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Chronic Toxicity of the Triazole Fungicide Tebuconazole on a Heterocystous, Nitrogen-Fixing Rice Paddy Field Cyanobacterium, *Westiellopsis prolifica* Janet

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This study explored the chronic effects of different doses of the triazole fungicide tebuconazole on the growth, and metabolic and enzymatic functions of the filamentous paddy field cyanobacterium, *Westiellopsis prolifica* Janet. The growth of the cyanobacterium was determined by an estimation of the change in pigment contents. Chlorophyll-*a*, carotenoids, and accessory pigments such as phycocyanin, allophycocyanin, and phycoerythrin were shown to decline over a 16-day period by a factor of 92%, 93%, 83%, 95%, and 100%, respectively, with increasing doses of the fungicide. Metabolic and enzymatic activities were also adversely affected. Over the 16 days, a gradual rise in total phenol content was recorded when *Westiellopsis prolifica* Janet was treated with 60 ppm of the fungicide, despite the reduction in carbohydrates, proteins, and amino acids by 96%, 92%, and 90%, respectively. Moreover, the enzymes nitrate reductase (NR), glutamine synthetase (GS), and succinate dehydrogenase (SDH) also registered reductions of 93%, 90%, and 98%, respectively. This study indicates that tebuconazole, although an important fungicide used extensively in rice fields, exhibits an inhibitory effect on the growth and metabolic activities of *Westiellopsis prolifica* Janet and hence possibly on other varieties as well.

Keywords: Chronic toxicity, enzymes, metabolites, pigments, tebuconazole, *Westiellopsis prolifica* Janet

Rice fields are one of the most extensive freshwater ecosystems on Earth. Nitrogen is a major factor in rice production. Among the indigenous nitrogen fixers, N_2-fixing cyanobacteria are responsible for most of the biological N_2 fixation in rice paddies [6]. The paddy field ecosystem provides an environment favorable for the growth of cyanobacteria with respect to their requirements for light, water, a high temperature, and the availability of nutrients. This may account for the higher abundance of cyanobacteria in paddy soils when compared with other cultivated soils. An additional benefit of cyanobacteria is their capacities to synthesize and liberate bioactive substances such as "auxins," "gibberellins," "cytokinins," "vitamins," "polypeptides," and "amino acids," thereby promoting plant growth and development [29].

Pesticides can have deleterious effects on algae by influencing soil algal growth, photosynthesis, nitrogen fixation, biochemical composition, and metabolic functions [3]. The effects of pesticides on the diazotrophic and nitrogenase activities of some anoxygenic phototrophic bacteria from paddy soils have been reported [4]. Algae have demonstrated a number of responses to pesticides, from complete inhibition to initial suppression followed by a gradual recovery and satisfactory growth [25]. Some observations have also been made on the effects of the herbicide isoproturon (CAS No. 34123-59-6) on aquatic organisms such as *Eichhornia crassipes*, *Ipomea*, aquatic planktons, and two test fish species [24]. It has been noted that fungicides pollute the environment, thereby having a detrimental impact on living organisms and reducing the population of the useful microorganisms in soil [12]. Reports [17] on the effects of fungicides on soil biota have provided further evidence for the reevaluation of the merits of fungicides.

Tebuconazole [((*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl) pentan-3-ol)] (Fig. 1) is a systemic triazole fungicide used widely in rice crop production to treat plant pathogenic fungi such as *Curvularia* spp., *Fusarium* spp., *etc*. However, in spite of its widespread use, comprehensive data assessing its biological impacts on cyanobacteria have been very limited and are almost completely lacking in the case of the triazole fungicides tebuconazole.

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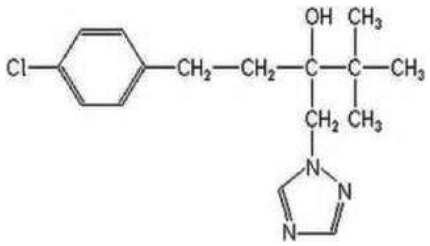

Fig. 1. Structure of the triazole fungicide tebuconazole.

