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ORIGINAL PAPER

Effect of Tricyclazole on morphology, virulence and enzymatic alterations in pathogenic fungi *Bipolaris sorokiniana* for management of spot blotch disease in barley

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Abstract *Bipolaris sorokiniana* synthesizes the 1,8-dihydroxynaphthalene (DHN) melanin via pentaketide pathway and promotes the development of aerial mycelia and conidia. A melanin biosynthesis inhibitor Tricyclazole (TCZ), brought changes when applied at 5–100 μg ml^{-1} concentration in the colony morphology, radial growth, mycelia weight, melanin content, antioxidant enzymes (SOD and CAT) and extracellular hydrolytic enzymes (cellulase, pectinase, amylase and protease) in black, mixed and white isolates of *B. sorokiniana*. A significant alteration was recorded in antioxidant enzymes in black and mixed isolates; however, non-significant alteration was recorded in white isolate. Isolates of *B. sorokiniana* exposed to 100 μg ml^{-1} TCZ showed significantly increased formation of superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)·H_2O_2 was detected significantly high in hyphae and conidia while, $O_2^{\cdot-}$ was found primarily in the conidia. Microscopic results suggest that TCZ damages not only the cell wall but also the cell membrane. The foliar application of TCZ (25, 50 and 100 μg ml^{-1}) decreases the area under disease progress curve, lesion development and spore formation on barley leaves thereby reducing potential for the disease development. In conclusion TCZ influences the pathogenic ability by damaging the cell structure of hyphae and conidia and also alters the antioxidant enzyme levels in *B. sorokiniana*. TCZ may therefore, works against to pathogen for better management of spot blotch disease in barley infected with *B. sorokiniana*.

Keywords Antioxidant · AUDPC · *Bipolaris sorokiniana* · Melanin · Spot blotch · Tricyclazole

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Introduction

Bipolaris sorokiniana is a well known plant pathogen and causative agent of several diseases of cultivated and wild plants including barley (Chand et al. 2003). A significant yield reduction was reported for spot blotch disease caused by *B. sorokiniana* in South Asian countries, and therefore it is considered a serious pathogen (Duveiller et al. 1998; Saari 1998). The infected cereal seeds are considered to be a major source of spot blotch disease (Chand et al. 2002; Chowdhury et al. 2013; Pandey et al. 2008). The risk of spot blotch epidemics are high in areas characterized by average temperature above17 °C during the cropping season with high relative humidity (Chaurasia et al. 1999).

During its development, the fungi interect with environmental factors and are constantly subjected to physical

and chemical stresses. Environmental factors like ionizing radiation (α, β, γ, and X-rays), UV radiation, temperature shifts and mechanical damage etc. significantly influences fungal development. In nature, the involvement of oxygen in metabolic processes of living organisms is coupled to its activation and formation of a number of highly reactive oxygen species (ROS) i.e. singlet state of oxygen ($1O_2$), superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), peroxide radical ($HO_2^{\cdot}$), peroxide ion (HO_2^-), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\cdot}$). At present, the main source of $O_2^{\cdot-}$ in the cell is the partial reduction of oxygen, releasing a H_2O molecule during the respiration process (Dröge 2002; Skulachev 1996). The toxicity of radicals and their role in pathological processes as well as aging are well documented (Dröge 2002; Longo et al. 2005; Zenkov et al. 2001). Reports are available on ROS mediated regulation on proliferation, differentiation, extracellular signal transduction, ion transport and immune response in organism (Chand et al. 2014; Dröge 2002). The population of *B. sorokiniana* produces black to mixed or white fluffy mycelia and belongs to black, mixed and white sub-populations (Bashyal et al. 2010; Chand et al. 2014; Poloni et al. 2009; Pandey et al. 2008).

Melanin is one of the most stable and resistant biochemical moieties insoluble in water (Dixon et al. 1991). Melanins are structurally very diverse and carry three types of polymers viz- eumelanin $(DOPA)_n$, pheomelanin (Cysteinyl $DOPA)_n$ and allomelanin $(DHN)_n$. Melanins are often complexed with protein and less often with carbohydrates (Cheng et al. 2004). In many phytopathogenic fungi melanin plays an important role in persistence of hyphae, conidia and formation of appressoria (Butler and Day 1998; Henson et al. 1999). Melanin is reported to be important for conidiogenesis in *B. sorokiniana* (Bashyal et al. 2010). The protective role of allomelanin against strong oxidants (O_2, H_2O_2, O_3, $OH^{\cdot}$, HO_2, etc), free radicals (NO and NO_2) and ROS is required for the virulence of several phyto-pathogenic fungi including *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Paracoccidioides brasilensis*, *Sporothrix schenckii* and *Exophialia* (Wangiella) *dermatitidis* (Dixon et al. 1991; Gomez et al. 2001; Romero-Martinez et al. 2000; Schnitzler et al. 1999). The regulation of peg penetration during infection is prevented due to high turgor pressure exerted by the melanized appressoria (Deising et al. 2000). Melanin also limits the secretions of lytic enzymes necessary for host tissue degradation and pathogenesis in *Verticillium dahliae*, *Alternaria alternata*, *Cochliobolus heterostrophus* (Bell and Wheeler 1986; Henson et al. 1999; Tanabe et al. 1995). Extracellular hydrolytic enzymes produced by melanin deficient white isolate of *B. sorokiniana* play important role in pathogenesis and disease development (Chand et al. 2014).

To overcome the problems of disease development, Tricyclazole (TCZ; $C_9H_7N_3S$) [5-methyl-1, 2,4,-triazolo (3,4,-b) (1,3) benzothiazole], is commonly used as fungicide in many countries (Chaube and Pundhir 2005; Chida and Sisler 1987; Thines et al. 2004). TCZ inhibits the DHN-melanin biosynthesis, but does not significantly affect the mycelia growth (Bashyal et al. 2010; Bashyal and Aggarwal 2013; Kunova et al. 2013), however it blocks the melanin biosynthesis pathway namely the reduction of 1, 3, 6, 8-THN to scytalone and 1, 3, 8-THN to vermelone (Huang 1981; Huang et al. 1993). TCZ is shown to have no inhibitory effect on the enzymatic dehydration of scytalone or vermelone (Wheeler 1982). Chattopadhyaya et al. (2013) reported that the foliar application of TCZ reduces the disease development caused by *B. sorokiniana*. The direct effect of TCZ on the capability of germinating conidia to penetrate host epidermis is well documented (Mares et al. 2004; Inoue et al. 1987; Woloshuk et al. 1983), whereas reduced secondary infection observed in the field was inferred to be due to reduced production of conidia or to lower virulence of conidia produced on tricyclazole-treated lesions (Kurahashi 2001; Kunova et al. 2013; Okuno et al. 1983; Zhang and Zhou 2004). The present work therefore, aims to study the effect of TCZ on *B. sorokiniana* with respect to colony morphology, melanin content, antioxidant, extracellular hydrolytic enzymes, infection process and damage to the cell structure for the management of spot blotch disease in barley caused by *B. sorokiniana*. Further, to evaluate the impact of TCZ treatment on barley spot blotch progress, the possible effects of TCZ on the infection efficiency of conidia, germination, AUDPC, lesion developments and spore formations on barley leaves have been studied.

