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Response of *Catharanthus roseus* Shoots to Salinity and Drought in Relation to Vincristine Alkaloid Content

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Abstract: The present study aims at investigation of the response of *Catharanthus roseus* shoots to salinity (control, 100 and 150 mM) and drought (control, two-weeks-regime and three-weeks-regime) for a period of 4 months. Total proteins, amino acids, proline and vincristine alkaloid contents were estimated before and after stress. Both salinity and drought reduced the amount of shoot total proteins while increased the amount of total amino acids which has been attributed to enhanced protein degradation and/or *de novo* synthesis of amino acids. Accumulation of proline after both stresses supported the previously recorded correlation between cellular proline levels and the capacity to survive environmental stresses. Salinity and drought resulted in increased amounts of the amino acids serine, methionine and arginine, which are considered precursors for the synthesis of glycinebetaine, nicotinamide and putrescine that are commonly encountered osmolytes that accumulates in plants under salinity and drought stresses. Vincristine alkaloid content increased with two peaks at 150 mM salinity at the 2nd month of treatment and at the 4th month of the highest drought level. The increase in vincristine content was attributed to the raised levels of arginine subsequent to stress that could derive the biosynthesis of putrescine. This polyamine was found to induce nitric oxide biosynthesis which acts as chemical elicitor for indole alkaloid production of *C. roseus* shoots.

Key words: *Catharanthus*, salinity, drought, vincristine, amino acids, proline, medicinal plants

INTRODUCTION

Secondary metabolites are a large array of plants natural product as part of plant defense system against pathogenic attack and environmental stresses. They also have their economic importance as pharmaceuticals, flavours, fragrances, insecticides, dyes, food additives, toxins, etc. (Verpoorte *et al*., 1997). Their production is frequently low and depends on the physiological and developmental stage of the plant and can be obtained from wild or cultivated plants (Verpoorte *et al*., 2002). *Catharanthus roseus* (L.) G. Don is a well known medicinal plant that possesses a large number of terpenoid indole alkaloids with over 130 compounds isolated and identified (Van der Heijden *et al*., 2004; Verpoorte *et al*., 1997). The leaves and stem are the sources of the natural dimeric alkaloids vinblastine and vincristine that are essential parts of most anti-cancer chemotherapies (Van der Heijden *et al*., 2004). The two compounds occur in very low concentrations (about 0.00025% of leaf dry weight; Van der Heijden *et al*., 2004; Misawa and Goodbody, 1996).

The growing needs for the natural anti-cancer therapeutics stimulates alternative approaches for increasing vincristine production in plants or plant cell lines. These approaches have focused on selection of high-alkaloid-yielding cell line, induction of salt tolerant mutants, employment of elicitors (fungal homogenates) or stress factors such as osmotic shock and salt stress (Misra and Gupta, 2006; Van der Heijden *et al*., 2004; Rai *et al*., 2003; Zhao *et al*., 2000; Datta and Srivastava, 1997; Misawa and Goodbody, 1996)

Drought and salinity are abiotic stresses that are becoming particularly widespread in many regions. The two stresses are often interconnected and may induce similar cellular damage. For example, drought and/or salinity are manifested as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano and Rodriguez-Navarro, 2001; Zhu, 2001) and denaturation of functional and structural proteins (Smirnoff, 1998). Thereby, they can activate similar cell signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001; Zhu, 2001, 2002), the production of stress proteins and accumulation of compatible solutes (Vierling and Kimpel, 1992; Zhu *et al*., 1997; Cushman and Bohnert, 2000).

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Various biological and technological limitations restrict commercial utilization of *C. roseus* cell cultures for production of vincristine (Moreno *et al.*, 1995) encouraging approaches dealing with *C. roseus* plants. The effect of different salinity levels and drought durations on growth as well as pigments, photosynthetic activity, transpiration rate and carbohydrate content of shoot system of *C. roseus* has been investigated by Elfeky *et al.* (2007). They concluded that *C. roseus* has tolerated salinity up to 150 mM NaCl and drought up to 3 weeks water-regime and the plant appeared to tolerate drought more than salinity. The present work aims at investigating how salinity and drought affect the total proteins and amino acids in relation to vincristine content of *C. roseus* shoots.

