



EFFECTS OF DROUGHT STRESS ON THE ALKALOID CONTENTS AND GROWTH PARAMETERS OF *Catharanthus roseus*

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ABSTRACT

In the present investigation *Catharanthus roseus*, an important herb used in traditional as well as modern medicine, exposed to water deficit stress and possible changes in chlorophyll contents, photosynthesis rate, transpiration rate, growth parameters as well as total alkaloid content and vincristine and vinblastine levels were studied. Seedlings subjected to 4 different water-regimes. Experimental samples irrigated once every week or every second and third week and the control irrigated every day. Drought had adverse effect on height, weight and relative water content of periwinkle. The photosynthetic activity and transpiration rate significantly decreased with increasing drought level. Total protein decreased to 77% and total chlorophyll decreased by 27%. Total alkaloid content significantly increased to maximum 187% compared to the control. Vincristine and vinblastine content of the seedlings grown under treatments³ increased to 175% and 171% compared to the control, respectively.

Keywords: *Catharanthus roseus* (periwinkle), drought, alkaloid content, chlorophyll, vinblastine, vincristine, water deficit.

INTRODUCTION

Catharanthus roseus (L.), Madagascar periwinkle, is a perennial tropical plant belonging to the family Apocynaceae that contain a good source of non-enzymatic and enzymatic antioxidants and anti-hypertension. Long before modern researcher learned of the plant's valuable and varied properties, people in faraway places were using the Madagascar periwinkle, for a host of medicinal purposes. In India, they treated wasp sting with juice from the leaves. In Hawai'i they prescribed an extract of the boiled plant to arrest bleeding. In Central America and parts of South America, they made a gargle to ease sore throats and chest ailments and laryngitis. In Cuba, Puerto Rico, Jamaica and other islands, an extract of the flower was commonly administered as eyewash for the eyes of infants. In Africa, leaves are used for menorrhagia and rheumatism (Dobelis, 1997; Walts, 2004).

Few plants have generated as much recent interest among scientist and medical communities as the, periwinkle. The interest began in the mid-1950, when researchers, hearing of a "periwinkle tea" that was drunk in Jamaica, began to study the plant for its reported antidiabetic properties. Periwinkle has been used against diabetes for many years. The antidiabetic effect is considered to be a result of the increase in glucose utilization (Singh, *et al.*, 2001).

Extracts of entire dried plant contain many alkaloids of medicinal use. Periwinkle is a valuable source of antitumor agents vinblastine and vincristine used in chemotherapy of leukemia and treatment of Hodgkin's disease (C. Jaleel, *et al.*, 2008). In the past few decades, a large number of publications have covered the improving knowledge on the antitumor alkaloids of *Catharanthus*. Vinblastine and vincristine are those substances which are used against cancer. They act by inhibiting proliferation in cancer cells. Vinblastine and vincristine bind to tubulin during mitosis. This inhibits kromatin threads drawn to a respective pole (Huxtable, 1992). This leads to inhibition of mitosis and cell start its programmed cell death,

apoptosis (Leveque and Jehl, 2007). Vinblastine, or vincal leukoblastine (vinblastine sulfate) is used experimentally for treatment of neoplasms, and is recommended for generalized Hodgkin's disease and resistant choriocarcinoma. Vincristine, formally known as leurocristine (vincristine sulfate), is a mitotic inhibitor, and is used in cancer chemotherapy. Vincristine is used in treatment of leukemia in children. Vincristine is created by the coupling of indole alkaloids vindoline and catharanthine in the vinca plant (Evans, Trease, and Evans, 2009). Using vinblastine and vincristine in combination chemotherapy has resulted in 80% remission in Hodgkin's disease, 99% remission in acute lymphocytic leukemia, 80% remission in Wilm's tumor, 70% remission in gestational choriocarcinoma, and 50% remission in Burkitt's lymphoma. There are over 70 other alkaloid that have been isolated from the plant in addition to vinblastine and vincristine (Dobelis, 1997; Walts, 2004).