An attempt has, therefore, been made in the present study to determine the chronic effects of the fungicide tebuconazole on pigments chlorophyll-*a* (chl-*a*), carotenoids, phycocyanin, allophycocyain, and phycoerythrin, as well as on the metabolic and enzymatic activities of the important nitrogen-fixing cyanobacterium *Westiellopsis prolifica* Janet. Chronic effect can be defined as an adverse effect on any living organism in which the symptoms develop slowly over time or recur frequently.

It is hoped that the results of this investigation will further contribute toward the more successful implementation of an effective pest management program for rice paddy fields and highlight the potential for the use of cyanobacteria in rice biofertilization programs.

MATERIALS AND METHODS

Growth Conditions and Pesticide Treatments

Axenic cultures of *Westiellopsis prolifica* Janet procured from the National Facility for Blue-Green Algae (IARI, New Delhi, India) were grown at 25±2°C in BG-11 medium [26] under an illumination of 3,000 lux light with a photoperiod of 14:10 (L/D).

Fungicide treatments were carried out on the cultures in logarithmic phase of growth, adding tebuconazole ((*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl) pentan-3-ol) (CAS No. 107534-96), common trade name Folicur (25.9% EC tebuconazole), obtained from Bayer Crop Science (Mumbai, India). Following a series of experiments to determine the LC_{50}, 2 ml of the inoculum from previously grown logarithmic cultures was introduced into a freshly prepared BG-11 medium in which tebuconazole had been added at final concentrations of 0 (control), 15, 30, and 60 ppm to make up a total volume of 20 ml. The LC_{50} can be defined as the standard measure of toxicity that leads to 50% reduction in the sample population of a specific test organism in a specified period of exposure to a compound. To prevent pesticide degradation, stock solutions were prepared just prior to each experiment by dissolving the fungicide in double distilled water. Each experiment was conducted in triplicate.

Pigment Analysis

The growth of the test organism was determined in terms of chlorophyll-*a*. Chlorophyll-*a* and carotenoids were measured spectrophotometrically in cell lysates after extraction in 80% acetone [7]. The cells were further suspended in 50 mM potassium phosphate buffer (pH 7.0) and the levels of phycobiliproteins phycocyanin, allophycocyanin, and phycoerythrin were measured spectrophotometrically at 562, 615, and 652 nm, respectively, after repeated freezing and thawing [2].

Biochemical Analysis

Biochemical studies included an estimation of the levels of carbohydrates, proteins, amino acids, and phenols. The culture medium was discarded through centrifugation and the cells were thoroughly crushed in a mortar and pestle with 80% ethanol. The supernatant obtained after centrifugation was used for biochemical analysis. The Anthrone method [27] was applied for total carbohydrate estimation using glucose as a standard. Total soluble proteins were determined using bovine serum albumin as the standard [13]. Amino acid content was estimated through the Ninhydrin method [11] and phenol levels were determined by the Folin–Coicalteau reagent method [14].

Enzymatic Assays

Estimation of *in vivo* nitrate reductase activity was made based on total nitrite formation [28]. Crude nitrate reductase (NR) enzyme preparation was obtained through the crushing of cells with cysteine buffer (pH 8.8). A reaction mixture containing 0.1 M PO_4 buffer, 0.1 M potassium nitrate, and 1 mM NADH with enzyme extract was used in each assay. Production of nitrite was assessed spectrophotometrically at 540 nm by adding 1% sulfanilamide and α-napthylethylenediamine to form an azo dye.

Ammonia-assimilating glutamine synthetase (GS) activity was determined through a reading of Mn^{2+} γ-glutamyl transferase activity [19]. The reaction mixture contained, in addition to enzyme extract, 50 mM Tris/HCl buffer, pH 7.5, 100 mM MnC1, 1 M sodium arsenate, 10 mM ADP (Na salt), 2 M hydroxylamine, and 100 mM glutamine). An incubation period of 10 min at 30°C with the absence of light was then given. The end product γ-glutamyl hydroxamate was measured spectrophotometrically at 540 nm. Succinate dehydrogenase (SDH), a major enzyme in the TCA cycle catalyzing the conversion of succinates to fumarates, was measured [10]. The samples were crushed in a 0.1 M phosphate buffer (pH 7.0) and centrifuged to obtain the supernatant in the crude enzyme extract. The enzyme assay was composed of 0.2 M sodium succinate and 0.1% triphenyl tetrazolium chloride (TTC). The reaction was terminated using pure acetone after an incubation period of 45 min at 30°C and measured spectrophotometrically at 460 nm. A standard curve using sodium hydrosulfite was run.

