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Original Article

EFFECT OF TRIAZOLE FUNGICIDE ON BIOCHEMICAL AND ANTIOXIDANT ENZYMES ACTIVITY IN OKRA (ABELMOSCHUS ESCULENTUS L.) PLANT UNDER DROUGHT STRESS

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Abstract

*Abelmoschus esculentus* L. was selected for present study under pot culture in Completely Randomized Block Design (CRBD) experiment, hexaconazole and tebuconazole are a triazole derivative, which have both fungicidal and plant growth regulator (PGR) properties and also protect plants from several types of abiotic stresses. The plants were subjected to 4 days interval drought stress and drought stress with hexaconazole (15mg l⁻¹) and tebuconazole (10mg l⁻¹). One-day-interval irrigation was kept as control. The plant samples were collected and separated into root, stem and leaf for estimating the amino acid (AA), proline (PRO) and glycine betaine (GB) contents and the activities of antioxidant enzymes. Drought stress and triazole treatments increased AA, PRO and GB contents, superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities when compared to control. Triazole treatment modified biochemical content and antioxidant enzyme activity. From the result of this investigation it can be concluded that the application of triazole caused a partial recovery of the damaging effect of drought stress by its influence on antioxidant system.

Key words: *Abelmoschus esculentus* L., Drought, Hexaconazole, Tebuconazole, Biochemical, Antioxidant etc

1. INTRODUCTION

*Abelmoschus esculentus* L. was selected for present study for its economic importance, mainly for the tropical zone of developing and non-developed countries. It is cultivated especially in India, Africa and Brazil, where it is also commonly known as Okra, Quiabo and lady’s finger, respectively. The tender pods are used as vegetable, ripe seeds, which are rich in protein (18-26%) are roasted and can be used as substitute for coffee. Immature pods are emollient, demulcent and diuretic and are employed in the form of decoction in catarrhal affections, dysuria and gonorrhrea. Seeds are stimulant, cordial and antispasmodic. Fatty fraction of fresh watery extract of seeds impaired cancerous cell growth in vitro (CSIR, 1985).

Water stress is a major problem in agriculture and the ability to withstand such stress is of immense economic importance. Plants are subjected to several environmental stresses that adversely affect growth, metabolism and yield (Lawlor, 2002). Drought, one of the environmental stresses, is the most significant factor restricting plant growth and crop productivity in the majority of agricultural fields of the world (Tas and Tas, 2007). Water deficit affects crop growth, depending on the stage of growth and the degree or intensity of water stress (Clavel et al., 2005).

Triazoles are a group of growth inhibitor chemical compounds that widely used as fungicides. The compounds contain three nitrogen atoms and a pentagonal ring. Triazole makes changes in plant by preventing the enzyme activity of CytP450 (Zhu, 2004). Application of PBZ partially alleviated the detrimental effects of rice senescence by modulating the activity of enzymatic antioxidants, and improving antioxidant system, which helped in sustaining plant growth. Therefore, spraying PBZ with 50 mg L⁻¹ or 6-BA with 30 mg L⁻¹ at the heading stage could increase grain yields and improve grain qualities in the two super hybrid rice. (Shenggang Pan et al., 2013). The application of TDM caused a partial recovery of the damaging effect of drought stress by its influence on antioxidant system. (Neda Mohamadi et al., 2013).

2. MATERIALS AND METHODS

Hybrid seeds *Abelmoschus esculentus* L. from Syngenta F1 Hybrid Okra variety OH-102 were used for this investigation. Plastic pots of 40 cm diameter and 45 cm
height size were used for the pot culture study. The pots were filled with 10 kg of soil mixture containing red soil; sand and farm yard manure at 1:1:1 ratio. 120 pots were arranged in Completely Randomized Block Design (CRBD). One set of 30 pots were kept as a control, two sets of 60 pots were used for four days interval of drought with triazole treatment and other one set was kept as four days interval drought treatment in order to impose drought stress. Hexaconazole (15mg/l-1) and tebuconazole (10mg/l-1) were used to determine the effect of these triazole compounds on *Abelmoschus esculentus* L... The treatments were given as soil drenching, 30 days after planting (DAP). The plants were allowed to grow up to 30 DAS on alternative day irrigation. From 30th to 60th day control plants were irrigated on every alternative day, drought treated and drought with triazole treated plants were irrigated at every 4 days interval. After the drought treatment all the pots were irrigated on alternate day were irrigated up to harvest. Plants were uprooted randomly on 40th, 50th and 60th DAS, washed with water and separated into root, stem and leaves for estimating biochemical, antioxidant enzyme activities.

