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Research article

Evidences for growth-promoting and fungicidal effects of low doses of tricyclazole in barley

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ABSTRACT

The effect of increasing concentrations (5–100 mg L^{-1}) of tricyclazole (TCZ), an important fungicide commonly used for control of spot blotch disease, was investigated for changes in physiological and biochemical parameters in 10 and 20-days-old barley plants (*Hordeum vulgare* L., *cv.* RD-2508). A 10 mg L^{-1} dose of TCZ supplemented with nutrient solution in barley plants reflected a lowered infection with a significant increase in plant growth, plant biomass, leaf chlorophyll level, altered reactive oxygen species (ROS) formation and altered activity of key antioxidant enzymes *viz*. superoxide dismutase (SOD, EC: 1.15.1.1), catalase (CAT, EC: 1.11.1.6), ascorbate peroxidase (APX, EC: 1.11.1.1) and guaiacol peroxidase (GPX, EC: 1.11.1.7). To our knowledge this is the first report that provides evidence for TCZ to act both as a fungicide as well as to have plant growth-promoting activity. The study suggests that this dual property of tricyclazole has a potential for integration in disease management programs in barley. Application of low doses of TCZ can fit in well with environment friendly strategies for sustainable barley crop production, more yield and minimal soil contamination.

1. Introduction

Barley (*Hordeum vulgare* L.) is an important cereal crop of family *Poaceae*, which is one of the first domesticated crops worldwide (Bothmer et al., 2003). Compared to the other crops, barley can be grown over a wide range of environmental conditions (Langridge and Barr, 2003). Barley is grown in many countries. It comes after maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*), occupying the fourth rank among all cereal crops in the World (Food and Agriculture Organization of the United Nations, 2006; Steven, 2010).

Fungicides are chemicals used to control fungal diseases, wherein, the primary disease risk depends mainly on variety, sowing date, location and local weather. Diseases caused by fungal pathogens have significant impact on cereal productions (Butt et al., 2001). Their use also led to significant reduction in the losses with improved crop yield (Steven, 2010). Tricyclazole (TCZ) ($C_9H_7N_3S$) [5-methyl-1, 2, 4,-triazolo (3,4,-b) (1,3) benzothiazole], has been observed to be one of the most effective chemical to control spot blotch disease among the commercially used melanin biosynthesis inhibitor formulations (Lee et al., 2003; Wheeler and Greenblatt, 1988; Butler et al., 2005). TCZ is advantageous over other fungicides as it provides long term protection during the entire growth period. Due to its long effectiveness the requirement of multiple applications is essentially ruled out (Froyd et al., 1976; Qamruzzaman and Abu, 2014). Moreover, it is readily absorbed by plant roots and translocated to leaves, where it provides residual disease control by inhibiting the synthesis of 1, 8- dihydroxy naphthalene melanin. Therefore TCZ application in agricultural field may be beneficial for crop improvement.

Earlier we reported that the loss of the melanin production in cell wall of *Bipolaris sorokinina*, reduces the spore production, spore size and number of septa in conidia in presence of TCZ (Chand et al., 2014). A decrease in virulence of pathogenic fungi *Bipolaris sorokiniana* upon foliar application of TCZ was also reported by our group (Kumar et al., 2014).

It is likely that application of fungicide/chemicals on barley plants may perturb the antioxidant apparatus including ROS and antioxidant enzymes *viz*. Superoxide dismutase (SOD), Catalase

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(CAT), Ascorbate peroxidase (APX) and Guaiacol peroxidase (GPX) (Habibzadeh et al., 2013; Singh and Shah, 2014), and/or induce *de novo* synthesis of defense related proteins that form part of plant defense machinery (Fischer et al., 1989; Kwon et al., 2008). It is the altered levels of these key players in plants that orchestrate the plant's ability to resist the TCZ induced stress which remains unexplored for integrated disease management of crop plants.

The present work therefore aims to study the effect of TCZ on barley plants with respect to change in plant growth, biomass, levels of ROS and antioxidant enzymes helps to establish the potential doses of TCZ tolerance in barley and its application in integrated disease management.

2. Materials and methods

2.1. Plant material and test conditions

Barley *cv.* RD-2508 seeds were obtained from Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. Seeds were surface sterilized with 0.1% sodium hypochlorite solution for 10 min, rinsed with distilled water and imbibed for 24 h in water. Ten seeds were sown in plastic pots filled with sterilized sand containing Hoagland nutrient (Hoagland and Arnon, 1950) solutions as control and supplemented with increasing doses (5, 10, 25, 50 and 100 mg L^{-1}) of TCZ (Sigma, USA) by initially solubilising in a drop of ethanol followed by dilutions with distilled water as per required for plant growth. Experiment was conducted in Complete Randomized Block Design with five concentrations and three replications each of TCZ doses.

Seedlings were grown in two replications for 10 and 20 days in a greenhouse at 18±1 °C under ~80–85% relative humidity with 12 h light/dark cycle (irradiance 170–576 μmol m^{-2} s^{-1}). Fresh weight (mg), shoot and root length (cm), leaf area (length and width in cm) of 10 plants from each replications were taken. The reactive oxygen species and activities of antioxidant enzymes SOD (EC: 1.15.1.1), CAT (EC: 1.11.1.6), APX (EC: 1.11.1.1) and GPX (EC: 1.11.1.7) were determined in uprooted seedlings at 10 and 20 days of growth.