Materials and methods

Experimental material

The three isolates of *B. sorokiniana* maintained on Sorghum grain (Chand et al. 2013), belonging to black (WPB-23), mixed (WPM-29) and white (WPR4 (87) subpopulations were used in this study. To study the various concentrations of TCZ (0, 5, 10, 25, 50 and 100 μg ml^{-1}) as food poison in medium for isolates were performed (Elliott 1995). 4 days old culture mycelia plug (5 mm diameter) inoculated and incubated at 25 ± 1 °C and data were recorded for colony color/morphology, radial growth (mm) and mycelia weight (mg) 7 days after inoculation. Experiment was laid in complete randomized block design in triplicate.

Extraction and quantification of melanin

The melanin pigment was extracted from 10 days old culture of *B. sorokiniana* according to the method of Gadd (1982). Melanin content (µg/g of mycelium) was determined by using standard melanin (Sigma Chemicals Co., St. Louis, USA).

Assay of extracellular hydrolytic enzymes

The cultures were grown in liquid media (6 g $NaNO_3$, 0.5 g KCl, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $ZnSO_4$, 0.01 g $FeSO_4$, one litre distilled H_2O) by adding different concentration of TCZ i.e. 0, 5, 10, 25, 50 and 100 µg ml^{-1} and substrates according to the assay of enzymes by a slightly modified method of Poloni et al. (2009). A single mycelia plug (5.0 mm diameter) from 4 days old cultures of *B. sorokiniana* isolates were transferred individually in 20 ml liquid media supplemented with enzyme specific substrates carboxymethyl cellulose (CMC) (1 %), pectin (1 %), starch (1 %) and gelatin (4 %) for cellulase, pectinase, amylase and protease respectively in triplicate and incubated at 25 ± 1 °C for 10 days. The culture was filtered through Buchner funnel using Whatman No.1 filter paper, centrifuged at 10,000×*g* for 10 min at 4 °C and supernatant transferred in fresh tubes for further study. Protein concentration in all the enzyme preparations was determined by the method of Bradford (1976), while the activities of extracellular hydrolytic enzymes (cellulase, pectinase, amylase and protease) were estimated according to previous studies (Miller 1959; Barnett and Fergus 1971; Dubey et al. 2010; Sarao et al. 2010).

Assay of antioxidant enzymes

Superoxide dismutase (SOD) activity was measured based on the inhibition of nitroblue tetrazolium chloride (NBT) reduction by $O_2^{\cdot-}$ under light. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction measured at 560 nm (Beauchamp and Fridovich 1971).

Catalase (CAT) activity was assayed by the method of Beers and Sizer (1952). About 200 mg of 10 days old mycelia were homogenized in 5 ml of 50 mM Tris–HCl buffer (pH 8.0) (0.5 mM EDTA, 0.5 % (v/v), Triton X-100 and 2 % (w/v) polyvinyl pyrrolidone using chilled mortar and pestle. The homogenates were centrifuged at 12,000×*g* for 10 min at 4 °C; supernatant was transferred in fresh tubes. The CAT activity was measured at 240 nm by decomposition of H_2O_2 (extinction coefficient of 0.036 mM^{-1} cm^{-1}) by observing decrease in absorbance using a double beam UV–VIS spectrophotometer (ELICO-SL 191). Enzyme specific activity is expressed as µmol H_2O_2 oxidized mg^{-1}protein min^{-1}.

Histochemical study for $O_2^{\cdot-}$ and H_2O_2 in *B. sorokiniana* exposed to Tricyclazole

NBT was used as a dye to localize superoxide anion ($O_2^{\cdot-}$) according to method of Frahry and Schopfer (2001). About 100 mg, 10 days old mycelia were scraped by sterilized blade and put in test tube. One ml of 6 mM NBT solution (prepared in 10 mM Na-Citrate buffer (pH 6.0) was added in tube and exposed under light for 8 h., washed thrice in PBS buffer (pH 7.2). Stains react with $O_2^{\cdot-}$ and formed blue coloured insoluble formazan deposits which were visualized under light microscope (model Nikon Eclipse E200MV R, Nikon Instruments Inc.) using a combination of eye piece and objective (12.5 × 25).

Hydrogen peroxide's localization (H_2O_2) in TCZ treated cultures was performed using 3, 3-diaminobenzidine (DAB; Amresco, Solon, OH, USA) according to Kumar et al. (2001), Thordal-Christensen et al. (1997). The mycelia were washed with distilled water and submerged in a solution containing 1 mg ml^{-1} DAB (dissolved in acidified water with HCl (pH 3.8) and incubated for 8 h to allow DAB uptake and its reaction with H_2O_2. The treated mycelia were further washed in saturated chloral hydrate solution. Visualization of processed samples under light microscope showed reddish brown color indicating the localization of H_2O_2.

Electron microscopy and ultrastructure of cell wall of *B. sorokiniana*

Melanin deposition and pores in cell wall were determined by transmission electron microscopy (TEM) (Money 1990; Brendan et al. 2003). The ultra structure for deposition of melanin contents in hyphae and conidial wall was investigated using 4-day-old TCZ (25 µg ml^{-1}) treated cultures of black and white isolates from PDA over the controls. The hyphae were fixed in 2.5 % glutaraldehyde solution. Sample mounting and viewing was performed at AIRF JNU, the images were examined on a JEOL USA JEM-2100F Transmission Electron Microscope.

