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# Influence of *Trichoderma viride* in modulating the antioxidant defenses in micropropagated plantlets of *Curcuma longa* during acclimatization

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## Abstract

*Curcuma longa* L. is a spice crop with enormous medicinal and cosmetic properties cultivated worldwide. It was propagated vegetatively by means of rhizomes, as these were underneath soil prone to soil-borne fungal diseases. Treatment of such diseases using chemical fungicides would hinder their nutritional and medicinal value. To overcome such challenges, there are a few alternatives; the major among them was the deployment of disease-free *in vitro* raised plantlets, and the other was biopriming of these plantlets with *Trichoderma viride*. Besides fungicidal action, *Trichoderma viride* has a prominent role in alleviating several biotic/abiotic stresses and it was more often used during acclimatization. In this study, we studied the role of *T. viride* on successful acclimatization of *in vitro C. longa* plantlets by modulating plant antioxidant defense systems. We determined the stress levels (MDA and chlorophyll contents) and monitored the antioxidant enzyme activities (CAT, APX and GR) in both *T. viride* treated and untreated micropropagated plantlets at four different time points (0, 15, 30, 45, 60 days) of acclimatization period. Our results depicted clear enhancement in the antioxidant enzyme activities in *T. viride* treated than untreated plantlets which signify the role of *Trichoderma viride* in activating defense mechanisms to combat oxidative stress. Thus, this study would give a clear understanding of the influence of *T. viride* on *in vitro* raised plantlets in sustaining adverse acclimatization conditions.

**Key words:** acclimatization, antioxidant defenses, *Curcuma longa,* oxidative stress, *Trichoderma viride*

## Introduction

*Curcuma longa* L. is an important rhizomatous perennial spice crop belonging to the family Zingiberaceae and distributed across the globe (Nair, 2019). *C. longa* is widely used as dye and drug with potential medicinal properties (Nayak *et al.*, 2011; Prasad *et al.*, 2014). For centuries, *C. longa* was included as a major contributing medicine in traditional Ayurveda, Sidda, Unani systems (Amalraj *et al.*, 2017). *C. longa* lacks seed formation; it hinders breeding efforts and, hence, is vegetatively propagated from recurrent rhizomes (Ray *et al.,* 2022). *C. longa* is conventionally cultivated from healthy buds of rhizomes as seed material, which needs to be retained at about 20-30% of the annual production to raise the next season's crop. Moreover, the multiplication rate of the rhizome is very low, and its yield is affected by biotic and abiotic stress. Thus, it has now become inevitable to adopt micropropagation techniques in raising *C. longa* (Sumathi *et al.,* 2008). Amongst all biotic factors, fungal diseases in *C. longa* constitute a significant concern as the rhizome is underneath the ground and controlled by chemical fungicides that interact with the medicinal components of *C. longa* (Sajid *et al.,* 2022).

To overcome this challenge, researchers need to look into alternative measures, including "adopting tissue culture raised plants for cultivation and minimizing the usage of chemical fungicides by complementing with bio fungicides such as *Trichoderma viride*. *In vitro* micropropagation has proven to be a successful vegetative propagation strategy for a variety of high-demand plant cultivars. Compared to conventional means, this technology is known to produce disease-free plantlets of improved mass-scale quality (Ali *et al.,* 2011; Ngomuo *et al.,* 2014). We from our laboratory have successfully established an efficient regeneration protocol from rhizome bud explants of *C. longa* (Unpublished data). Micropropagated plantlets will be prone to high relative humidity and poor gaseous exchange between the micro and macro environment, which in turn induces physiological imbalances and thus makes them vulnerable to adapt upon sudden transfer into *ex vitro* conditions (Chakrabarty and Datta, 2008; Dias *et al.,* 2011; Talla *et al.,* 2022). This swift relocation of plantlets results in the accumulation of excess reactive oxygen species (ROS), ultimately resulting in oxidative stress and might be a possible reason for high rates of mortality during acclimatization. Therefore, the adequate pool of antioxidant metabolites and a well-developed antioxidant defense system of *in vitro*-raised plantlets is quite essential for successful acclimatization, which immediately persuades their survival and performance (Batkova *et al.,* 2008; Talla *et al.,* 2022).