### 2.1 BIOCHEMICAL ANALYSIS

#### 2.1.1 ESTIMATION OF TOTAL FREE AMINO ACID CONTENT

Total free amino acids were extracted and estimated by following the method of Moore and Stein (1948).

**Extraction**

Five hundred milligrams of fresh plant material was homogenized in a mortar and pestle with 10 ml of 80% boiled ethanol. The extract was centrifuged at 800 g for 15 minutes and the supernatant was made up to 10 ml with 80% ethanol and used for the estimation.

**Estimation**

In 25 ml test tube, one millilitre of ethanol extract was taken and neutralized with 0.1 N NaOH using methyl red indicators. To which, 1 ml of ninhydrin reagent was added. The contents were boiled in a boiling water bath for 20 minutes, and then 5ml of diluting solution was added, cooled and made up to 25 ml with distilled water. The absorbance was read at 570 nm in a Spectrophotometer (Hitachi) against an appropriate blank. The standard graph was prepared by using leucine as standard and the amino acid content was calculated using the standard graph and the results are expressed in milligram per gram dry weight.

**Ninhydrin Reagent preparation**

Solution I: 80 mg of stannous chloride in 50 ml citrate buffer at pH 5.0.

**Solution II:** 2 grams of ninhydrin in 50 ml methyl cellosolve, both solutions were mixed freshly.

**Diluting reagent**

Distilled water and n-propanol mixed in equal volume (1:1 v/v).

#### 2.1.2 DETERMINATION OF PROLINE CONTENT

Proline was extracted and estimated following the method of Bates *et al.* (1973).

**Extraction**

Five hundred milligrams of fresh plant material was homogenized in a mortar and pestle with 10 ml of 3% aqueous sulfsalicyclic acid. Then the homogenate was filtered through Whatmann No.1 filter paper. The residue was re-extracted and pooled and the filtrates were made up to 20 ml with aqueous sulfsalicyclic acid and this extract was used for the estimation of proline.

**Estimation**

To 2 ml of proline extract, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added. The mixture was incubated for an hour at 100 °C in a boiling water bath. Then the test tubes were transferred to an ice bath to terminate the reaction. Then 4 ml of toluene was added and mixed vigorously using a test tube stirrer for 20 seconds and the toluene containing the chromophore was separated from the aqueous phase with the help of a separating funnel and the absorbance was measured at 520 nm in a Spectrophotometer using a reagent blank. The proline content was determined from a standard curve with proline and the results are expressed in milligrams per gram dry weight.

**Reagents**

- **Acid-ninhydrin reagent**
  
  To 1.25 gms of ninhydrin, 30 ml warm glacial acetic acid, 20 ml of 6 M phosphoric acid were added with agitation.

**2.1.3 ESTIMATION OF GLYCINE BETAIN CONTENT**

The samples were extracted and estimated following the method of Grieve and Grattan (1983).

**Extraction**

Five hundred milligrams of finely ground dry plant sample were mechanically shaken with 20 ml of distilled water for 24 hours at 25 °C. Time required for this step was determined by extracting the plant samples for 4, 8, 16, 24 and 48 hours. The samples were then filtered through Whatmann No.1 filter paper and the filtrates were made up to 20 ml with deionized water and used for estimation immediately.

**Estimation**

One millilitre of the extract was diluted with one millilitre of 2 N H₃SO₄ and 0.5 ml of this acidified extract was cooled in ice water for 1 hour. Later 0.2 ml of cold potassium tri iodide solution was added and mixed gently with a Vortex mixture and the tubes were stored at 4°C for 15 minutes and then centrifuged at 10,000 g for 15 minutes. The supernatant was aspirated with a fine tipped glass tube. The per iodide crystals were dissolved in 9.0 ml of 1, 2-dichloroethane with vigorous vortexing. After 2.5 hours the absorbance was measured at 365 nm in a Spectrophotometer. Reference standard of glycine betaine was prepared in 1 N H₂SO₄ and used for estimating the glycine betaine content and the results are expressed in micrograms per gram dry weight.

**Preparation of Reagent**

- **Potassium tri iodide reagent**

  15.7 grams of iodine and 20 grams of potassium iodide were dissolved in 100 ml of distilled water and gently stirred in a vortex mixture.

**2.2 ANTIOXIDANT ENZYMES**

#### 2.2.1 ASCORBATE PEROXIDASE (APX) (EC: 1.11.1.11)

Ascorbate peroxidase was extracted and estimated by the...