Effect of Tricyclazole on aggressiveness of *B. sorokiniana* on barley

The experiment was conducted in polyhouse under 80–95 % humidity at 27 ± 2 °C. 40 days old barley plants (cv. RD-2508) were inoculated by *B. sorokiniana* spore suspension (10^4 spores/ml) for the disease reactions and after specific time intervals (0, 24, 48 and 72 h.) inoculated plants were treated by spraying of TCZ (25, 50 and

100 μg ml^{-1}). The control plant sets were also maintained by sterile water treatments. Initial data was collected 72 h. after inoculation and then subsequently three readings were taken after every 4 days. Percent disease severities were calculated according to Saari and Prescott (1975). The AUDPC was calculated using percent disease severity value corresponding to the disease ratings according to Shaner and Finney (1977).

$$\text{AUDPC} = \sum_{i=1}^{n} [\{Yi + (Yi + 1)\}/2\{(ti + 1) - ti\}]$$

where Yi = disease level at time ti, (ti + 1)−ti = time (days) between two lesion scores, n = no of observations (score).

10 days after inoculation, the infected leaves were taken and lesions were excised (1 cm^2) from five leaves randomly selected and used for: (a) visualization of spore positions on the infected portions directly placing under light microscope and (b) numbers of spores/leaf by dislodging the spore from infected leaves pieces in 1 ml of water and visualized under light microscope. The infection process and spore germination of *B. sorokiniana* on barley leaves from above treatments were determined by histopathological studies according to Sillero and Rubiales (2002). 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).

Statistical analysis

Statistical analysis was carried out using one-way ANOVA for determination of significant differences (separately for radial growth, mycelia weight, melanin, spore production, number of septa and spore size) by statistical analysis software (SAS) using PROC GLM and PROC CORR (version 9.2; SAS Institute Inc., Cary, NC 2010). All the experiments were carried out in triplicate. *P* values ≤0.05 were considered as statistically significant.

Results

Effect of Tricyclazole on *B. sorokiniana*

TCZ treated black and mixed isolates turned reddish brown or whitish with increasing concentration of TCZ in the growth media as compared to control (Fig. 1). The significant variation among black, mixed and white isolates of *B. sorokiniana* were recorded for radial growth, mycelia weight, melanin content, antioxidant enzymes (SOD and CAT) and extracellular hydrolytic enzymes (cellulase, pectinase, amylase and protease) under the TCZ exposure (5–100 μg ml^{-1}). The mean melanin content (0.58 μg/g), SOD (12.29 U mg^{-1} protein) and CAT (21.59 μmol H_2O_2 oxidized mg^{-1} protein min^{-1}) were significantly ($P \geq 0.05$) higher in the black isolate followed by mixed and white. However, the mean radial growth (55.33 mm), mycelia weight (71.72 mg), cellulase (15.17 U mg^{-1} proteins), pectinase (10.98 U mg^{-1} proteins), amylase (7.20 U mg^{-1} proteins) and protease (3.49 U mg^{-1} protein) were significantly ($P \geq 0.05$) higher in the white isolate followed by mixed and black (Table 1).

In black isolate the significant growth reduction was recorded at 100 μg ml^{-1} TCZ, in mixed isolate it was recorded at 25–100 μg ml^{-1} TCZ, however in white isolate the growth reduction was noted at 10–100 μg ml^{-1} TCZ compare to controls (Table 2). Similarly, the significant reduction in mycelia weight was recorded when TCZ was applied in black and mixed isolates, but in white isolate a non-significant increase in mycelia weight was noted (Table 2). Black and mixed isolates produced melanin in the culture media and were drastically affected by the application of TCZ. The melanin content was affected on exposure of increasing concentrations of TCZ (5–100 μg ml^{-1}) by 2.04–45.00 folds in back and 2.19–11.25 folds in mixed isolate (Table 2).

Effect of Tricyclazole on antioxidant enzyme activity and release of extracellular hydrolytic enzymes

A greater impact of TCZ on antioxidant enzymes (SOD and CAT) were recorded in black, mixed and white isolates. The inhibition in activity of SOD was recorded to be 1.07–3.97 folds in black, 1.08–2.64 folds in mixed and 1.04–1.35 folds in white isolate upon TCZ exposure (5–100 μg ml^{-1}) in the culture media. Similarly, the activity of CAT also declined significantly in 10–100 μg ml^{-1} TCZ treatments; however a 5 μg ml^{-1} TCZ treatment led to a non-significant reduction in black, mixed and white isolate (Table 2).

The exposure of TCZ (5–100 μg ml^{-1}) in culture media significantly enhanced the production of cellulase in black (2.72–6.50 folds) and mixed (1.59–1.55 folds) isolates over the controls, however in white isolate the cellulase production declined by 1.35–1.55 folds and it may be due to the toxic effect of TCZ. An increase in pectinase activity was recorded in black isolate by 5.89–11.64 folds but, declined in mixed 1.01–1.52 folds and 1.21–1.75 folds in white isolate upon 5–100 μg ml^{-1} TCZ exposures. Similarly, the amylase was increased by 1.41–1.92 folds in black isolate. However, decline in mixed and white isolates, but a non-significant alteration was recorded in mixed isolate upon exposure of TCZ. The release of protease was also recorded increased in white isolate by 1.13–1.41 folds in black and 1.43–2.01 folds in mixed isolates; whereas in

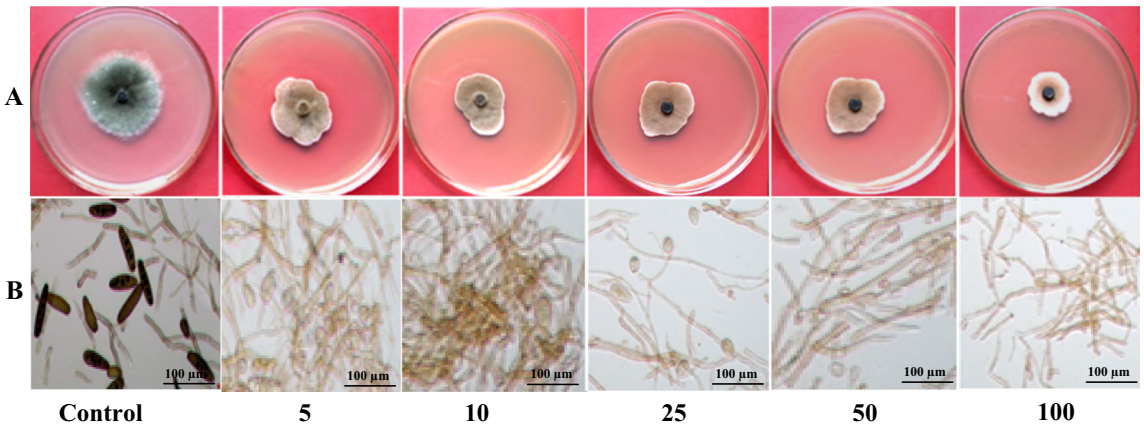

Fig. 1 The effect of increasing concentrations of Tricyclazole (5–100 µg ml^{-1}) on colony morphology (**a**: *upper plates*) and conidia (**b**: *lower plates*) of *black* isolate (WPB-23). The microscopic view **b** indicates reduced conidia size with increasing Tricyclazole levels as compared to controls (*scale bar* 100 µm)