RESULTS AND DISCUSSION

The growth of *Westiellopsis prolifica* Janet was measured in terms of the level of the chl-*a*. The results clearly revealed that the levels of pigments of the broth of *Westiellopsis prolifica* Janet decreased with rising tebuconazole concentrations in the culture medium (Fig. 2–6). The decline in the pigments with respect to increasing exposure periods (days) was more prominent and highly significant at higher doses as compared with lower doses (Tables 1–4). The pigments chl-*a*, carotenoids, and phycobiliproteins (phycocyanin, allophycocyanin, and phycoerythrin) of the organism decreased continuously with increasing fungicide concentrations. The percentage reductions at the highest tebuconazole

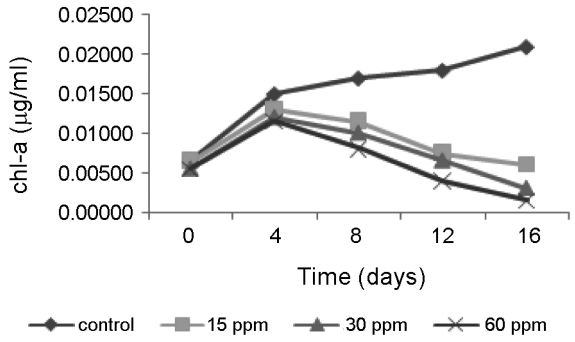

Fig. 2. Concentration (µg/ml) of chl-*a* content in *W. prolifica* at different doses of tebuconazole.

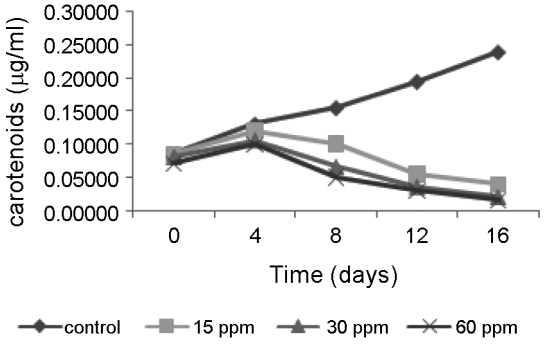

Fig. 3. Concentration (µg/ml) of carotenoid content in *W. prolifica* at different doses of tebuconazole.

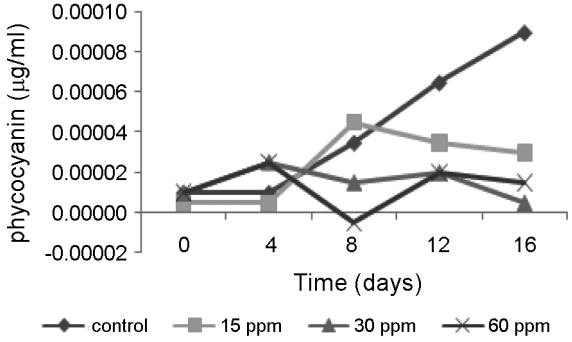

Fig. 4. Concentration (µg/ml) of phycocyanin content in *W. prolifica* at different doses of tebuconazole.

concentration (60 ppm) were 92%, 93%, 83%, 95%, and 100% for chl-*a*, carotenoids, phycocyanin, allophycocyanin, and phycoerythrin, respectively, by the end of 16 days. The results are in consonance with the deleterious effects of clotrimazole fungicides on chl-*a*, carotenoids, and phycobiliproteins within marine microalgal communities [21].