**Extraction**

Five hundred milligrams of fresh plant tissue was ground in a pestle and mortar under liquid nitrogen and 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 per cent PVP and 1 mM ascorbic acid. The homogenate was filtered through a double layered cheese cloth and centrifuged at 15,000 rpm for 20 minutes at 4 °C. The supernatant was used as source of enzymes.

**Estimation**

One ml of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H$_2$O$_2$ and 200 µl of enzyme extract. The absorbance was read as decrease at 290 nm against the blank, correction was done for the low, non-enzymatic oxidation of ascorbic acid by H$_2$O$_2$ (extinction coefficient 2.9 mM$^{-1}$ cm$^{-1}$). The enzyme activity was expressed in µg per gram dry weight.

2.2.2 SUPEROXIDE DISMUTASE (SOD) (EC: 1.15.1.1)

Crude enzyme extract was prepared, for the assay of superoxide dismutase by the method (Hwang et al., 1999).

**Extraction**

One gram of fresh tissue was homogenized with 10 ml of ice-cold 50 mM sodium phosphate buffer containing 1 mM PMSF. The extract was filtered through double-layered cheesecloth. The extract was centrifuged at 12,500 rpm for 20 minutes at 4°C. The supernatant was saved and made up to 10 ml with extraction buffer and used for estimation of the SOD enzyme activity.

**Estimation**

Superoxide dismutase activity was assayed as described by Beauchamp and Fridovich (1971). The reaction medium was prepared and to 3 ml reaction medium, 1 ml of enzyme extract was added. The reaction mixture contained 1.17 x 10$^{-6}$ M riboflavin, 0.1M methionine, 2x10$^{-5}$ potassium cyanide and 5.6 x 10$^{-5}$ M nitroblue tetrasodium salt (NBT), dissolved in 0.05 M sodium phosphate buffer (pH 7.8). The mixture was illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes. Illumination started to initiate the reaction at 30°C for one hour. Those without illumination saved as blank and kept in dark. The absorbance was read at 560 nm in the spectrophotometer against blank. Superoxide dismutase activity was expressed in units. One unit is defined as the amount of change in the absorbance by 0.1 per hour per milligram protein under the assay condition (Cherry, 1963).

2.2.3 CATALASE (CAT, EC: 1.11.1.6)

Catalase activity was assayed as described by Chandlee and Scandalios (1984).

**Extraction**

Five hundred milligrams of frozen material was homogenized in 5 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.5) containing in 1 mM PMSF. The extract was centrifuged at 4°C for 20 minutes at 12,500 rpm. The supernatant was used for enzyme assay. The enzyme protein was determined by Bradford (1976) method.

**Assay**

The activity of enzyme catalase was measured using the method of Chandlee and Scandalios (1984) with modification. The assay mixture contained 2.6 ml of 50 ml of 50 mM potassium phosphate buffer (pH 7.0) 0.4 ml, 15 mM H$_2$O$_2$ and 0.04 ml of enzyme extract. The decomposition of H$_2$O$_2$ was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units 1 mM of H$_2$O$_2$ reduction per minute per mg protein.

3. STATISTICAL ANALYSIS

The pot culture was carried out in completely randomized design (CRBD). The data are expressed as mean ± SE for seven samples in each group.

4. RESULTS AND DISCUSSION

4.1 BIOCHEMICAL PARAMETERS

4.1.1 AMINO ACID

Drought stress increased the amino acid content when compared to control in *Abelmoschus esculentus* L. The amino acid content increased under drought condition in sunflower (Manivannan et al., 2007a), in *Arachis hypogaea* (Asha and Rao, 2002); Radix *astragali* (Tan et al., 2006). Accumulated amino acid may be occurring in response to the change in osmotic adjustment of their cellular contents (Shao et al., 2007). Amino acids accumulation plays a very important role in drought tolerance, probably through osmotic adjustment in different plant species, such as, *Radix astragali* (Tan et al., 2006). Amino acids and other soluble nitrogenous compounds play an essential role in plant metabolism being the primary product of inorganic nitrogen assimilation and precursors of protein and nucleic acids. Because of the importance of soluble nitrogenous compounds, there has been much interest in the influence of environmental stress on their metabolism. A common response of plants to environmental stress is an accumulation of amino acids (Aspinall and Paleg, 1981). Triazole compound treatment to the drought stressed plants lowered the amino acid content when compared to drought stress but it was higher than that of control. Similar results were observed in paclobutrazol and triacontanol in olive varieties under water stress (Thakur et al., 1998) and paclobutrazol treated wheat seedlings under low temperature stress (Berova et al., 2002). Amino acids accumulation plays a very important role in drought tolerance, probably through osmotic adjustment in different plant species, such as, *Radix astragali* (Tan et al., 2006).