Table 1 Cumulative effect of Tricyclazole (including controls) on radial growth, mycelia weight, melanin content, antioxidant enzymes (SOD and CAT) and extracellular hydrolytic enzymes (cellulase, pectinase, amylase and protease) in black, mixed and white isolates of *B. sorokiniana*

Isolates	Radial growth (mm)	Mycelia weight (mg)	Melanin content (µg)	Antioxidant enzymes		Extracellular enzymes (U mg^{-1} protein)			
				SOD (U mg^{-1} protein)	CAT (µmol H_2O_2 oxidized mg^{-1} protein min^{-1})	Cellulase	Pectinase	Amylase	Protease
Black	45.67	54.46	0.58	12.29	21.59	9.00	5.05	4.73	2.09
Mixed	50.34	64.58	0.30	9.64	19.51	12.06	8.19	5.56	2.74
White	55.33	71.72	0.00	10.28	16.15	15.17	10.98	7.20	3.49
LSD (0.05)	1.16	1.61	0.07	0.59	1.01	0.54	0.57	0.60	0.28

LSD least significance difference ($P \geq 0.05$)

white isolate a significant decline was recorded by 1.14–1.40 folds (Table 3).

Table 4 shows the effect of TCZ on all of the 9 parameters on black, mixed and white isolates of *B. sorokiniana*. The activities of extracellular hydrolytic enzymes and mycelia weight were positive correlated with radial growth upon application of TCZ. However, melanin content and antioxidant enzymes (SOD and CAT) were negative correlated with radial growth.

Effect of Tricyclazole on formation of H_2O_2 and $O_2^{\cdot-}$ in black isolate of *B. sorokiniana*

The microscopic studies was done for formation of H_2O_2 and $O_2^{\cdot-}$ in *B. sorokiniana* grown under 5–100 µg ml^{-1} TCZ-PDA media 7 day after inoculation (Fig. 2). The localization of $O_2^{\cdot-}$ was carried out using NBT that resulted in a dark blue formazan product (Fig. 2). TCZ treatments significantly increased the dark blue spots as compared to untreated wherein the stain for $O_2^{\cdot-}$ levels was much intense in *B. sorokiniana* exposed to 100 µg ml^{-1} TCZ. Formation of H_2O_2 in *B. sorokiniana* was detected as reddish brown stain resulting from reaction between DAB and H_2O_2. The 100 µg ml^{-1} TCZ treated *B. sorokiniana* had more H_2O_2 by the appearance of dense reddish-brown colour (Fig. 2). H_2O_2 was largely located in the conidia and hyphae, while $O_2^{\cdot-}$ was found primarily in the conidia (Fig. 2).

Electron microscopy for Tricyclazole treated isolates

Figure 3 shows the transmission electron micrograph for cell wall of *B. sorokiniana*. The TCZ treatments showed damage in cell wall and cell membrane of the *B. sorokiniana* isolates, the thick, deeply corrugated cell wall contained two distinct layers in non treated black isolate (Fig. 3a). However in white isolate these layers were not clear and showed pores in cell wall. These pores may be helpful to release of the extracellular hydrolytic enzymes (Fig. 3b). In TCZ (25 µg ml^{-1}) treated cultures the cell wall and cell membrane integrity were affected and damaged (Fig. 3c, 4d).

The white isolate hyphae are melanin deficient on outermost cell wall, while black isolate hyphae and conidia

Table 2 Effect of increasing concentration of Tricyclazole (0–100 μg ml^{-1}) on radial growth, mycelia weight, melanin content and antioxidant enzymes activities (SOD and CAT) in black, mixed and white isolates of *B. sorokiniana*

Treatment (μg ml^{-1})	Radial growth (mm)				Mycelia weight (mg)				Melanin content (μg)				Antioxidant enzymes							
													SOD (U mg^{-1} protein)				CAT (μmol H_2O_2 oxidized mg^{-1}protein min^{-1})			
	B	M	W	Mean	B	M	W	Mean	B	M	W	Mean	B	M	W	Mean	B	M	W	Mean
0	47.33	53.00	59.33	53.22	56.67	65.33	73.00	65.00	1.80	0.90	0.00	0.90	18.44	13.37	11.57	14.46	23.81	23.71	22.49	23.34
5	46.33	52.87	57.67	52.29	55.83	65.17	72.67	64.56	0.88	0.41	0.00	0.43	17.26	12.34	11.12	13.57	22.61	22.46	21.27	22.11
10	46.00	52.87	55.00	51.29	54.13	64.83	72.00	63.65	0.48	0.21	0.00	0.23	16.21	11.56	11.01	12.93	22.00	21.34	19.76	21.03
25	45.67	50.20	54.33	50.07	53.57	64.50	71.33	63.13	0.15	0.12	0.00	0.09	10.42	8.94	10.16	9.84	20.96	18.43	15.32	18.24
50	45.33	46.87	54.00	48.73	53.33	63.83	70.67	62.61	0.10	0.10	0.00	0.07	6.76	6.53	9.26	7.52	20.56	16.98	9.71	15.75
100	43.33	46.20	51.67	47.07	53.23	63.83	70.67	62.58	0.04	0.08	0.00	0.04	4.64	5.07	8.57	6.10	19.61	14.16	8.33	14.03
LSD (0.05)	3.77	1.49	2.20	1.64	2.61	5.11	5.35	2.28	0.17	0.30	0.00	0.10	1.54	1.64	0.82	0.83	2.51	3.32	2.10	1.43

B black (WPB-23), *M* mixed (WPM-29), *W* white (WPR4(87)), *LSD* least significance difference ($P \geq 0.05$)