Biosphere's continued exposure to abiotic stress, e.g. drought and salinity, cause imbalance in the natural status of the environment. Thus growth and development of plants, as immobile organisms, are harmfully affected by a variety of abiotic and biotic stress factors. Plants are frequently exposed to many stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity (Amirjani, 2010a, 2011a, 2011b, 2012b; Mahajan and Tuteja, 2005). Drought and heat are important biomass-limiting stress factors (Araus, Slafer, Reynolds, and Royo, 2002) in the field causing the suppression of cultivated plants in growth and in crop production. During water deficit many morphological features and physiological processes associated with plant growth and development are affected (Rahbarian, Khavari-Nejad, Ganjeali, Bagheri, and Najafi, 2011). These changes include reduction of water content (RWC), diminished leaf water potential (Ψ_w) and turgor loss, closure of stomata and a decrease of cell enlargement and plant growth. Water stress inhibits cell enlargement more



than cell division. It reduces plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters (Farooq, Wahid, and Kobayashi, 2009; Cheruth Abdul Jaleel, Gopi, Sankar, Gomathinayagam, and Panneerselvam, 2008).

Drought stress reduces plant growth by affecting photosynthesis, respiration, the membrane stability index and nutrient metabolism (C. A. Jaleel, Gopi, and Panneerselvam, 2008). In drought stress conditions, plants close their stomata to avoid further water loss. During drought stress net photosynthetic rate is decreased. The reduction of CO₂ fixation partially results from the closure of stomata due to water deficit, since decrease of stomatal conductance (gs) is the most capable way to diminish water loss, and parallel with this the CO₂ distribution into the leaves is restricted, resulting in a decrease in intercellular CO₂ concentration and inhibition of ribulose-1, 5-bisphosphate carboxylase/oxygenase enzyme activity and ATP synthesis under drought stress (Dulai, *et al.*, 2006). Photosystem II (PSII) is extremely sensitive to abiotic stresses and its function was inhibited to a much greater extent than that of photosystem I (PSI). Recent studies have also indicated that stresses exert multiple effects on both donor and acceptor sites of PSII. On the donor site the oxygen evolving cycle and, consequently, oxygen evolution is inhibited; on the acceptor site, electron transfer from Q_A⁻ to Q_B⁻ is inhibited (Sigfridsson, Bernat, Mamedov, and Styring, 2004). Maximum photosystem efficiency (Fv/Fm) and electron transport rate (ETR) are affected during stress (Amirjani, 2010b, 2012a, 2012b).

With mild water deficiency, plants are usually slow growing and stunted. Some plant leaves turn from shiny to dull at first signs of stress. Grasses, which are the first to show the loss of water in the landscape, will show signs of wilt. Footprints in wilted grass persist instead of disappearing as grass blades spring upright. Under long term water stress, plants might permanently wilt or stop growing; they may have diminished crops and discolored leaves, flower buds and flowers. Plants may eventually die. Bare spots will appear in ground covers. Water-stressed plantings may show the effects of weeds, insect pests, and diseases. Drought symptoms can be very confusing, and can vary with different types of plants. Woody plants under drought stress can have many symptoms including yellowing, wilting leaves that develop early fall color and burning or scorching on edges of leaves. Plants may drop some or all of their leaves and appear dead.

The water needs of different plants vary greatly. Some factors include the species and age of the plant, the type of soil in which it is planted, and its exposure. The symptoms of drought stress may be similar to the symptoms of over watering or even to some pest and disease problems. It is important to identify the causes of the problem in order to take corrective steps. In the present study the effects of drought on the growth, photosynthetic performance, transpiration as well as contents of total

alkaloid, vinblastine and vincristine of *Catharanthus roseus* were examined.