4.1.2 PROLINE

In *Abelmoschus esculentus* L. drought stress caused increased accumulation of proline content at all stages of growth. Water stress resulted in an increase in proline accumulation in sorghum (Yadav et al., 2005). The similar results were observed in wheat (Nayar, 2006), soybean (Heerden and Kruger, 2002). Proline content increased in a large variety of plants under stress, up to 100 times the normal level, which makes upto 80 per cent of the total amino acid pool. Proline was known to accumulate in plants under water stress (Hsiao, 1973). Proline accumulation was maximum at flowering stage and minimum at vegetative stage. Proline content is effective in increasing osmotic status of the plant. The accumulation of proline increased when water stress was followed by simultaneous increase in leaf water potential in chickpea (Gupta et al., 2000). Proline accumulation in plants might be a scavenger and acting as an osmolyte. The reduced proline oxidase may be the reason for increasing proline
accumulation (Jaleel et al., 2008), proline content increased in both drought stress and with TDM treatments when compared to control. TDM treatments decreased proline content in plants under drought stress compared with the plants that had received only water stress treatment. (Neda Mohamadi et al., 2013).

4.1.3 GLYCINE BETAINES

Drought stressed Abelmoschus esculentus L. plants showed an increase in glycine betaine content when compared to control. The glycine betaine content increased under drought stress in Radix astragali (Tan et al., 2006), in barley (Nakamura, 2001) and in higher plants (Jun et al., 2000). Glycine betaine is considered to be one of the most abundant quaternary ammonium compounds produced in higher plants under stressful environment (Yang et al., 2003). Glycine betaine has been shown to protect enzymes and membranes and also to stabilize PSI protein pigment complexes under stressful conditions (Papageorgiou and Morata, 1995). Glycine betaine, an important quaternary ammonium compound, is considered to be one of the most predominant and effective osmoprotectants. It is well established that its exogenous application might have some advantages as it improves drought tolerance in plants (Iqbal et al., 2008). It has been also reported earlier that rate and timing of GB application significantly affects drought tolerance ability of sunflower (Iqbal et al., 2008 and 2009). Triazole compound treatment to the drought stressed plants decreased glycine betaine content but it was higher than that of control. Similar results were observed in Arachis hypogaea (Girija et al., 2002). The accumulation of glycine betaine might serve as an intercellular osmoticum and it can be closely correlated with the elevation of osmotic pressure (Kavikishore et al., 1995).

4.2 ANTIOXIDANT ENZYMES

4.2.1 ASCORBATE PEROXIDASE (APX)

APX activity increased in Abelmoschus esculentus L. under drought condition and in all the treatments. Increased APX activity was reported in Phaseolus acutifolius under drought stress (Turkan et al., 2005). APX found in organelles is believed to scavenge H2O2 produced from the organelles, whereas the function of cytosolic APX is probably to eliminate H2O2 that is produced in the cytosol or apoplast and that has diffused from organelles. In the chloroplast, H2O2 can be detoxified by the ASA–GSH–NADPH system, which has been catalyzed by APX (Jaleel et al., 2006). Drought stress with triazole treatment decreased APX activity in drought stressed plants, and increased it in control plants. Triazole treatment increased APX activity when compared to the case of control and drought-stressed plants. Similar results were obtained by many workers in many higher plants under drought stress (Manivannan et al., 2007b). Drought stress induced generation of active oxygen species is well recognized at the cellular level and is tightly controlled at both the production and consumption levels through increased antioxidant systems (Reddy et al., 2004), also Kentucky bluegrass (Liu et al., 2008). Ascorbate peroxidase is one of the most important antioxidant enzymes of plants that detoxify hydrogen peroxide using ascorbate for reduction. APX reduces H2O2 to water by ascorbate as specific electron donor (Gara et al., 2003). In trifoliate orange, under water stress, an increased APX activity was not significant. Variation in APX activity at mild water deficit in maize and wheat were observed (Nayyar and Gupta, 2006).