Table 3 Effect of increasing concentration of Tricyclazole (0–100 μg ml^{-1}) on release of extracellular hydrolytic enzymes (cellulase, pectinase, amylase and protease) in black, mixed and white isolates of *B. sorokiniana*

Treatment (μg ml^{-1})	Enzyme activities (U mg^{-1} protein)															
	Cellulase				Pectinase				Amylase				Protease			
	B	M	W	Mean	B	M	W	Mean	B	M	W	Mean	B	M	W	Mean
0	2.34	6.00	20.69	9.68	0.76	10.22	13.86	8.28	1.04	5.78	8.03	4.95	1.75	1.75	4.18	2.56
5	6.36	9.53	15.27	10.39	4.48	10.08	11.43	8.66	4.64	5.75	7.98	6.12	1.97	2.50	3.66	2.71
10	7.61	13.05	14.14	11.60	5.04	7.70	11.24	7.99	5.18	5.63	7.44	6.08	2.01	2.61	3.54	2.72
25	9.97	13.49	13.97	12.48	5.44	7.65	10.83	7.97	5.33	5.58	7.23	6.05	2.11	2.91	3.44	2.82
50	12.51	14.21	13.59	13.44	5.70	6.76	10.63	7.70	5.86	5.41	7.20	6.16	2.24	3.13	3.12	2.83
100	15.22	16.10	13.35	14.89	8.85	6.72	7.91	7.83	6.33	5.23	5.30	5.62	2.47	3.51	2.98	2.99
LSD (0.05)	1.16	1.52	1.06	0.76	1.12	2.18	1.26	0.81	1.59	1.78	1.34	0.84	0.54	0.92	0.55	0.40

B black (WPB-23), *M* mixed (WPM-29), *W* white (WPR4(87)), *LSD* represents least significance difference ($P \geq 0.05$)

contained melanin contents. The melanin pigment decreased in TCZ (100 μg ml^{-1}) treated hyphae and conidia (Fig. 3). Variations were found in the pore size of black and white isolate and varied from 4 to 50 nm in white isolate, while pore sizes were relatively smaller and less in number in black isolate. No effect was recorded in pore size in the cell wall of white isolate after the TCZ treatment. Although, the melanin erosion from cell wall and cell membrane in *B. sorokiniana* isolates were also visualized (Fig. 3).

Effect of Tricyclazole on aggressiveness of *B. sorokiniana*

Tricyclazole influenced the disease development in term of AUDPC, lesions developments/leaf and spore formation/leaf at different inoculation hours in black (WPB23), mixed (WPM29) and white (WPR4 (87)) isolates of barley. A significant reduction was noted in AUDPC, lesion formation/development and per leaf spore production under TCZ applications (25–100 μg ml^{-1}) in comparison to controls.

Table 4 Correlation coefficient of radial growth, mycelia weight, melanin content, antioxidant enzymes (SOD and CAT) and extracellular hydrolytic enzymes (cellulase, pectinase, amylase and protease) in black, mixed and white isolates of *B. sorokiniana*

Parameters	Isolates	Radial growth	Mycelia weight	Melanin content	SOD	CAT	Cellulase	Pectinase	Amylase	Protease
Radial growth	B	1.00								
	M	1.00								
	W	1.00								
	BMW	1.00								
Mycelia weight	B	0.61	1.00							
	M	0.27	1.00							
	W	0.35	1.00							
	BMW	0.84	1.00							
Melanin content	B	0.38	0.64	1.00						
	M	0.48	0.15	1.00						
	W	–	–	–						
	BMW	−0.26	−0.39	1.00						
SOD	B	0.42	0.62	0.80	1.00					
	M	−0.81	−0.25	0.70	1.00					
	W	−0.76	−0.42	–	1.00					
	BMW	−0.11	−0.11	0.73	1.00					
CAT	B	0.21	0.44	0.68	0.75	1.00				
	M	0.77	0.27	0.68	0.89	1.00				
	W	0.75	0.34	–	0.91	1.00				
	BMW	−0.08	−0.38	0.49	0.54	1.00				
Cellulase	B	−0.40	−0.64	−0.90	−0.94	−0.74	1.00			
	M	−0.69	−0.21	−0.85	−0.81	−0.81	1.00			
	W	0.56	0.28	–	0.57	0.53	1.00			
	BMW	0.36	0.50	−0.84	−0.70	−0.42	1.00			
Pectinase	B	−0.48	−0.56	−0.85	−0.75	−0.67	0.89	1.00		
	M	−0.18	0.04	0.32	−0.13	−0.17	−0.03	1.00		
	W	0.81	0.39	–	0.82	0.81	0.72	1.00		
	BMW	0.63	0.72	−0.63	−0.41	−0.27	0.75	1.00		
Amylase	B	−0.49	−0.49	−0.71	−0.44	−0.44	0.61	0.71	1.00	
	M	−0.15	−0.02	0.28	−0.08	−0.09	0.01	0.26	1.00	
	W	0.08	−0.07	–	0.13	0.03	0.34	0.09	1.00	
	BMW	0.39	0.50	−0.60	−0.33	−0.35	0.61	0.68	1.00	
Protease	B	−0.15	−0.35	−0.51	−0.48	−0.45	0.59	0.63	0.51	1.00
	M	−0.38	−0.08	−0.58	−0.46	−0.36	0.41	−0.69	0.03	1.00
	W	0.09	0.17	–	0.18	0.07	0.58	0.35	0.36	1.00
	BMW	0.52	0.67	−0.56	−0.33	−0.43	0.68	0.55	0.57	1.00

B black (WPB-23), *M* mixed (WPM-29), *W* white (WPR4(87)), *BMW* cumulative correlation in black, mixed and white isolates