The use of microbes (bacteria and fungi) for biotic and abiotic stress management was performed by several researchers (Zaidi and Singh, 2013; Misra and Ansari, 2021). Our study focused on stress tolerance in plants by deploying *T. viride* during acclimatization, an eco-friendly bio-control agent that has been a role model for sustainable agricultural practice for many years (Singh *et al.,* 2015; Al-Ani, 2018). It is quite familiar

as plant growth-promoting fungi (PGPF) and also a potential fungicide with antagonist function on several soil-borne fungal pathogens (Martínez-Medina *et al.,* 2011; Barari, 2016; Khan *et al.,* 2017; Castillo *et al.,* 2019). Several studies revealed an enhancement in plantlet survival rate upon *T. viride* treatment during acclimatization in various plant species (Gutiarrez-Miceli *et al.,* 2008).

Based on this background, in this study, we aimed to investigate the influence of *T. viride* on antioxidant defense mechanisms to combat oxidative stress caused during acclimatization of *in vitro* regenerated *C. longa* plantlets. We determined the MDA level and chlorophyll content to assess stress intensity. Further, the antioxidant enzyme activities were evaluated in both *T. viride* treated and untreated plantlets on different days of the acclimatization period. The present study revealed the pronounced role of *T. viride* in the induction of antioxidant enzyme activities to counteract the oxidative stress caused during acclimatization. Our study is the first of its kind, revealing the role of *T. viride* in improving antioxidant defenses of micropropagated *C. longa* plantlets against oxidative stress in acclimatization conditions.

## Materials and methods

**Plant material**: Rhizomes of *C. longa* cv. Duggirala were surface sterilized and bud parts were excised for *in vitro* culture establishment. The rhizomes were collected from the Turmeric Research Station, Kammarpally, Nizamabad. The surface sterilization and regeneration was standardized in our laboratory and the data is unpublished.

**Acclimatization:** The *in vitro* raised *C. longa* plantlets with well-developed root and shoot system (approx.15 cm) were collected and thoroughly washed to ensure there was no adherent agar on the surface of plantlets and shifted to portrays (40 well) with soil: coco-peat: manure (1:1:1) mixture. From day 1, plantlets were treated with only 10 mL *T. viride* (5 g/L) to avoid excess moisture, which may lead to rotting of roots. The acclimatization for the first 15 days was performed by placing the plantlets covered with a transparent polythene sheet under light with photoperiod conditions of 16:8 h and relative humidity (80–85 %). Next, the polythene sheets were removed, and the plantlets were transferred to pots containing soil and vermi compost (1:1) and were grown in a greenhouse until the 60$^{th}$ day.

**Biopriming:** *T. viride* was provided by SLN Biologicals LLP, Nizamabad, which contains a spore count measuring CFU 2 x $10^{6-8}$. The *in vitro* plantlets were treated regularly throughout the acclimatization period with *T. viride* (5 g/L water), and the application was given to soil at the rhizosphere.

**Sample collection:** The fresh healthy leaf samples (from *in vitro* raised plantlets which were under different days of acclimatization periods 0, 15, 30, 45 and 60$^{th}$ day) were used for assessment of MDA, total chlorophyll contents, and antioxidant enzyme activity.

**Measurement of malondialdehyde:** The determination of lipid peroxidation was performed by quantifying the amount of malondialdehyde (MDA) content produced by the thiobarbutyric acid (TBA) reaction with minor modifications as per Heath and Packer (1968). The leaf sample of 1 g was homogenized and ground to a fine powder using liquid nitrogen in a mortar and pestle by adding 1 mL of 0.5 % trichloroacetic acid (TCA). The obtained homogenate was collected and centrifuged at 13,000 g for about 20 min at 4 $^{o}$C, and to the supernatant (0.5 mL), 2.5 mL of 20 % TCA containing 0.5% TBA was added and incubated at 95 $^{o}$C for 30 min then cooled immediately on an ice bath. This solution was centrifuged at 10,000 g for 10 min at 4 $^{o}$C, and the MDA content was determined from the resulting supernatant. The MDA level was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) using the extinction coefficient (ε) of absorbance 155 $mM^{-1}$ $cm^{-1}$.

**Determination of chlorophyll content:** The modified protocol of Lichtenthaler (1987) was followed to determine the total chlorophyll (Chl a+b). To the leaf extract, 5 mL of 80% (v/v) acetone is added, then filtered with Whatmann No.1 filter paper and kept in darkness until the sample's colour completely turned colourless. The Chl a and Chl b were estimated using a ultraviolet–visible–visible (UV–Vis) spectrophotometer at 663 nm and 645 nm (Lab India UV-3000) as per Lichtenthaler and Wellburn (1983).

