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ORIGINAL PAPER

Interactive effects of triadimefon and salt stress on antioxidative status and ajmalicine accumulation in *Catharanthus roseus*

Cheruth Abdul Jaleel · Ragupathi Gopi · Ashok Kishorekumar · Paramasivam Manivannan · Beemarao Sankar · Rajaram Panneerselvam

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Abstract The ability of triadimefon (TDM), a triazole group of fungicide, to ameliorate salinity stress was studied in *Catharanthus roseus* (L.) G. Don. plants subjected to sodium chloride (NaCl) treatment. NaCl treatment at 80 mM level decreased overall growth of this plant and reduced the chlorophyll contents, protein, antioxidant enzymes such as peroxidase (POX), superoxide dismutase (SOD) and polyphenol oxidase (PPO). The root alkaloid ajmalicine got increased under salt treatment. When these stressed plants were treated with TDM at 15 mg l^{-1} concentration minimized the inhibitory effects of NaCl stress by increasing the root, shoot growth and leaf area and increased dry weight (DW), chlorophyll, protein contents and the activities of antioxidant enzymes like POX, SOD and PPO, thereby paved the way to overcome the salinity injury. The quantity of ajmalicine was again increased with the TDM treatment when compared to both control and NaCl treated plants. From these results, it is proved that the fungicide TDM have great role in the enhancement of plant antioxidative enzymes and the enhanced scavenging of potentially harmful free radicals, as a mechanism of protecting plants against noxious oxidative stress from the environment and also in the enhancement of active principles.

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C. A. Jaleel · R. Gopi (✉) · A. Kishorekumar · P. Manivannan · B. Sankar · R. Panneerselvam
Stress Physiology Lab, Department of Botany, Annamalai University, Annamalainagar 608 002, Tamil Nadu, India
e-mail: suriyagopi@yahoo.co.in

C. A. Jaleel
e-mail: abdul79jaleel@rediffmail.com

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Introduction

Soil salinity is one among the several environmental stresses causing drastic changes in the growth, physiology and metabolism of plants. Saline environment can induce a wide number of responses in plants ranging from readjustment of transport and metabolic processes to growth inhibition (Azooz et al. 2004). Most plants sensitive to saline environment due to a combination of adverse osmotic gradients and inhibitory effects of salts and ions on cell metabolism and of nutrient imbalance and secondary stresses such as an oxidative stress linked to the production of toxic reactive oxygen intermediates (Hasegawa et al. 2000). NaCl induces the generation of OH^- and H_2O_2 suggesting that activated oxygen species could be involved in mechanisms of salt injury (Miszalski et al. 1998). ROS scavenging is one among the common defense response against abiotic stresses (Lin and Kao 2000). ROS scavenging depends on the detoxification mechanism provided by an integrated system of non-enzymatic reduced molecules like ascorbate and glutathione and enzymatic antioxidants (Mittova et al. 2002). The major ROS scavenging activities includes complex non-enzymatic (ascrobate, glutathione, α-tocopherol) and enzymatic (CAT, APX, GR, SOD, etc.) responses (Misra and Gupta 2006). Antioxidant mechanisms may provide a strategy to enhance salt tolerance in plants (Jaleel et al. 2007a).

Salinity affects the dry matter production in relation to physiological processes, biochemical reactions or a combination of these factors (Jaleel et al. 2007b). Improving plant resistance to salt, although not a final solution, may provide

field stability in subsistence agriculture (Flowers and Yeo 1995). A possible alternative is to induce the capability within plants to successfully face the detrimental situation by treatment with growth regulators. Application of growth regulators has been reported to mitigate the adverse effects of salinity (Jaleel et al. 2007b). The use of plant growth regulators results in a significant increase in the growth and yield of many crops under stress condition (Singh et al. 1995).