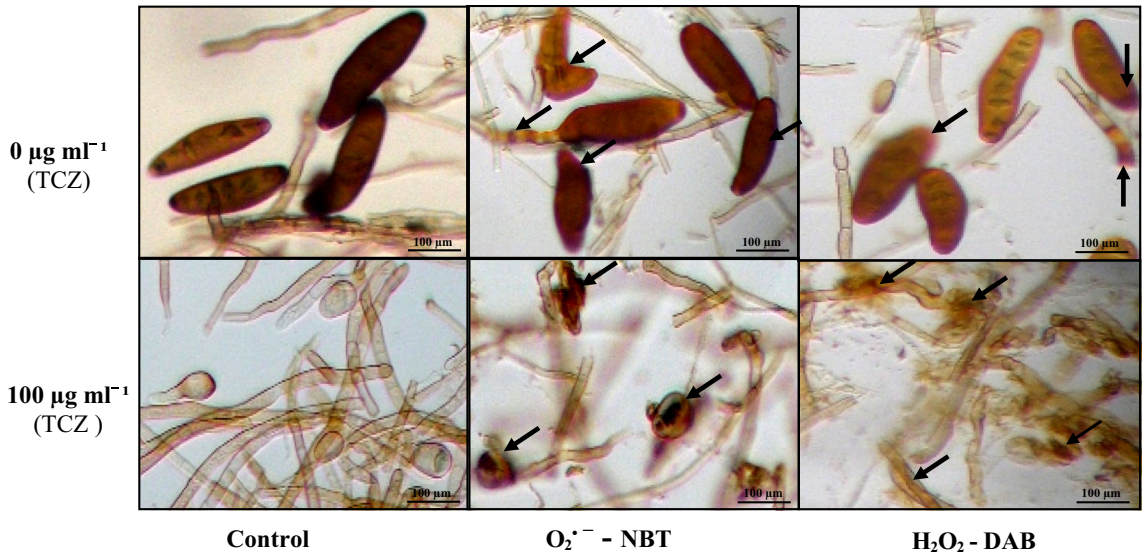

Fig. 2 Localization of $O_2^{\cdot-}$ and H_2O_2 stained by NBT and DAB in hyphae and conidia in *black* isolate (WPB-23) of *B. sorokiniana*. The conidia size drastically reduced by the exposure of increasing concentrations (5–100 µg ml^{-1}) of Tricyclazole, without staining hyphae and conidia serve as control for the formation of $O_2^{\cdot-}$ and H_2O_2 (*scale bar* 100 µm)

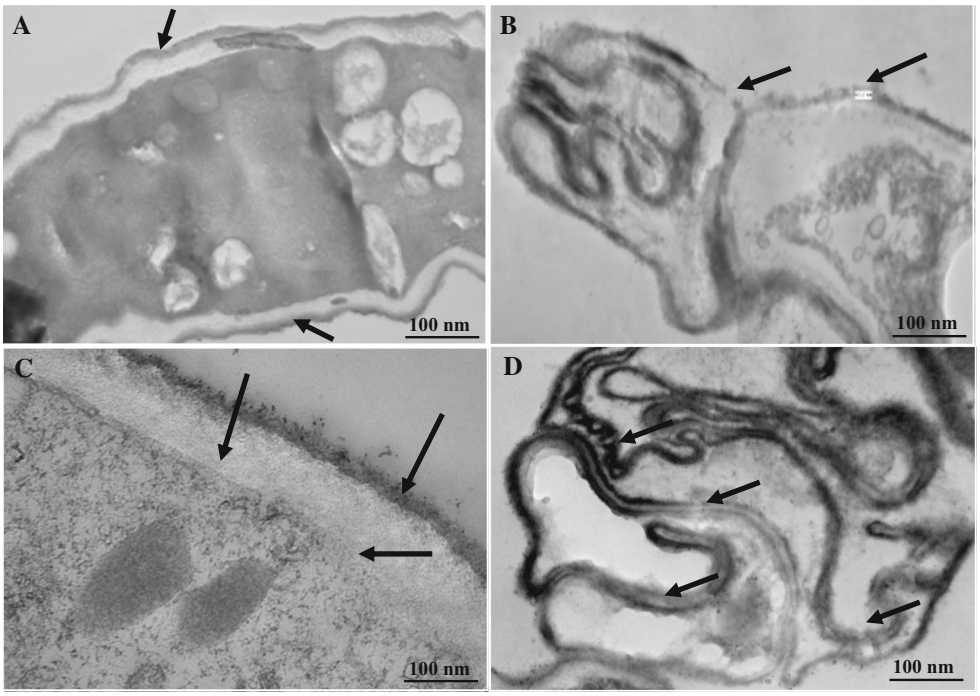

Fig. 3 Transmission electron micrograph of longitudinal section showing instance zones of melanin content with smooth surface in *black* isolate (**a**) and pores in diffused membrane visualized in *white* isolate (**b**), the cell wall and cell membrane damage in *black* (**c**) and *white* (**d**) isolate of *B. sorokiniana* under Tricyclazole (25 µg ml^{-1}) exposure (*scale bar* 100 nm)

Table 5 shows that the significant difference in AUDPC value with respect to LSD (5 %) for the fungicide dose signified that 100 µg ml^{-1} (AUDPC value = 106.22) was most effective after spore inoculation on host at 0 h. as compared to 24, 48 and 72 h. The AUDPC was maximum for white isolate and minimum for black isolate while, mixed isolate scored medium AUDPC. A significant difference in the AUDPC by a particular isolate for different fungicide dose at a constant time of inoculation after spraying. There appeared to be a non significant interaction between isolate × fungicide doses (Table 5). Similar observations were also made for lesion development and