MATERIALS AND METHODS

Plant material and treatments

Seeds of *Catharanthus roseus* (L.) (Family: Apocynaceae) were surface sterilized in 0.1% HgCl₂ for 10 min and rinsed thoroughly in distilled water to remove HgCl₂. Surface-sterilized seeds were sown in plastic pots, with dimensions 15 cm width × 1 cm height, filled with soil mixture containing clay and perlite (3-1). During the study, average temperature was 24/19°C (maximum/minimum) and relative humidity (RH) varied between 60-75 per cent. Seedlings were grown under a photo synthetically active photon flux density (PPFD) of 250-350 μmol m⁻² s⁻¹ in 16 h of white light/8 h dark condition. 30 day-old seedlings were used for analysis (the seedling age was determined from the day of sowing).

Drought stress

Some pots were irrigated with water 1-day interval as a control and irrigation of other three water-regimes were applied as: one week regime (irrigation every week, treatment 1), 2 weeks regime (irrigation every 2 weeks, treatment 2) and three weeks regime (irrigation every 3 weeks, treatment 3) for a period of 4 months. The pots were arranged in randomized complete blocks where all drought durations were represented in each block. Five samples were collected from each treatment every month up to 4 months. Immediately after sample collection growth criteria (shoot length and shoot fresh weight), chlorophyll content, net photosynthesis and respiration rate were measured.

Growth parameters

Morphological parameters (shoot length and fresh weight) were measured in fresh samples. Samples were oven-dried at 80°C for three days and the dry weight was calculated.

Gas exchange measurement

Photosynthesis and transpiration of seedlings were measured. Leaf gas exchange was measured on the second using a LI-6400[®] portable (LI-COR Inc., USA) photosynthesis system.

Total protein estimation

Protein extracts of plant material prepared by a method described by Böddi *et al.* (Amirjani, 2010a; Böddi, Evertsson, Ryberg, and Sundqvist, 1996). Oven dried (100 mg) leaves were ground at 95°C in an extraction buffer of 10% (v/v) glycerol, 4% (w/v) sodium dodecyl sulphate (SDS), 0.3 M dithiothreitol, 0.001% bromophenol blue and 250 mM Tris-HCl, pH 6.8. The proteins were quantified by a colorimetric assay for protein determination using the Bio-Rad[®] DC Protein Assay kit based on the well-documented Lowry assay (Bio-Rad, Richmond, CA). The absorption values were read at 750



nm with a Perkin Elmer Lambda® 900 UV/VIS spectrophotometer.

Chlorophyll content

Fresh leaves sample (0.1 g) were analyzed for pigment contents. The leaves were sliced and ground to a fine powder in liquid nitrogen using a pestle and mortar. Pigments were extracted in 3 ml cold 80% acetone as described by Brouers and Michel-Wolwertz (1983). Acetone extracts were centrifuged at 3000 g for 10 min and the resulting pellets were extracted in cold 80% acetone. This operation was repeated three times. The successive supernatants were pooled and centrifuged at 4000 g for 5 min for clarification. The absorbance of the acetone extracts was recorded at 626, 647, 664 nm and (for background correction) 720 nm using a Perkin Elmer Lambda 900 UV/VIS spectrophotometer (Perkin-Elmer® Corp. Norwalk, CT, USA). The amounts of Chl were calculated according to Brouers and Michel-Wolwertz (1983) by putting the obtained value on the following formulas:

$$\text{Chl a } (\mu\text{g/ml}) = 12.68 \text{ OD}_{664} - 2.65 \text{ OD}_{647} + 0.30 \text{ OD}_{626}$$

$$\text{Chl b } (\mu\text{g/ml}) = -4.23 \text{ OD}_{664} + 23.62 \text{ OD}_{647} - 3.26 \text{ OD}_{626}$$

Relative water content (RWC)