Triazole compounds such as triadimefon (TDM), paclobutrazol (PBZ), uniconazole (UCZ), etc. are widely used as fungicides and they also possess varying degrees of plant growth regulating properties and have been called 'plant multi-protectants' because of their ability to induce tolerance in plants to environmental and chemical stresses (Fletcher et al. 2000; Kishorekumar et al. 2006; Jaleel et al. 2007b). Protection of plants from apparently unrelated stress by triazole is mediated by a reduction in free radical damage and increase in the antioxidant potential (Wu and Tiedemann 2001). The plant growth regulating properties of TDM [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazole-1-yl)-2-butanone] are mediated by interference with the isoprenoid pathway and subsequent shift in balance of important plant hormones, including gibberellins, ABA and cytokinins (Fletcher and Hofstra 1985). There is evidence that triazoles can increase cold and heat resistance (Asare-Boamah and Fletcher 1986), drought resistance (Manivannan et al. 2007) in various plants and can increase alkaloid content in medicinal plants like *Catharanthus roseus* (Jaleel et al. 2006a).

Catharanthus roseus (Family: Apocyanaceae) is one of the most important medicinal plants, being a valuable source of antitumour agents vinblastine and vincristine used in chemotherapy of leukemia and treatment of Hodgkin's disease and also a popular ornamental plant with pink or white flowers (Jaleel et al. 2006b). In the past few decades, a large number of publications have covered the improving knowledge on the antitumor alkaloids of *Catharanthus*. On the contrary, the salinity tolerance nature of this plant, as a biological approach to saline soil reclamation is attracted little attention (Jaleel et al. 2007c). Previous works revealed the influences of TDM on the antioxidant metabolism and ajmalicine production (Jaleel et al. 2006a), PBZ mediated growth regulation (Jaleel et al. 2006b), salinity problems (Jaleel et al. 2007d), salt stress protection by PBZ (Jaleel et al. 2007c) and drought effects (Jaleel et al. 2007e, f) in this medicinal plant. However, the influential mechanism of TDM on medicinal plants under salinity stress is not much studied. In this context, the present study investigated the effectiveness of fungicide TDM as a salt stress-ameliorating agent in *C. roseus* plants, with specific emphasis on the free radical scavenging metabolism in plants and active principle contents, hence it is a model system representing the physiological processes of medicinal plants.

Materials and methods

Plant culture, salt stress induction and TDM application

Seeds of *Catharanthus roseus* (L.) G. Don. were collected from Botanical garden, Annamalai University, Tamil Nadu, India and surface sterilized with 0.2% $HgCl_2$ solution for 5 min with frequent shaking and then thoroughly washed with deionised water. The seeds were pre-soaked in 500 ml of deionised water (control), 80 mM NaCl and 80 mM NaCl + 15 mg l^{-1} TDM (25% WP BAYLETON-Registered Trademark, Bayer, India Ltd., Mumbai) solutions for 12 h. Seeds were sown in plastic pots (300 mm diameter) filled with 3 kg of soil mixture containing red soil, sand and farmyard manure (FYM) at 1:1:1 ratio. Before sowing the seeds, the pots were irrigated with the respective treatment solutions and the electrical conductivity (EC) of the soil mixture was measured and the EC level was found to be 0.10 dS m^{-1} (control), 12.00 dS m^{-1} (80 mM NaCl) and 10.00 dS m^{-1} (80 mM NaCl + 15 mg l^{-1} TDM), respectively. Four seeds were sown per pot and the pots were watered to the field capacity with deionized water up to 90 days after sowing (DAS) and every care was taken to avoid leaching. The initial EC level of the soil was maintained by flushing each pot with required volume of corresponding treatment solution on 45, 60 and 75 DAS.

The position of each pot was randomized at 4 days intervals to minimize spatial effects in the greenhouse, where the temperature 28°C during the day and 22°C at night and the relative humidity (RH) varied between 60–70%. The seedlings were thinned to one per pot on the 10 DAS. Plants were harvested randomly on 90 DAS and analysed for estimating the growth, biochemical parameters, antioxidant status and ajmalicine content.

Growth parameters

Morphological parameters like root length, plant height were measured in fresh samples. The total leaf area was calculated with LICOR photoelectric area meter (Model LI-3100, Lincoln, USA). Fresh weight (FW) and dry weight (DW) were calculated from samples.