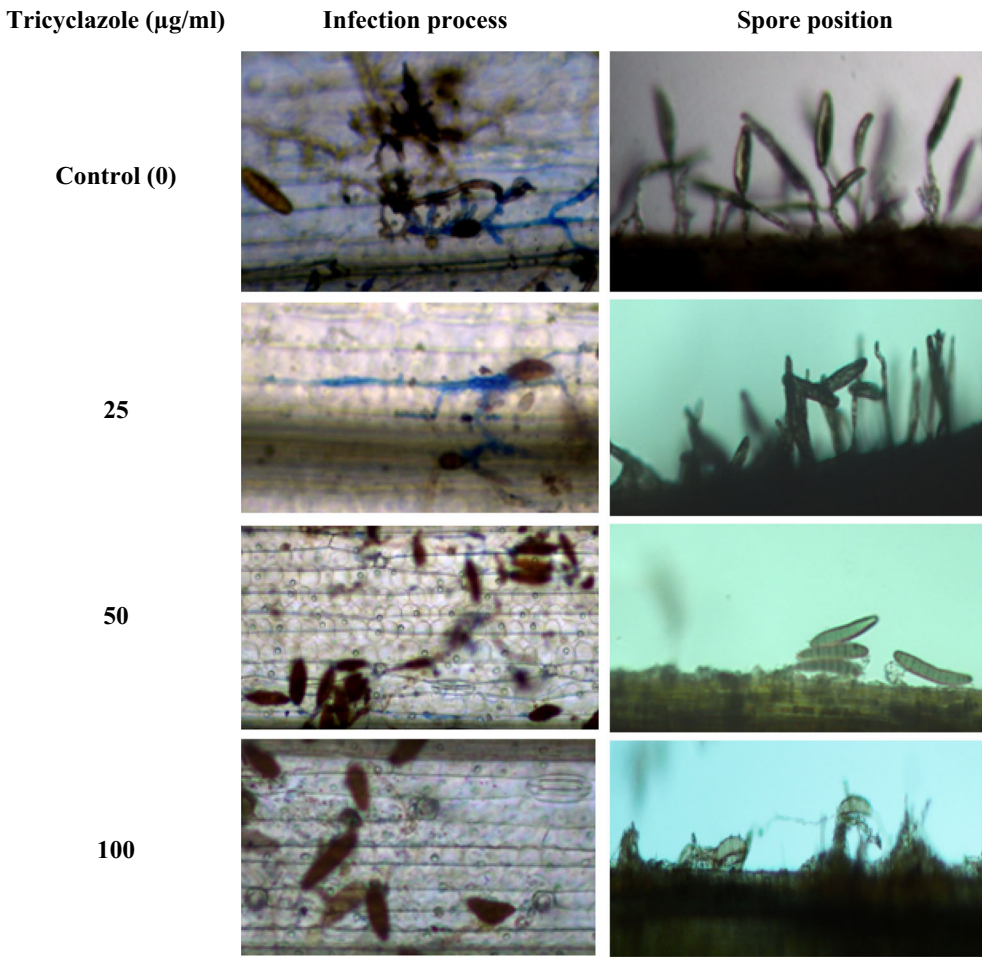

Fig. 4 Effect of different doses of Tricyclazole on spore germination and infection process (*left*) and spore position (*right*) of *B. sorokiniana* on infected barley leaves (cv. RD 2508)

number of spores formed per leaf. The isolates, isolates × inoculation hours and fungicide dose nonsignificantly affected the lesion development. The inoculation hours isolates × fungicide dose and isolates × inoculation hours × fungicide dose depicted the significant effects lesion numbers after TCZ spraying on barley plants (Table 5). The isolates, inoculation hours and isolates × inoculation hours are significantly affected by TCZ. However, the fungicide dose, inoculation hours and isolates × inoculation hours × fungicide dose depicted nonsignificant effect on sporulation after TCZ treatment on barley plants (Table 5). The TCZ inhibited the spore germination, growth and their stability on the infected portion of the host surface (Fig. 4).

Discussion

B. sorokiniana synthesizes 1,8- Dihydroxynaphthalene (DHN)-melanin via pentaketide pathway (Bell and Wheeler 1986) and melanin has been reported to promote the development of aerial mycelia and conidia of the fungus (Butler and Day 1998; Frederick et al. 1999; Henson et al. 1999). Similarly, a melanin deficient mutant of *A. alternata* had smaller conidia and reduced number of septa/conidia (Kawamura et al. 1999). Bashyal et al. (2010) reported the role of melanin in differentiation of secondary hyphae, coniodiophores and conidia. The lower dose of TCZ had non-significant effect on spore germination, but the higher dose became toxic to fungus (Chattopadhyaya et al. 2013). Eliahu et al. (2007) reported that *C. heterostrophus* mutant produces contrast albino conidia over the melanized wild type *C. heterostrophus*. The increasing doses of TCZ, reduced the melanin content, sporulation and number of septa/conidia over the control (Bashyal et al. 2010; Chattopadhyaya et al. 2013).

In this study the activity of antioxidant enzymes and damage in ROS system was recorded by the application of TCZ. Results of present study indicate dual role of TCZ, first on antioxidant enzymes and second on melanin

Table 5 Effect of increasing concentration of Tricyclazole (25, 50 and 100 μg ml^{-1}) on AUDPC, lesion development and spore formation on infected barley (cv. RD-2508) leaves

Treatment (μg ml^{-1})	Isolates	AUDPC				Mean of individual isolates	Cumulative Mean	Lesion No.				Mean of individual isolates	Cumulative Mean	Spore No.				Mean of individual isolates	Cumulative Mean
		0	24	48	72			0	24	48	72			0	24	48	72		
Control (0)	WPB23	83.67	85.33	95.00	141.00	101.25		11.33	17.33	23.00	37.00	22.17		308.00	948.67	1350.00	3039.00	1411.42	
	WM29	78.00	85.00	90.67	195.00	112.17	112.81	10.67	18.00	24.67	35.00	22.09	22.75	286.67	960.33	1280.67	3030.00	1389.42	1375.34
	WPR4(87)	76.67	85.67	86.67	251.00	125.00		11.67	22.33	24.33	37.67	24.00		219.67	842.67	1331.67	2906.67	1325.17	
25	WPB23	77.00	80.00	95.00	137.00	97.25		12.67	19.00	22.00	36.00	22.42		264.67	926.33	1333.33	3049.00	1393.33	
	WM29	77.00	86.33	91.00	193.00	111.83	111.08	9.00	19.67	28.33	35.33	23.08	22.75	300.00	932.67	1330.00	3033.33	1399.00	1359.19
	WPR4(87)	75.67	82.67	85.33	253.00	124.17		11.00	21.00	22.67	36.33	22.75		182.33	812.00	1233.33	2913.33	1285.25	
50	WPB23	76.33	78.00	90.00	133.00	94.33		9.67	17.67	29.00	36.00	23.09		293.00	894.33	1336.67	3039.00	1390.75	
	WM29	84.00	85.00	90.00	183.00	110.50	108.53	10.33	17.67	21.00	34.67	20.92	22.72	305.67	920.33	1251.67	3033.33	1377.75	1887.58
	WPR4(87)	77.67	81.67	84.67	239.00	120.75		12.00	23.33	28.33	33.00	24.17		194.67	764.33	1320.00	2916.67	1298.92	
100	WPB23	72.33	77.00	88.00	131.00	92.08		13.67	18.00	22.67	32.33	21.67		262.33	903.67	1333.33	3049.00	1387.08	
	WM29	83.33	86.00	89.00	179.00	109.33	106.22	18.33	19.33	21.33	33.67	23.17	21.78	221.33	862.00	1319.00	3026.67	1357.25	1350.42
	WPR4(87)	78.33	82.00	83.67	225.00	117.25		13.00	16.33	24.33	28.33	20.50		192.33	815.33	1306.67	2913.33	1306.92	
Mean		**78.33**	**82.89**	**89.08**	**188.33**			**11.95**	**19.14**	**24.31**	**34.61**			**252.56**	**881.89**	**1310.53**	**2995.78**		
Factors		Least Significance Difference (**LSD at** $P \geq 0.05$)																	
Isolates		2.35						NS						19.91					
Inoculation hrs.		2.71						1.40						22.99					
Isolates × Inoculation hrs.		4.69						NS						39.82					
Fungicide dose		2.71						NS						NS					
Isolates × Fungicide dose		NS						2.43						NS					
(Inoculation hrs. × Fungicide dose)		5.42						2.81						NS					
(Isolates × Inoculation hrs. x Fungicide dose)		NS						4.86						NS					

