be explored.

CONCLUSION

In the present study we indicated increased production of total alkaloids in *C. roseus* cell suspension culture system under the salinity and water stress conditions. There was fluctuating but higher biomass fresh and dry weight with significantly enhanced content, production, productivity and the yield of alkaloids with the threshold tolerance limit of 150mM NaCl or the 12% PEG (6000). While, in the higher concentration of any of the stress there was significant reduction of all the observable parameter. The enhanced level of fresh and dry weight, along with increased alkaloid content, production, productivity and the yield might be due to accumulation of compatible solute and the other secondary metabolites to compensate and to overcome the detrimental effect of reactive oxygen species (ROS) under the salinity and water stress conditions. Further experimentation are in progress to confirm the accumulation of various soluble sugars, free amino acids, enzymes system, alkaloids and other secondary metabolites in suspension cultures of *C. roseus* following stress treatments. Finally, the present study concluded that the cell suspension culture system could effectively improved the alkaloids contents and other secondary metabolites by inter playing with various abiotic stresses and many other in vitro conditions in *C. roseus* and could be extended to other medicinal plants for industrial production of various pharmaceuticals.

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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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