A remarkable degree of carbohydrate inhibition was observed with increasing concentrations of tebuconazole at 96% reduction of total carbohydrates by the end of 16 days (Fig. 7). This observation is in agreement with other reported findings [23]. Moreover, some reports [8] also

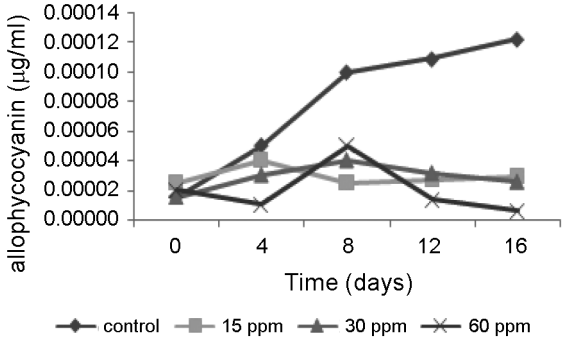

Fig. 5. Concentration (µg/ml) of allophycocyanin content in *W. prolifica* at different doses of tebuconazole.

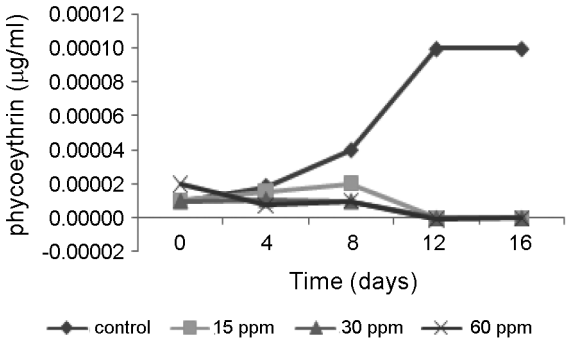

Fig. 6. Concentration (µg/ml) of phycoerythrin content in *W. prolifica* at different doses of tebuconazole.

emphasize the concentration- and time-dependent retardation of carbohydrate levels in cyanobacterium. Further application of tebuconazole to *Westiellopsis prolifica* Janet suppressed the total protein content in comparison with the control, the effect being more pronounced at higher doses (Fig. 8). A considerable reduction (92%) in the total protein content was registered at the end of 16 days at 60 ppm. Kapoor *et al.* [9] reasoned that the interruption of protein synthesis could be due to the inhibition of enzymes and structural proteins essential for growth of the organism. A gradual reduction of up to 90% of the total amino acids content at the end of 16 days was recorded when the cells were treated with 60 ppm of tebuconazole (Fig. 9). When reflecting upon a decline in protein and amino acids content as a result of fungicide use, Ochoa-Acuña *et al.* [18] suggested that fungicides directly impact algae. In addition, the amino acids produced in algal cells differed quantitatively and qualitatively according to the type of alga and conditions of cultivation [16]. Another report suggests that the changes in amino acid concentrations may be due to the synthesis from endogenous precursors or by the inhibition of normal catabolism.

Phenols are important aromatic molecules formed during stress conditions, which in turn trigger various biochemical processes within organisms. Phenol content in response

Table 1. Statistical analysis between various pigments for control.

Tebuconazole					
Pigments	Control (0 ppm)				
	Df[b]	R^{2b}	R^{*2b}	R[b]	P[b]
Chl-*a*[a]	4	0.853	0.804	0.923	≤0.05
Car[a]	4	0.992	0.990	0.996	≤0.05
PC[a]	4	0.750	0.660	0.866	≤0.05
APC[a]	4	0.500	0.330	0.707	≤0.05
PE[a]	4	0.750	0.666	0.866	≤0.05

[a]Meanings of abbreviations: Chl-*a*, Chlorophyll-a; Car, Carotenoids; PC, Phycocyanin; APC, Allophycocyanin; PE, Phycoerythrin.
[b]Meanings of symbols: Df, Degree of freedom; R^2, Coefficient of determination; R^{*2}, Adjusted R^2; R, correlation coefficient; P, Probability.

Table 2. Statistical analysis between various pigments for 15 ppm tebuconazole treatment.