Biochemical analysis

Photosynthetic pigments like chlorophyll was extracted from leaf and estimated following the method of Arnon (1949). Protein was extracted and estimated by following the method of Bradford (1976).

Antioxidant enzymes

POX (EC: 1.11.1.7) activity was assayed by the method of Kumar and Khan (1982). The enzyme activity is expressed in units mg^{-1} protein. One unit (U) is defined as change in 0.1 absorbance min^{-1} mg^{-1} protein under the assay condition. SOD (EC: 1.15.1.1) activity was assayed as described by Beauchamp and Fridovich (1971). SOD activity is expressed in U mg^{-1} protein (U = change in 0.1 absorbance h^{-1} mg^{-1} protein). PPO (EC: 1.10.3.1) activity was assayed by the method of Kumar and Khan (1982). PPO activity is expressed in U mg^{-1} protein (U = change in 0.1 absorbance min^{-1} mg^{-1} protein). For all the enzymatic calculations protein was determined by the method of Bradford (1976), using bovine serum albumin (BSA, Sigma, USA) as the standard.

Ajmalicine extraction and quantification

Ajmalicine was extracted from the roots by following the standard extraction method of Zhao et al. (2000) with small modification (Jaleel et al. 2006a).

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The values are mean ± SD for six samples in each group. P values ≤ 0.05 were considered as significant.

Results and discussion

Morphological parameters

NaCl treatment decreased the root length plant height and total leaf area to a large extent when compared to control plants. TDM treatment to NaCl stressed *C. roseus* plants increased the root length, plant height and total leaf area. However, it was lower than that of control. A decrease in whole plant FW and DW was noted as a result of salt stress. NaCl treatments combined with TDM increased the FW and DW when compared with NaCl stressed plants and even control plants (Table 1).

Reduced root growth under NaCl salinity was observed in *C. roseus* (Jaleel et al. 2007c). This decrease may be due to the ability of salinity to effect external water potential, ion toxicity or imbalance (Hasegawa et al. 2000) and salinity can reduce biophysical restraints to cell wall expansion, which in turn, can inhibit root growth (Singh et al. 1995). Increasing of root growth by TDM is associated with increased level of endogenous cytokinin (Fletcher and Arnold 1986). Salt stress can negatively affect the growth performance of plants by decreasing the leaf and root growth and other parameters, as reported in *C. roseus* (Misra and Gupta 2006). The increased ABA content and decreased gibberellic acid (Fletcher et al. 2000) may be the reasons for the increased stem growth under TDM treatment.

Salinity injury caused a reduction in leaf area, which might be due to inhibition of cell division and cell expansion under salt stress (Aspinall 1986). A decrease in leaf size under unfavourable conditions allows the conservation of energy, thereby launching the appropriate defence response and also reducing the risk of heritable damage (Sankar et al. 2007). TDM treatment reversed the inhibition of leaf growth caused by NaCl stress. The TDM treatment to NaCl stressed *Catharanthus* plant increased the FW and DW which is correlated with the increased ABA content which in turn induces stomatal closure (Fletcher et al. 2000).

Biochemical parameters

A reduction in total chlorophyll contents has been observed in the NaCl stressed *Catharanthus* plants when compared to control. TDM treated NaCl stressed plants showed

Table 1 Effect of TDM on the root length, plant height, leaf area, fresh weight (FW), dry weight (DW), chlorophyll and ajmalicine contents of *C. roseus* on 90 DAS