The relative water content (RWC) was determined. Five plants from each treatment were randomly selected and the method described by Whetherley and Turner was followed (Turner, 1981; Whetherley and Barrs, 1962). About 0.1 g leaf sample was cut into smaller pieces and weighed to determine initial weight (W_i). The leaf samples were then floated in freshly de-ionized water for 12 h and weighed thereafter to determine fully turgid weight (W_f). The sample was oven-dried at 80°C for 3 days and the dry weight was obtained (W_d). The relative water content (RWC) was determined using the following formula:

$$\text{RWC} = (W_i - W_d) / (W_f - W_d) \times 100.$$

Total alkaloid content

Total alkaloid content was estimated in the dry leaf-powder as described by Misra and Gupta (2006). Freshly harvested leaves were oven-dried at 70°C for 72 h and powdered. Five hundred milligram of fine powder of leaves was extracted in 90% ethanol. The mixture was then filtered, and concentrated to be dried. Dried residue was re-dissolved in ethanol and diluted with the same volume of water, followed by addition of diluted (3%) HCl. The mixture was extracted (3 times) by transferring to a separating funnel, to which hexane was added. This mixture was shaken for 15-20 min. The lower water layer was decanted in a beaker and was made slightly basic by adding 3% ammonium hydroxide to pH 8.5 and cooled to 10°C. Subsequently, the mixture was again transferred into a separating funnel adding chloroform (three times). The content was shaken for 15-20 min. The lower layer was discarded and the upper chloroform layer was decanted. Decant transferred to a pre-weighed dry porcelain dish and

then it was evaporated till dryness. The weight of this dish was then taken again. Total alkaloids were expressed as percent alkaloid content in the dry leaves.

Vincristine and vinblastine content

Fresh leaf tissues of control and treated plants were used for spectrophotometrically determination of vincristine and vinblastine using the method described by Idrees *et al.* (2011). Fresh leaves (0.2 g) were sliced and ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder was homogenized in 1 ml MeOH and 2 ml of 5 M hydrochloric acid. The suspension was filtered through Whatman® filter paper No.1 and the filtrate were collected in a volumetric flask. The flask, containing 3 ml of 0.1% 4-nitroaniline, was cooled in an ice bath followed by the addition of 0.5 ml of 0.5% sodium nitrite. To it, 0.5 ml sulphamic acid was added and then the reaction mixture was cooled for 5 min with occasional shaking. Thereafter, the sample (leaf-filtrate sample) was transferred to the contents of the flask followed by heating the content on a boiling water bath for 10 min. The flask-content was cooled and diluted with distilled water. The absorbance of the resulting yellow-coloured azo product of vincristine and vinblastine was recorded at 440 and 430 nm, respectively. A blank was run simultaneously with each set of determination. Standard curve was plotted using graded dilutions of vincristine and vinblastine (Sigma-Aldrich). The absorbance of each sample was compared using the calibration curve and vincristine and vinblastine was expressed in terms of percentage on the fresh weight basis.

Statistical data analysis

Statistical analysis was performed using SPSS 16. The data represent means calculated from five replicates. The analysis of variance procedure (ANOVA) followed by Duncan's multiple range Test (DMRT) used to compare the effect of drought. The values are mean \pm S.D. for five samples in each group and statistical significance was set at $P < 0.05$.

RESULTS

Growth parameters

The data presented in Figure-1 indicates that drought had adverse effect on the height of periwinkle. Irrigation of once a week had no significant ($p \leq 0.05$) decreasing effect on the plant height but when irrigated once every 2 and or 3 weeks the decrease became significant.

Fresh and dry weights were affected by drought. The highest drought stress (irrigation every 3 weeks) was the most injurious treat which reduced the fresh and dry weight of periwinkle by 84% and 87%, respectively (Table-1). Treatments 1 and 2 decreased fresh weight by 24% and 51%, respectively, compared to the control. Dry weight was decreased by 33% and 59% when treatment 1 and 2 were applied, respectively.



Relative water content (RWC)

Drought stress decreased the relative water content at all three treatments (Table-1). RWC of seedlings exposed to drought treatments 1, 2 and 3 decreased to 87%, 73% and 65% that of control, respectively.