4.2.2 SUPEROXIDE DISMUTASE (SOD)

The activity of SOD increased under water stress in Abelmoschus esculentus L. Super oxide dismutase activity increased under drought stressed higher plants (Reddy et al., 2004). SOD activity increased under drought stress in Oryza sativa (Chandrashekar Reddy et al., 1998), maize (Jiang and Zhang, 2002), Euphorbia esula (Davis and Swanson, 2001), Cassia angustifolia (Agarwal and Pandey, 2003), wheat (Singh and Usha, 2003; Shao et al., 2005), rice (Wang et al., 2005), P. acutifolins (Turkan et al., 2005) and the SOD activity was higher under salinity stress in C. roseus (Misra and Gupta, 2006), while subjecting to water deficit stress, the SOD activity was increased in higher plants (Reddy et al., 2004). Triazole treatment decreased SOD activity when compared to drought-stress and increased it in the control. Triazoles increased the antioxidant potential in oxidative stressed plants under treatment when compared to control (Sankar et al., 2007). It was reported that SOD enhances water-stress tolerance in plants. The cytosolic Cu/Zn–SOD was induced strongly by stress, while Cu/Zn–SOD remained largely unaffected (Jaleel et al., 2008). TDM treatment increased the SOD activity in drought stressed as well as in control plants. (Neda Mohamadi et al., 2013). Furthermore, it was observed that spraying PBZ or 6-BA could increase super oxide dismutase (SOD). (Shenggang Pan et al., 2013). SOD activity increased in drought treatment when compared to control.

4.2.3 CATALASE (CAT)

Drought stress has increased the catalase activity in all the parts of the plants to a larger extent under all the treatments in Abelmoschus esculentus L. Similar results were observed in wheat (Lin and Wang, 2002; Gong et al., 2005). Catalase activity increased under drought stress in Oryza sativa (Chandrashekar Reddy et al., 1998), maize (Pastori et al., 2000), Zea mays (Jiang and Zhang, 2002), Allium schoenoprasum (Egert and Tevini, 2002), wheat (Dalmia and Sawhney, 2004; Shao et al., 2005) and P. acutifolius (Turkan et al., 2005). An increase in catalase activity was reported in higher plants under drought stress (Reddy et al., 2004). Similar results were found in Lotus corniculatus (Borsani et al., 2001) and rice (Wang et al., 2005). Catalase activity increased in drought stress and with TDM treatments compared with control. Enzyme activity in stressed plants treated with TDM showed no significant increase compared with control. (Neda Mohamadi et al., 2013). Triazole treatment decreased catalase activity when compared to drought stress and increased it in controls. This result is in accordance with the findings in Catharanthus roseus (Jaleel et al., 2006). The combined action of CAT and SOD converts the toxic O2·−, H2O2 into water and molecular oxygen, averting the cellular damage under unfavourable conditions like water stress (Manivannan et al., 2007b).
BIOCHEMICAL PARAMETERS

Fig. 1-3. Effects of drought and drought with Triazole combination on the amino acid (AA) content of *Abelmoschus esculentus* L. Values are mean ± SE of seven replicates.

**Fig -1**

![Amino Acid Content - Root](image)

**Fig -2**

![Amino Acid Content - Stem](image)

**Fig -3**

![Amino Acid Content - Leaf](image)

Fig. 4-6. Effects of drought and drought with Triazole combination on the Proline (PRO) content of *Abelmoschus esculentus* L. Values are mean ± SE of seven replicates.

**Fig -4**

![Proline Content - Root](image)

**Fig -5**

![Proline Content - Stem](image)

**Fig -6**

![Proline Content - Leaf](image)

Fig. 7-9. Effects of drought and drought with Triazole combination on the Glycine betaine content of *Abelmoschus esculentus* L. Values are mean ± SE of seven replicates.

**Fig -7**

![Glycine Betaine Content - Root](image)

**Fig -8**

![Glycine Betaine Content - Stem](image)

**Fig -9**

![Glycine Betaine Content - Leaf](image)

**ANTIOXIDANT ENZYMES**

Fig 10. Effects of drought and drought with Triazole combination on the Ascorbate peroxidase content of *Abelmoschus esculentus* L. Values are mean ± SE of seven replicates.

**Fig -10**

![Ascorbate Peroxidase Activity](image)
5. CONCLUSION

Plants are highly regulated by triazole compounds, drought stressed plants under triazole treatment maintain a balance between formation and detoxification of activated oxygen species, leading to partial improvement of their response to drought-induced oxidative stress. It can be concluded that triazole such as hexaconazole and tebuconazole may be useful to trigger drought avoidance mechanisms. The triazole treatment mitigated the adverse effects of drought stress by modifying biochemical and antioxidants and there by paved the way for overcoming drought stress in Okra (Abelmoschus esculentus L.).

REFERENCE


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