***Determination of antioxidant enzymes***

**Extract preparation:** For the determination of antioxidant enzyme activities, fresh leaf samples of 0.5 g were homogenized using liquid nitrogen in 1 mL of extraction buffer (50 mM potassium phosphate buffer (pH 7.5) comprising 1 % polyvinyl pyrrolidone (PVP), 1 % Triton X-100, and 0.1 g of EDTA). The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 14,000 rpm for 20 min. The resulting supernatant was used for enzyme assays (catalase, ascorbate peroxidase and glutathione reductase), and the total soluble protein content in the enzyme extracted was quantified according to Lowry's method (1951) using bovine serum albumin as standard.

**Catalase (CAT):** CAT (EC: 1.11.1.16) activity was determined from the rate of $H_2O_2$ decomposition as monitored by the decrease of absorbance at 240 nm following the procedure of Aebi (1974). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 100 µg of enzyme extract in a total volume of 3 mL, and the reaction started by adding 10 mM $H_2O_2$. The activity was calculated by using the extinction coefficient of absorbance 0.036 $mM^{-1}$ $cm^{-1}$.

**Ascorbate peroxidase (APX):** APX (EC: 1.11.1.11) activity was measured by recording enzyme breakdown of ascorbate and decrease in absorbance at 290 nm due to oxidation of ascorbic acid (ε = 2.8 $mM^{-1}$ $cm^{-1}$) to dihydro ascorbate by $H_2O_2$ (Nakano and Asada, 1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate, 0.1 mM $H_2O_2$, 0.1 mM EDTA and 100 µg of enzyme extract.

**Glutathione reductase (GR):** GR (EC: 1.6.4.2) activity was recorded as described by Foyer and Halliwell (1976), on glutathione-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. The assay mixture comprised 50 mM phosphate buffer (pH 7.5), 1.0 mM EDTA, 0.2 mM NADPH, and 0.5 mM glutathione disulfide (GSSG). The correction was made for any GSSG oxidation in the absence of NADPH. The enzyme extract of 100 µg was added to initiate the reaction and was allowed to run for 3 min. The GR activity was measured by using an extinction coefficient of NADPH at the absorbance of 340 nm (ε ¼ 6.2 $mM^{-1}$ $cm^{-1}$).

**Data analysis**: The data presented in the given study are the average mean values (± SE) of results obtained from triplicates and data was subjected to one way ANOVA (Duncan's Multiple Range Test) using SigmaPlot Version 12.0.

## Results and discussion

*C. longa* has enormous economic demand across the world due to its active ingredient, curcumin, which is widely used in pharmaceuticals, cosmetics and dyes (Jyotirmayee and Mahalik, 2022). But, the quality and quantity of *C. longa* components were adversely affected by several abiotic and biotic stress factors (Sumathi *et al.,* 2008). Adapting trait improvement strategies such as establishing an efficient regeneration protocol of *C. longa* and acclimatization with low mortality, healthy plantlets is mandatory. It was quite evident from several reports that biopriming of *T. viride* to *in vitro* raised plantlets has improved the acclimatization process (Rawat *et al.,* 2011). Several demonstrated reports suggested that *T. viride* colonizes at the rhizosphere and synthesizes secondary metabolites, which stimulate the plant's antioxidant defense systems to combat oxidative stress during acclimatization (Sood *et al.,* 2020). Until now, there have been scanty studies on improving acclimatization, which is a key step of any successful *in vitro* regeneration protocol. In *C. longa* there are no such efforts undertaken. Hence, this study focused on acclimatization improvement strategies assisted by the deployment of *T. viride*.

It was quite familiar that malondialdehyde (MDA), a product of lipid peroxidation, is considered as an indicator of free radical damage to cell membranes caused by oxidative stress. In this study, we examined MDA to evaluate stress levels because the sudden transfer of plantlets from *in vitro* to *ex vitro* conditions during acclimatization leads to oxidative stress (Talla *et al.,* 2022). We quantified the MDA levels during different acclimatization periods of micropropagated plantlets with and without priming of *T. viride*. During the initial days of acclimatization, there observed a significant increment of MDA levels in both treated and untreated plantlets, this increase was prolonged in control from 0 to 30 days. On the whole, the plantlets treated with *T. viride* have exhibited significantly low levels of MDA content compared to the control (Fig. 1a). The decrement of MDA accumulation in *T. viride* treated plantlets might be due to the increased activity of antioxidant enzymes responsible for minimizing the excess ROS, thus suppressing the lipid peroxidation levels. Our results are in line with several reports that demonstrated a decrease in MDA levels upon biopriming of plantlets with *T. viride* (Rawat *et al.,* 2013; Dief *et al.,* 2021; Metwally and Soliman, 2022).