Parameters	Control	NaCl	NaCl + TDM
Root length (cm $plant^{-1}$)	24 ± 0.888[a]	13 ± 0.464[b]	16 ± 0.533[c]
Plant height (cm $plant^{-1}$)	62 ± 2.696[a]	50 ± 1.851[b]	56 ± 2.074[c]
Leaf area (cm^2 $plant^{-1}$)	165 ± 6.111[a]	101 ± 3.258[b]	132 ± 4.400[c]
FW (g $plant^{-1}$)	28.46 ± 0.948[a]	26.11 ± 0.967[b]	28.01 ± 0.903[a]
DW (g $plant^{-1}$)	3.62 ± 0.116[a]	3.20 ± 0.102[b]	3.60 ± 0.129[a]
Chlorophyll '*a*' (mg g^{-1} FW)	0.036 ± 0.001[a]	0.024 ± 0.001[b]	0.080 ± 0.003[c]
Chlorophyll '*b*' (mg g^{-1} FW)	0.092 ± 0.003[a]	0.061 ± 0.002[b]	0.121 ± 0.004[c]
Total chlorophyll (mg g^{-1} FW)	0.128 ± 0.005[a]	0.085 ± 0.003[b]	0.201 ± 0.007[c]
Ajmalicine (mg g^{-1} DW)	0.468 ± 0.018[a]	0.489 ± 0.019[a]	0.613 ± 0.021[b]

Values are given as mean ± SD of six experiments in each group. Values are not sharing a common superscript (a,b,c) differ significantly at $P \leq 0.05$ (DMRT)

higher amount of photosynthetic pigments, even above that of control in 90 DAS (Table 1). The reduction in leaf chlorophyll under salinity has been attributed to the destruction of the chlorophyll pigments and the instability of the pigment protein complex (Levitt 1980). TDM increased the chlorophyll content, with a concomitant increase in alkaloid ajmalicine content, due to the increased production of cytokinin under TDM treatment. Cytokinin accelerated chlorophyll differentiation and stimulation of chlorophyll production (Fletcher and Arnold 1986).

The content of root alkaloid ajmalicine got increased to a slight extent under NaCl stress when compared to unstressed plants (Table 1). The addition of TDM again increased the content of this alkaloid to a significant level. The abiotic stress rose from NaCl caused the increase in secondary compounds. The plant growth regulating properties of TDM may be the reason for increased alkaloid content under treatment (Jaleel et al. 2006a). An enhancement in the production of ajmalicine under cadmium treatment (Zheng and Wu 2004) and nitrogen fertilization (Sreevalli et al. 2004) was previously reported. We have reported an increase in total indole alkaloid content in *C. roseus* plants under drought stress and *Pseudomonas fluorescence* treatment (Jaleel et al. 2007e, f).

The total soluble protein content decreased in the root, stem and leaves by NaCl stress and slightly increased under triadimefon treatments (Fig. 1a). The protein degradation under saline environment has been attributed to the decrease in protein synthesis, accelerated proteolysis, decrease in availability of amino acid and denaturation of enzyme involved in protein synthesis (Levitt 1980).

Antioxidant enzymes

The SOD activity has been lowered by the NaCl stress to a larger extent in root, stem and leaves of *Catharanthus* plants. As a stress signaling mechanism, SOD catalyses the dismutation of superoxide anion radical (O_2^-) with great efficiency, resulting in the production of H_2O_2 and O_2 (Lin and Kao 2000). TDM treatment to the NaCl stressed plants increased the SOD activity to a level even higher than that of control (Fig. 1b). Increased SOD activity was reported previously in *C. roseus* plants under TDM treatment (Jaleel et al. 2006a).

The POX activity has been reduced by the NaCl stress in all parts of the *Catharanthus* plants when compared to control (Fig. 1c). The results obtained in this study were in accordance with that found in roots of rice seedlings (Lin and Kao 1999). The inhibition of POX activity by salinity may interfere with the regulation of auxin levels and also with cell wall biosynthesis (Mittova et al. 2002). Low basal rate and decreased POX activity seems to indicate that this enzyme does not take a crucial part in defence mechanisms against oxidative stress or that, suffering POX for salt toxicity, a cooperation is activated between different antioxidants enzymes for establishing a proper H_2O_2 homeostasis (Chaparzadeh et al. 2004). The TDM application increased the POX activity in root, stem and leaves, even above the control plants. These results are in agreement with those of the previous works, were the activity of POX in fungicide treated plants was about two times higher than the untreated plants (Wu and Tiedemann 2001). These results also coincide with the previous findings in salt treatment (Zaidi and Singh