Non-destructive, measurements of chlorophyll content in leaves were recorded as SPAD (Soil Plant Analysis Development) values in relative units using chlorophyll meter (SPAD 502, Minolta, Japan). Five measurements were recorded for SPAD value corresponding to leaf chlorophyll levels at different positions starting from tip to base of each leaf (Lombard et al., 2010; Rosyara et al., 2010).

2.2. Assay of antioxidant enzymes in leaves and roots of barley seedlings exposed to TCZ

The enzyme SOD was determined in 10 and 20-days-old barley seedlings (Misra and Fridovich, 1972; Shah et al., 2001). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of nitroblue tetrazolium (NBT) reduction measured at 560 nm wavelength. The specific activity of CAT was determined by spectrophotometer adopting the method of Beers and Sizer (1952) at 240 nm and expressed as μmol H_2O_2 oxidized mg^{-1} protein min^{-1} using an extinction coefficient of 0.036 mM^{-1} cm^{-1}. The GPX activity was determined according to the method of Egley et al. (1983) at 420 nm and expressed as μmol H_2O_2 reduced mg^{-1} protein min^{-1} using an extinction coefficient of 26.6 mM^{-1} cm^{-1}. APX activity was assayed according to Nakano and Asada (1981) at 290 nm and expressed as μmol ascorbate oxidized mg^{-1} protein min^{-1} using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. Lower, middle and upper leaves from 10 to 20-day-old barley seedlings were used in all enzymatic preparations and protein was measured according to the method of Bradford (1976).

2.3. Histochemical study of H_2O_2 and $O_2^{\bullet -}$ formation in barley leaves and roots

Superoxide anion ($O_2^{\bullet -}$) was detected in plant leaves according to Frahry and Schopfer (2001) using the dye NBT. Dark blue insoluble formazan produced by the reaction of NBT with $O_2^{\bullet -}$ was examined under light microscope.

The H_2O_2 formation was determined using the 3, 3′-diaminobenzidine (DAB; Amresco, Solon, OH, USA) according to Thordal-Christensen et al. (1997) formed due to perhydroxides precipitate or brown DAB-H_2O_2 polymer, and visualized as reddish brown coloration under light microscope.

Hydrogen peroxide was also detected in roots using fluorescent dye dichlorodihydrofluoresce in diacetate (DCFH-DA, Sigma, USA) according to Kristiansen et al. (2009). Root tips (5 mm) were excised from 20-day-old barley seedlings and submerged in 10 μmol DCFH-DA in 5 ml dimethyl sulfoxide (DMSO) for 3 h. The root tips were washed three times in 50 mM sodium phosphate buffer (pH 7.4) and visualized under the fluorescent light with a Nikon Eclipse & E 200 MV fluorescence microscope (Nikon Inc. Japan). The formation of H_2O_2 was observed as light green zones in roots.

2.4. Effect of TCZ on infection process in barley leaves

The experiment was conducted in greenhouse under 80–95% humidity at 27 ± 2 °C. Two sets of 20-day-old barley plants (*cv.* RD-2508) were taken for experiments, plants without TCZ treatment (control) and plants exposed to 10 mg L^{-1} TCZ treatment. The barley plants were inoculated by spraying the *B. sorokiniana* spore suspension (10^4 spores/ml) and kept with leaf surface wetted for 12 h. Two days after inoculation histopathological studies were performed for studying the reaction of TCZ on disease control, as described by Sillero and Rubiales (2002). However, 10-days after inoculation the leaves were also used for studying the spore formation and their position on the leaf surface under light microscope. The suitable photographs were taken under a combination of eye piece and objective (12.5 × 25) by the Nikon Eclipse E200MV R microscope (Nikon Corporation, Tokyo, Japan).

2.5. Statistical analysis

Statistical analysis was carried out using one-way ANOVA for determination of significant differences between treatments (separately for shoots and roots) by Tukey's test (SPSS; version 16.0, software). P values ≤ 0.05 were considered as significant. All the experiments were carried out in triplicate.

3. Results

3.1. Effect of TCZ on growth and biomass of barley seedlings

Fig. 1 shows significant increase in length and width of leaves in barley seedlings grown for 10 and 20 days in 5 and 10 mg L^{-1} TCZ in the growth medium. At 25–100 mg L^{-1} TCZ treatments, a gradual decrease in length and width of leaves were recorded (Table 1). The SPAD values corresponding to chlorophyll content increased significantly with increasing dose of TCZ (5–100 mg L^{-1}) over controls (Table 1). The barley seedlings show a similar or an increased root/shoot biomass under 5–25 mg L^{-1} of TCZ at both 10 and 20 days of growth. Barley seedlings grown in 50–100 mg L^{-1} of TCZ, significantly reduced seedling biomass compared to control (Table 2). The shoot and root length of barley seedlings significantly increased in presence of 5 mg L^{-1} TCZ by 8.6% and 11.7% whereas in 10 mg L^{-1} TCZ by 20.1% and 17.8%, respectively at 20 days of growth