In general, the chlorophyll complexes will be the major target of any oxidative stress which is due to damage of cell membranes by excess ROS, resulting in a decrease of chlorophyll contents, ultimately retarding overall plant growth (Singh and Gautam, 2013). During the acclimatization process, the leaves of *in vitro* plantlets turn dark green due to mesophyll cell differentiation thereby leading to alterations in the synthesis of pigments (Pospisilová *et al.,* 1999), thus limiting the photosynthetic apparatus development (Muller and Munné-Bosch, 2020). The study conducted by Zhang *et al.* (2016) demonstrated decreased chlorophyll content in wheat seedlings upon NaCl stress, but chlorophyll content recovered when treated with *T. viride*. This

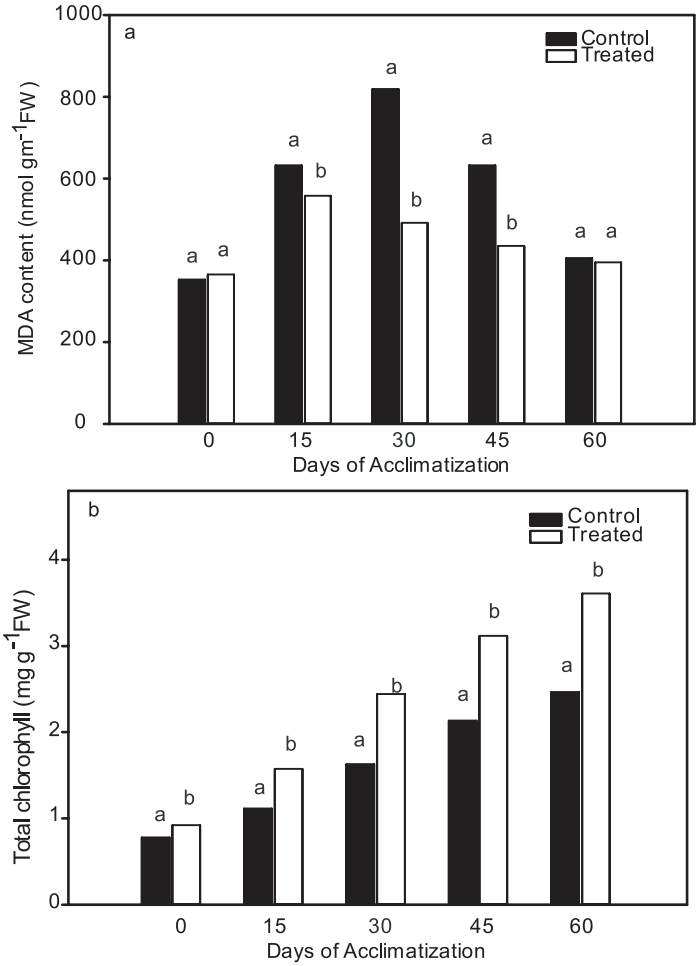


Fig.1. Lipid peroxidation (a) and total chlorophyll content (b) in control and *Trichoderma viride* treated micropropagated plantlets at different days of acclimatization periods (0 to 60$^{th}$ day). Different letter on bar pair indicate that the differences ($P < 0.05$) between the different experimental samples are statistically significant as determined by one way ANOVA (Duncan's Multiple Range Test).

might be due to the inhibition of ROS production (Qi *et al.,* 2012). Similarly, even in our study, the *in vitro* regenerated plantlets, both *T. viride* treated and untreated, exhibited a significant increase in the content of photosynthetic pigments till 60$^{th}$ day acclimatization period (Fig. 2b). The total chlorophyll contents were significantly high in *T. viride* treated plantlets when compared to untreated ones (Fig. 2b).

In plants, the excess production of ROS is generally considered an oxidative stress that needs to be minimized; if not, it leads to several deleterious effects (Younis *et al.,* 2010). In order to alleviate the damage caused by excess ROS, plants have evolved to develop a wide range of enzymatic defense mechanisms (Li *et al.,* 2011). Similar to the other abiotic stresses, there is an enhancement in the antioxidant enzyme activities during the acclimatization process of *in vitro* raised plantlets (Dias *et al.,* 2011; Ahmed and Anis, 2014). Evidence demonstrated that the application of *T. viride* stimulates the antioxidative enzyme activities to minimize the elevated ROS levels produced during stressed conditions (Ahmad *et al.*, 2015; Zhang *et al.*, 2016). Even in our study, there observed apparent enhancement in all three antioxidative enzymes (CAT, APX and GR) in both *T. viride* treated and control plantlets during acclimatization process (Fig. 2a–c). This increase in enzyme activities was significantly higher

in *T. viride* treated compared to control. These findings suggest the pronounced role of *T. viride* in alleviating the oxidative stress caused during acclimatization by induction of antioxidant defense systems.