Chlorophyll contents

The effect of drought treatment on the chlorophyll amount of leaves was examined (Figure-1). The chlorophyll a and chlorophyll b contents respond differently to the drought. Plants growing with treatments

1 or 3 (irrigation every week or every third week) possessed 81% or 70% chl *a*. Treatment 2, however, are statistically similar to treatments 1 and 3 but still significant to the control. Chl *b* did not have any significant changes with treatments 1 and 2 but decreased significantly under treatment 3. Total chlorophyll content of leaves was decreased significantly ($P \leq 0.05$) by increasing the drought. Total chlorophyll content decreased approximately by 27% at the treatment 3. The ratio of chl *a* to chl *b* was changed similarly ($P \leq 0.05$) at all treatments which were lower than the control.

Table-1. Effect of water stress on height, fresh weight, dry weight and relative water content (RWC), of *Catharanthus roseus* seedlings leave. Data are the mean value \pm SD of five individual experiments.

	Treatment (Week between irrigations)			
	Control	1	2	3
Height (cm)	25, 8 \pm 1, 6	23 \pm 1,8	18, 8 \pm 1, 9	12, 1 \pm 2, 1
Fresh weight (g)	21, 6 \pm 1, 9	16,3 \pm 1,9	10, 4 \pm 0, 6	3, 36 \pm 0, 6
Dry weight (g)	4, 82 \pm 0, 6	3,2 \pm 0,5	1, 97 \pm 0, 4	0, 62 \pm 0, 08
RWC (%)	77, 8 \pm 2, 6	67,4 \pm 3,1	57, 9 \pm 2, 7	50, 8 \pm 2, 4

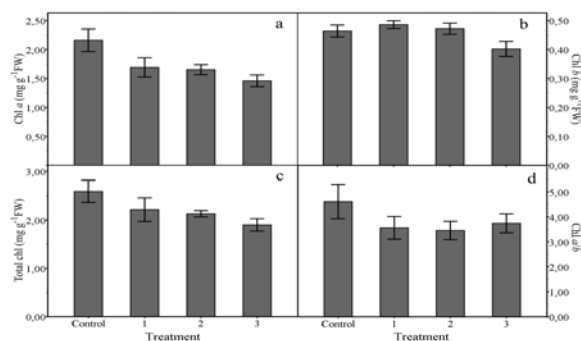


Figure-1. Effect of water stress on amount of chlorophyll *a*, *b*, total and ratio of pigments in *Catharanthus roseus* seedlings. Data are the mean value \pm SD of five individual experiments.

Photosynthetic activity and transpiration

The photosynthetic activity significantly decreased with increasing drought level and the period of irrigation (Figure-2). The lowest activity were evident with the treatment 3, which was 62% of the control, followed by treatment 2 and 1 which showed 44% and 26% of photosynthesis rate, respectively compared to the control (Figure-2). Transpiration rate also decreased with increasing drought level. Greatest reduction occurred with treatment 3 in which 35% decrease was seen. Treatment 1 did not affect transpiration rate significantly ($p \leq 0.05$). Statistical analysis showed, however, transpiration rate in plant treated by treatments 2 and 3 had no significant differences (Figure-2).

Protein content

Two drought treatments (1 and 2) resulted in similar but significant compared to control. Exposure of

seedlings to drought treatments 1 or 2 decreased the protein content to 82% and 77% of the control, respectively, during the experiment period (Figure-2).

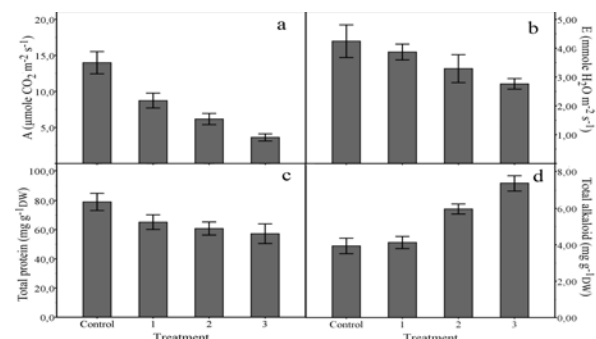


Figure-2. Effect of water stress on photosynthesis rate, *a*, transpiration rate, *b*, total protein, *c*, and total alkaloid, *d*, in *Catharanthus roseus* seedlings. Data are the mean value \pm SD of five individual experiments.