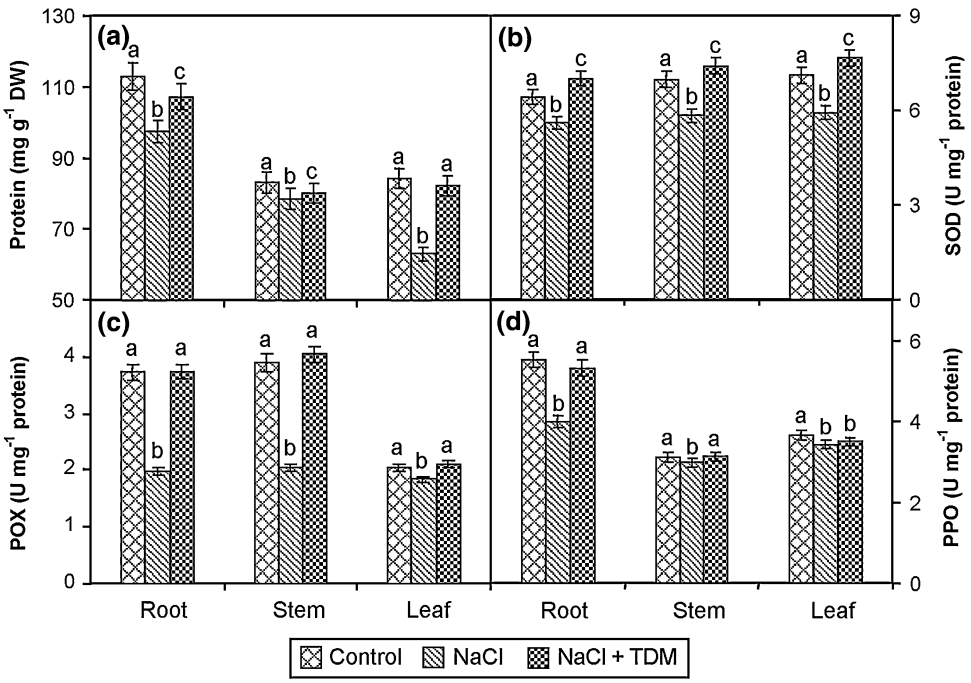

Fig. 1 Effect of TDM on the protein content (**a**), SOD (**b**), POX (**c**) and PPO (**d**) activities of *C. roseus* on 90 DAS. Values are given as mean ± SD of six samples in each group. Bar values are not sharing a common superscript (a,b,c) differ significantly at $P \leq 0.05$ (DMRT)

1995). The increased POX activity under TDM treatment in the NaCl stressed *Catharanthus* plants might be due to the ability of triazole in reduction of free radical damage and increased antioxidant potentials (Jaleel et al. 2007c).

The PPO activity decreased due to NaCl treatment in root, stem and leaves of *Catharanthus*. Similar results were obtained in mungbean (Sakuja and Chawla 1994). TDM treatment to the NaCl stressed plants increased the PPO activity to a large extent when compared with NaCl stressed and control plants (Fig. 1d). This increased PPO activity may decrease the phenol content thereby protecting the level of IAA and this increased IAA can increase cell and cell wall growth thereby inducing the growth in the NaCl stressed and unstressed *Catharanthus* plants. The increased PPO activity is well correlated with the increase in the growth of triadimefon treated plants.

With increasing realization of the health hazards and toxicity of modern synthetic medicine, more and more people are interested in the use of plants and plant-based drugs revived throughout world. Exploration of medicinal plants became more and more popular. As far as is known medicinal plants like other field crops, will meet various stress like salinity during their growth stages. So it seems significant to test important medicinal plants for their salinity tolerance nature and methods to overcome salinity with specific emphasis to secondary metabolite content. The data of this work demonstrated that the TDM utilization in *C. roseus* cultivation have a promising role in increasing salinity tolerance mechanisms and economically important alkaloid, ajmalicine content.

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