MATERIALS AND METHODS

Plant materials: Two-month-old *C. roseus* plantlets were planted in 150 pots with dimensions 15 cm width × 10 cm height (2 individuals per pot) filled with soil (3 clay: 1 perlite). The pots were divided into two sets and salinity and drought stresses were separately applied for each set. Salinity stress: Three levels of NaCl solution were used: 0 (control), 100 and 150 mM. Pots of each treatment were irrigated every two weeks for a period of 4 months.

Drought stress: Three water regimes were applied as: control (3 days), two-weeks-regime (irrigation every 2 weeks) and three-weeks-regime (irrigation every 3 weeks) for a period of 4 months.

Quantitative estimation of total proteins: Samples (0.1 g) of dried shoot powder were vortexed with 1 mL borate buffer (pH 8.8), centrifuged and the supernatants were collected in fresh tubes. Protein content was quantitatively estimated according to Bradford (1976).

Estimation of amino acids: Dried tissues (0.1 g powder of plant shoot system) were deprotenized with 5% sulfosalisylic acid. The filtered samples (0.025 mL) were dried 10-15 min then dried again with 0.03 mL drying solution (0.2 mL methanol, 0.2 mL of 0.2 M sodium acetate and 0.1 mL triethylamine). The dried sample were mixed with 0.03 mL of the freshly prepared derivatization reagents (0.35 mL methanol, 0.05 mL HPLC grade water, 0.05 mL triethylamine and 0.05 mL phenyl-thiocyanate) and allowed to react. After 20 min, 0.03 mL of HPLC grade methanol was added to the tube and left for 15 min thereafter 0.1 mL of the sample was transferred to the injection vials. The standard amino acids solutions were treated typically as the sample. Amino acids were analyzed by HPLC according to the method of Weibull *et al.* (1990).

Colorimetric estimation of proline: Proline was measured as described by Bates *et al.* (1973). Five hundredmilligram of fresh shoot was homogenized in 10 mL of 3% sulphosalicylic acid and the residue was removed by filtration through Whatman (No. 2) filter paper. Two milliliter of the extract was reacted with 2 mL glacial acetic acid and 2 mL acid ninhydrin (1.25 g ninhydrin warmed in 30mL glacial acetic acid and 20 mL 6 M phosphoric acid until dissolved) for 1 h at 100°C and the reaction was then terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene. The chromophore-containing toluene was warmed to room temperature and its optical density was measured at 520 nm. Proline concentration was determined from a standard curve and calculated on fresh weight basis as (μ moles g^{-1} fresh weight).

Quantitative estimation of vincristine alkaloids: For extraction of alkaloids, 10 g powder of dried shoot systems were mixed overnight with 25 mL acidulated-ethyl alcohol by continuous stirring overnight. This step was repeated 3 times till complete extraction of alkaloids. The previous extract was filtered, poured into a separating funnel, mixed with 25 mL chloroform, alkalinized (pH 9) with 10% ammonium hydroxide solution and shaken. The addition of chloroform was repeated till exhaustion as tested with Mayers reagent. The combined chloroformic extracts were dried with anhydrous sodium sulphate, filtered and evaporated to dryness. Alkaloid contents were assayed according to Bruneton (1999). The alkaloids residue obtained above was dissolved in 1 mL of acidulated methanol for HPLC analysis. Samples (20 μL each) were injected in HPLC column C18 with flow rate 0.5 mL min^{-1} of solvent MeOH:H_2O (1:1) and detected by UV detectors at λ256. The concentrations of alkaloids were determined using calibration curve prepared from Sigma vincristine authentic sample.