Alkaloid content

Total alkaloid content significantly increased in plants challenged with drought treatments 2 and 3. Total alkaloid content of seedlings grown under treatment 2 and 3 were increased by 52% and 87%, respectively. Treatment 1, however, had no significant effect on total alkaloid content (Figure-2).

Vincristine and vinblastine content

A highly significant increase in vincristine content ($p \leq 0.05$) was observed in the shoots of drought treated plants compared to control sample (Figure-3). vincristine contents of seedlings grown under treatments 1, 2 and 3 increased to 112%, 131% and 175% compared to



the control. The contents of vinblastine, however, increased only in response to treatment 3 which was reached to 171% of the control. Treatment 1 and 2 had no significant effect on vinblastine content.

DISCUSSIONS

The present research showed that water deficient decreases the height and fresh and dry weight. It is well-known that drought stress is a very important limiting factor at the initial phase of plant growth and establishment. Drought stress affects both elongation and expansion growth (Shao, Chu, Shao, Jaleel, and Mi, 2008). Water deficient significantly suppresses cell growth and expansion due to the low turgor pressure. 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). Stress inhibits the efficiency of the translocation and assimilation of photosynthetic products (Xiong and Zhu, 2002) and might have caused reduction in shoot growth.

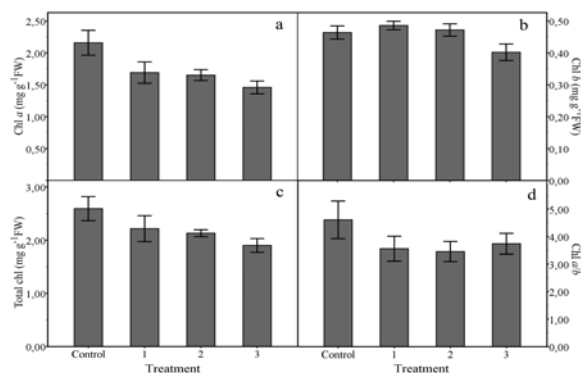


Figure-3. Effect of water stress on vincristine and vinblastine contents of *Catharanthus roseus* seedlings. Data are the mean value \pm SD of five individual experiments.

The photosynthetic activity and transpiration rate has adversely been affected by drought stress. Water deficit stress mostly reduced leaf growth and in turn the leaf areas in many species of plant (Farooq, *et al.*, 2009; Zhang and Sharkey, 2009). Pigmentation reflects photosynthetic properties of phototrophic organisms as it indicates the size of light harvesting capacity. In the present study both chlorophyll a and chlorophyll b were reduced in *C. roseus* plants subjected drought with more reduction in chlorophyll a. Other researchers have shown that the photosynthetic activity significantly increased in plants treated for one and two weeks of drought for one and two months then significantly decreased in all drought regimes (Elfeky, Osman, Hamada, and Hasan, 2007).

Proteins 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 drought treatments resulted in reduction of total protein content. Similar findings were observed by Misra and Gupta (2006) and Osman *et al.* (2007).

The total alkaloid accumulation in shoot of *C. roseus* was found increased significantly under drought stress. 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). The leaves and stem are the sources of the natural dimeric alkaloids vinblastine and vincristine that are essential parts of most anti-cancer chemotherapies (Heijden, Jacobs Denise, Snoeijer, Hallard, and Verpoorte, 2004). The results showed that vinblastin was not so sensitive to drought and only affected by a high level of drought while vincristine was more sensitive and has been affected even by a slight drought treatment. Increasing of alkaloids may be a defense response of periwinkle.

On the basis of present findings, it can be concluded that all the physiological, biochemical and growth parameters were significantly reduced under the applied drought stress on *C. roseus*. Under drought stress the height, fresh and dry weight of plants has been reduced. The chlorophyll amount is also reduced. But results showed that chl a is more sensitive to drought than chl b. The results of the study showed, however, drought stress increased the level of total alkaloid, vinblastine and vincristine.

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