The present study reports a crucial role of *T. viride* in stimulating antioxidant defense mechanisms to counteract the oxidative stress caused during acclimatization. This study enlightens the versatile roles of *T. viride* by protecting both from biotic/abiotic stress which is encompassed by sensitive *in vitro* plantlets face during adaptation to macro environments. The innovative component of this work lies in amalgamating biotization methods to the acclimatization process in improvising the survival rates of *in vitro* plants in external adverse environments.

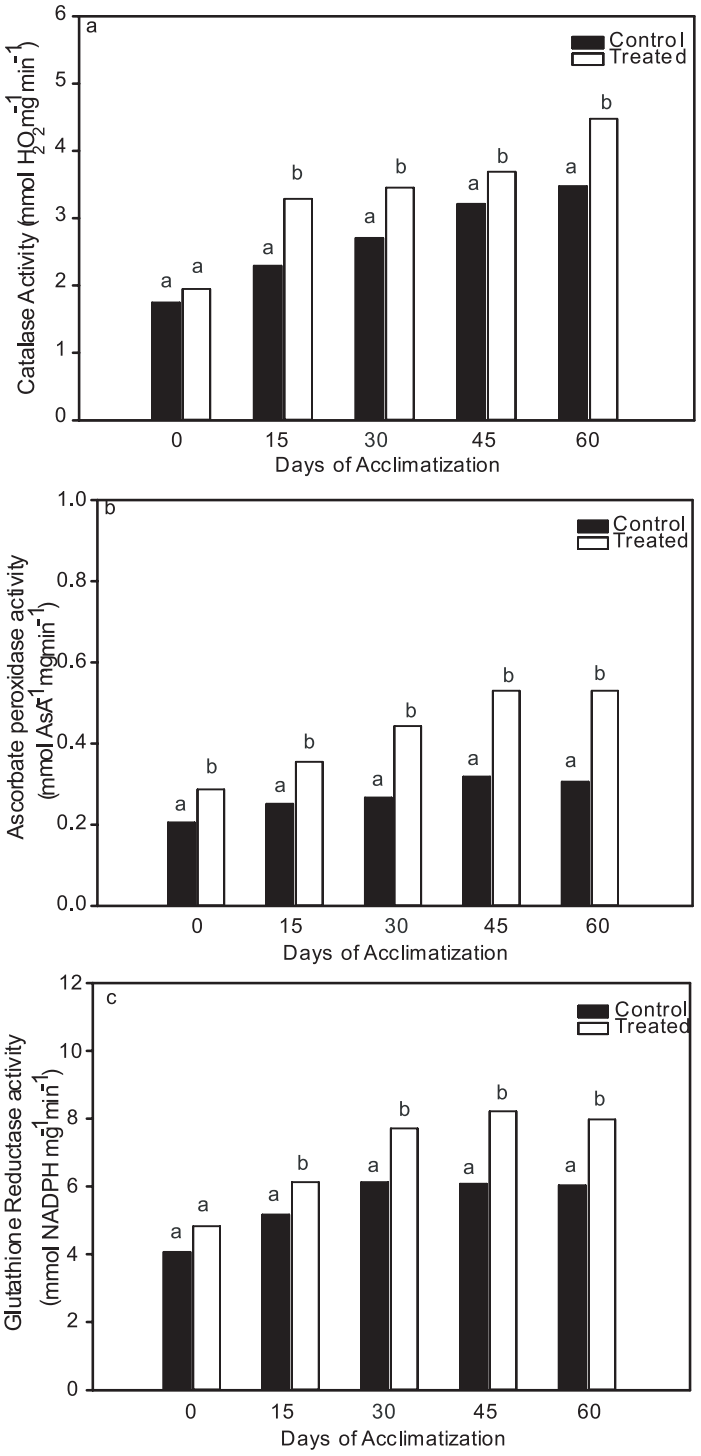


Fig. 2. Activities of catalase (a), APX (b) and GR (c) enzymes in control and *Trichoderma viride* treated micropropagated plantlets at different days of acclimatization periods (0 to 60$^{th}$ day). Different letter on bar pair indicate that the differences ($P < 0.05$) between the different experimental samples are statistically significant as determined by one way ANOVA (Duncan's Multiple Range Test).

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