Data analysis: For both salinity and drought treatment, the pots were arranged in randomized complete blocks where all salinity levels and drought durations were represented in each block. Six samples were collected from each treatment at the second, third and fourth months. Data were statistically analyzed using (ANOVA) for randomized complete blocks using MINITAB-13 for windows (MINITAB, 2000).

RESULTS

Protein content: Compared to control, the two NaCl concentrations (100 and 150 mM) resulted in highly significant decrease in protein content during the course of experiment period (Table 1). On the other hand, protein content was insignificantly reduced in plants subjected to two-week-water regime for 3 and 4 months. The

remarkable significant decrease was observed in plants subjected to 3-week-water regime throughout the experimental periods.

Proline content: In the third month of treatment, the proline content increased significantly with the maximum value 1.41 μmol g^{-1} f. wt. observed at salinity level 150 mM. In the fourth month of treatment, the proline content followed the same trend with increasing salinity level, yet it tended to decrease than the level it reached the last month and ranged from 0.3 to 0.43 μmol g^{-1} f. wt compared to control sample. On the other hand, the proline content mildly increased with increasing drought duration at the different growth stages. The maximum increase in proline content (0.28 and 0.25 μmol g^{-1} f. wt.) was observed in plants subjected to three-week-water regime in the third and fourth month, respectively (Table 1).

Amino acids content: Generally, the amount of total amino acids significantly increased with increasing salinity level and drought duration, revealing 2 fold increase at 3 week water regime after 2 and 4 months duration and 100 mM salinity for 4 months.

All salinity levels and drought durations resulted in the accumulation of the amino acids glutamic acid, serine, therionine, aspartic acid, tyrosine and phenylalanine reaching the peak after 2 months treatment with 3 week water regime and after 4 months salinity treatment. Arginine content continued to significantly elevate throughout the course of treatment with salinity and drought. The amount of alanine increased only after two month duration to both drought regimes and after 4 month treatment with 150 mM NaCl and 2 week water regime. Insignificant changes in the amounts of glycine, isoleucine, lysine and methionine were observed throughout the experimental course with salinity and drought. The only significant increase in the amount of cystine was observed after 2 month treatment with 150 mM NaCl. (Table 2).

Vincristine alkaloid content: A highly significant increase in vincristine content (287.4 μg mL^{-1}, Table 1) was observed in the shoots of plants treated with 150 mM NaCl for 2 months compared to control sample (13.31 μg mL^{-1}). On the other hand, a highly significant decline in vincristine content was observed with increasing salinity level in the third and fourth month. Vincristine reached its minimum value (3.67 μg mL^{-1}) with the level 150 mM in the fourth month of treatment, compared to control sample (10.07 μg mL^{-1}).

Table 1: Effect of salinity and drought on total proteins, proline and vincristine alkaloids of *C. roseus* shoots during different treatment periods

	Total protein (g/100 g dry weight)			Proline (μmol g^{-1} f.wt)			Vincristine content (μg mL^{-1})		
Treatments	2nd month	3rd month	4th month	2nd month	3rd month	4th month	2nd month	3rd month	4th month
Control	6.28	6.64	7.80	0.09	0.12	0.08	13.31	22.68	10.07
Salinity									
100 mM	4.80	4.50	4.63	0.12	1.10	0.30	15.00	16.85	3.22
150 mM	4.30	4.36	3.60	0.16	1.41	0.43	87.40	6.96	3.68
Drought									
Two week	5.57	6.20	6.10	0.10	0.25	0.17	49.00	16.42	8.50
Three week	4.80	4.20	6.10	0.13	0.28	0.25	3.66	36.67	196.95

Table 2: Effect of salinity and drought on amino acids content of *C. roseus* shoot