NS Non significant

content in *B. sorokiniana*. According to the earlier reports melanin has the property to bind with the drugs and antifungal compounds and reduces the diffusion of toxic chemical inside the hyphae (Eisenman et al. 2005; Nosanchuk and Casadevall 2006). Thus, ROS production in the TCZ treated isolates reduced to the antioxidant activities and melanin content in *B. sorokiniana*. The earlier reports shows that the melanin inhibitors based fungicides i.e. TCZ, Pthalide and Probenazole are nontoxic to fungi and stimulate $O_2^{\cdot-}$ production (Aver'Yanov et al. 1997). Melanin in fungal cell resists ROS and also plays an important role in disease development (Jacobson et al. 1995; Jacobson 2000). In *A. nidulans* the H_2O_2 is considered to be one of the most important metabolites in all respiring cells and provoked gene transcription (Pocsi et al. 2005), as well as sclerotial differentiation in *Sclerotium rolfsii* (Sidery and Georgiou 2000), increased expression of genes of carotenogenesis in *N. crassa* (Iigusa et al. 2005), and promoted transition of filamentous growth and development of its pathogenicity in *U. maydis* (Leuthner et al. 2005). Melanin in the conidial wall restricted the influx of water and solutes from the external environment, thus reducing spore germination (Chand et al. 2002). Contrary to this, our result suggests that TCZ leads to damage the melanin deposition in cell wall and facilitates the release of extracellular hydrolytic enzymes (Chand et al. 2014).

TCZ minimizes the pathogenesis in fungi and triggers a series of deleterious processes, including lipid peroxidation, degradation of proteins and DNA damage in the cell (Scandalios 1993). The over production of ROS as observed herein causes oxidative damage as also evident from electron microscopic studies of membrane, release of extracellular hydrolytic enzymes, etc. SOD and CAT play an important role in the defence of aerobic organisms against oxidative stress by converting ROS into nontoxic molecules (Tosi et al. 2010; Apel and Hirt 2004). Among these enzymes, SOD plays an important role in scavenging of $O_2^{\cdot-}$ by catalyzing the dismutation of two molecules of $O_2^{\cdot-}$ into O_2 and H_2O_2 and serve as first line of defence against toxic $O_2^{\cdot-}$ (Wang et al. 2005). We found that increasing concentrations of TCZ (>10 μg ml^{-1}) declined the activity of SOD and CAT. Changes in the activity of SOD are used as an indicator of changes in $O_2^{\cdot-}$ production (Wang et al. 2005). The significant difference in the lesion development was reported by the application of TCZ in black, mixed and white isolate Chattopadhyaya et al. 2013). White isolate is more aggressive in causing infection than black isolate (Chand et al. 2014). Results of this work also indicate that the application of TCZ on white and black isolate resulted in similar infection.

In a recent report it was found that the melanin deficient white isolate of *B. sorokiniana* produce much more extracellular hydrolytic enzymes and play an important role in pathogenecity than melanized black and mixed isolates (Chand et al. 2014). Another study on *A. alternata* also supported that melanin is not essential for the pathogenicity, because melanin deficient mutants have the same capacity to produce necrotic lesions on host tissue as the parent wild-type strain (Kawamura et al. 1999; Tanabe et al. 1995). However, the pigment does protect conidia from UV radiation and is likely to promote survival of the pathogen in field conditions (Kawamura et al. 1999). In the present study TCZ treated isolates also produce more extracellular hydrolytic enzymes (cellulose, pectinase, amylase and protease) in black isolate. The increased production of cellulose and protease was recorded under increasing concentrations of TCZ treated mixed isolate. However, the decline production of extracellular hydrolytic enzymes (cellulose, pectinase, amylase and protease) was noted in white isolate under increasing concentrations of TCZ exposure. The reduced aggressiveness in black isolate on barley leaf tissues was perhaps due to more damage caused due to reduced antioxidative enzyme activity with increased concentrations of TCZ.

Chattopadhyaya et al. 2013 reported a significant reduction in disease development in term of area under lesion progress curve at different fungicide dose for black, mix and white isolates suggest that varying degree of aggressiveness. Similarly the AUDPC is reduces by the application of TCZ. This indicated that TCZ affect the disease progress and colonization in pathogen on plant tissue. Kunova et al. (2013) reported that the efficacy of TCZ towards inhibition of sporulation and secondary infection indicates an additional possible mode of action of this fungicide that is different from inhibition of melanin biosynthesis. Herein, TCZ behave as fungicidal molecule in plant system by restricting pathogen growth. The previous report suggests that the TCZ deposited on the host leaves by their foliar applications and provides the defence to the host against pathogen (Ishiguro et al. 1992, Kunova et al. 2013).

Earlier studies showed that the melanised cells arrested cell growth and provide resistance to the fungi against lysis and protect the pathogens against environmental, chemical and physical stress (Bloomfield and Alexander 1967). TCZ damage to the infection behaviour and pathogenicity of *A. alternata* and also reported that the electron dense materials (melanin) accumulated in the spore of cell wall of parent spore but not in albino (Tanabe et al. 1995). We also report that TCZ damages to the key enzymes of melanin biosynthesis pathway in *B. sorokiniana* (Chand et al. 2014).

Although the application of TCZ has been studied for many years, most of the studies have been reported by the foliar application of TCZ on disease control in rice and other cereal crops. This study is one of the pioneer studies wherein TCZ has been applied to culture media to study its

direct toxic effects on *B. sorokiniana*. From the results it can be concluded that TCZ influences the pathogenic ability of *B. sorokiniana* by damaging the cell structure of hyphae and conidia and reducing the melanin content concurrent with the altered antioxidant enzyme activities and can therefore be employed for management of the disease in barley plants.

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Conflict of interest All the authors have contributed equally to this work and they have no conflict of interest.

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