	2nd month					4th month				
		Salinity		Drought			Salinity		Drought	
Amino acid	Control	100 mM	150 mM	2 weeks	3 weeks	Control	100 mM	150 mM	2 weeks	3 weeks
Glycine	1.390	1.15	1.78	1.66	2.21	1.56	2.04	1.4	1.41	1.48
Alanine	9.730	8.02	10.16	13.72	11.84	8.59	6.49	15.97	9.56	6.59
Valine	11.990	7.45	15.22	7.02	15.97	8.62	11.92	10.65	12.93	10.80
Leucine	4.500	3.78	5.57	4.92	6.36	3.26	6.15	5.36	4.24	4.50
Isoleucine	3.650	2.70	3.84	2.7	3.42	2.19	3.92	4.81	3.15	2.96
Serine	14.060	16.46	21.84	17.43	22.79	14.86	25.20	21.84	16.53	22.86
Therionine	20.200	21.56	29.01	25.09	30.74	21.38	36.25	31.42	22.19	32.89
Aspartic	15.600	17.43	16.69	28.22	28.52	9.05	14.03	21.97	11.77	19.22
Glutamic	3.770	4.58	7.86	5.06	8.07	3.69	6.92	6.46	4.53	7.38
Lysine	3.310	3.42	2.47	1.94	6.47	2.87	5.17	4.26	6.53	5.17
Arginine	15.630	25.35	22.47	22.53	66.18	57.63	118.29	77.61	100.52	152.11
Histidine	3.160	1.96	4.54	4.07	6.76	2.74	3.21	2.62	4.74	6.52
Cystine	0.880	0.75	9.46	0.18	0.18	0.25	0.75	0.75	0.18	0.44
Methionine	0.470	0.83	0.71	0.71	0.94	0.59	1.18	0.47	1.18	0.47
Tryosine	3.870	4.72	5.46	8.34	9.56	2.48	4.81	4.75	4.40	5.71
Phenylalanine	8.480	8.87	11.77	14.78	14.94	4.55	10.05	6.57	9.75	11.77
Total	121.730	130.38	170.97	159.97	236.79	146.46	259.60	148.28	215.32	292.53

On the contrary to salinity treatment, the sharp increase in vincristine content (196.95 μg mL^{-1}) was observed in the fourth month of treatment with the highest drought level (three weeks water regime) compared to control sample (10.07 μg mL^{-1}). In the second month of treatment, the two-week-water regime increased the vincristine content up to (49 μg mL^{-1}) compared to control sample (13.3 μg mL^{-1}), while, the three-week-water regime increased vincristine content up to (36.6 μg mL^{-1}) in the third month of treatment compared to control sample (10.07 μg mL^{-1}).

DISCUSSION

Nitrogen containing compounds were suggested to have important roles during stress as osmotic adjustment and available sources of carbon and nitrogen (Misra and Gupta, 2006). In the present study, both salinity and drought treatments resulted in reduction of total protein content (Table 1). Similar findings were observed by Misra and Gupta (2006) and Gilbert *et al.* (1998). On the other hand, both salinity and drought significantly increased proline content (Table 1) with a peak at 3 months salinity treatment with 150 mM while maintaining values higher than those of control in other salinity and drought treatment.

Several authors have reported a strong correlation between cellular proline levels and the capacity to survive both water deficit, high salinity and other environmental stresses (Munns, 2005; Khedr *et al.*, 2003; Ashraf and Harris, 2004). Increased levels of proline were recorded to correlate with enhanced water deficit stress tolerance in *Phaseolus vulgaris* L. (Jimenez-Bermont *et al.*, 2006), *Pringlea antiscorbutica* (Hennion *et al.*, 2006), sorghum (Yadav *et al.*, 2005), *Lathyrus sativus* (Tyagi *et al.*, 1999) and transgenic tobacco (Yonamine *et al.*, 2004).

It has been also established that low concentrations of proline could act as a component of signal transduction pathways that regulate stress responsive genes in *Arabidopsis* (Kiyosue *et al.*, 1996) and *Pancratium maritimum* (Khedr *et al.*, 2003). Besides, intermediates of proline biosynthesis and catabolism, such as glutamine and δ-1-pyrroline-5-carboxylic acid were found to increase the expression of several osmotically regulated genes in rice (Iyer and Caplan, 1998). In addition, proline was found to function as radical scavenger, electron sink, stabilizer of macromolecules and a cell wall component (Matysik *et al.*, 2002).

The remarkable feature of salt and drought stressed *C. roseus* in the present study was the accumulation of total amino acids with two peaks at 100 mM salinity level and 3-weeks water regime after 4 months (Table 2). Amino acid were observed to be among metabolites that are involved in osmotic adjustment under water deficit (Yadav *et al.*, 2005; Asha and Rao, 2002) and can act as sinks for excess N in relation to the decreased growth occurring during the imposed stress (Gilbert *et al.*, 1998). Several explanations for the accumulation of free amino acids under stress have been suggested. These include stimulated synthesis, inhibited degradation of amino acids, impaired protein synthesis and/or enhanced protein degradation (Yadav *et al.*, 2005; Asha and Rao, 2002; Ranieri *et al.*, 1989). Gilbert *et al.* (1998) deduced that accumulation of amino acids in *Coleus blumei* during salinity stress was due *to de novo* synthesis since ^{14}C has been rapidly incorporated into the basic fraction of the stressed plants as compared to the control plants. Since the increase in total amino acids was correlated with reduction in protein content (Table 1, 2) *de novo* synthesis of amino acids and/or enhanced protein degradation in *C. roseus* shoots could be suggested.

Accumulation of specific amino acid was clearly observed in shoots of *C. roseus* after salinity and drought stress (Table 2). Besides being incorporated into proteins, free amino acids can serve as precursor for polyamines. A large number of reports have demonstrated accumulation of polyamines under a variety of environmental stresses, such as salt, drought, extreme temperature, acidity, ozone and heavy metals (Legocka and Kluk, 2005; Kuthanová *et al.* 2004; Kakkar and Sawhney, 2002). Serine, methionine and arginine are precursors for the synthesis of glycinebetaine, nicotinamide and putrescine that are commonly encountered osmolytes that accumulates in plants under salinity and drought stress (Sharma and Dietz, 2006; Liu *et al.*, 2006; Papadakis and Roubelakis-Angelakis, 2005; Kakkar and Sawhney, 2002). Besides, glutamic acid was considered as precursor for glutathione that is involved in oxidative defense (Sharma and Dietz, 2006). Based on these reports, the accumulation of these amino acids in *C. roseus* shoot subsequent to salinity and drought could be considered as stress adaptability through deriving the biosynthesis of polyamines.

In the present study, an increase in vincristine was observed with the level 150 mM after two months of treatment and thereafter there was a sudden depression in its value in the third and fourth month compared to control. Studies on alkaloid composition of the roots and leaves of *C. roseus* indicated that NaCl salinity exerts a remarkable influence on individual alkaloids of these plant parts which were considered as adaptability of the plant to these conditions (Misra and Gupta, 2006).

On the contrary, the increase in vincristine content with the drought treatment was in the fourth month with the highest drought level. In this regard, Saenz *et al.* (1993) observed two fold increase in the alkaloid content of the mature leaves of *C. roseus* under severe water

stress. Plants which have been previously exposed to water stress show an improved capacity to tolerate subsequent periods of water stress through increases in solute levels and a decrease in osmotic potential (Virk and Singh, 1990).

The results of the present study can suggest a mechanism by which drought and salinity increased the vincristine alkaloid levels. This suggestion is based on the observation that both stresses raised the levels of amino acids (e.g., arginine). The increased amounts of these amino acids under stress could derive the biosynthesis of polyamines (e.g., putrescine). The polyamines were found to induce nitric oxide biosynthesis (Tun *et al*., 2006) that can move freely through the membranes of plant cells acting as a potentially efficient chemical elicitor for indole alkaloid production of *C. roseus* cells (Neill *et al*., 2003; Xu and Dong, 2005).

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