Tebuconazole					
Pigments	15 ppm				
	Df[b]	R^{2b}	R^{*2b}	R[b]	P[b]
Chl-*a*[a]	4	0.106	−0.19	−0.33	≤0.05
Car[a]	4	0.565	0.42	−0.75	≤0.05
PC[a]	4	0.484	0.313	0.696	≤0.05
APC[a]	4	0.014	−0.31	−0.12	≤0.05
PE[a]	4	0.386	0.181	−0.62	≤0.05

[a]Meanings of abbreviations: Chl-*a*, Chlorophyll-*a*; Car, Carotenoids; PC, Phycocyanin; APC, Allophycocyanin; PE, Phycoerythrin.
[b]Meanings of symbols: Df, Degree of freedom; R^2, Coefficient of determination; R^{*2}, Adjusted R^2; R, correlation coefficient; P, Probability.

Table 3. Statistical analysis between various pigments for 30 ppm tebuconazole treatment.

Tebuconazole					
Pigments	30 ppm				
	Df[b]	R^{2b}	R^{*2b}	R[b]	P[b]
Chl-*a*[a]	4	0.213	−0.05	−0.46	0.05
Car[a]	4	0.773	0.697	−0.87	0.05
PC[a]	4	0.09	−0.21	−0.3	0.05
APC[a]	4	0.144	−0.14	0.379	0.05
PE[a]	4	0.746	0.662	−0.86	0.05

[a]Meanings of abbreviations: Chl-*a*, Chlorophyll-*a*; Car, Carotenoids; PC, Phycocyanin; APC, Allophycocyanin; PE, Phycoerythrin.
[b]Meanings of symbols: Df, Degree of freedom; R^2, Coefficient of determination; R^{*2}, Adjusted R^2; R, correlation coefficient; P, Probability.

to increasing concentrations of the fungicide has been represented (Fig. 10). An increase in the phenol content was registered throughout the treatment. This could be due to the possible conversion of primary metabolites into phenols as well as to the accumulation of fungicide during stress conditions, which could corroborate other findings [15].

Table 4. Statistical analysis between various pigments for 60 ppm tebuconazole treatment.

Tebuconazole					
Pigments	60 ppm				
	Df[b]	R^{2b}	R^{*2b}	R[b]	P[b]
Chl-*a*[a]	4	0.409	0.212	-0.64	0.05
Car[a]	4	0.723	0.63	-0.85	0.05
PC[a]	4	0.004	-0.33	0.069	0.05
APC[a]	4	0.05	-0.27	-0.22	0.05
PE[a]	4	0.087	0.743	-0.9	0.05

[a]Meanings of abbreviations: Chl-*a*, Chlorophyll-*a*; Car, Carotenoids; PC, Phycocyanin; APC, Allophycocyanin; PE, Phycoerythrin.
[b]Meanings of symbols: Df, Degree of freedom; R^2, Coefficient of determination; R^{*2}, Adjusted R^2; R, correlation coefficient; P, Probability.

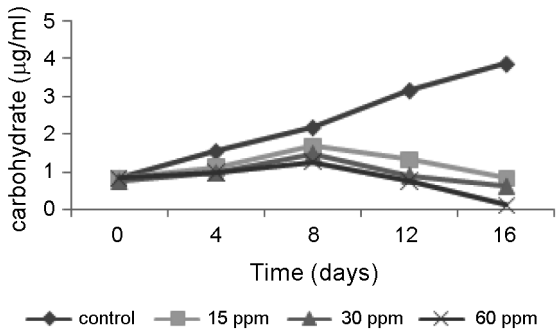

Fig. 7. Concentration (µg/ml) of carbohydrate content in *W. prolifica* at different doses of tebuconazole.

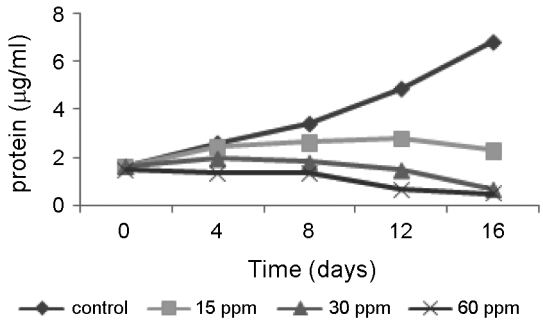

Fig. 8. Concentration (µg/ml) of protein content in *W. prolifica* at different doses of tebuconazole.

Tebuconazole treatments of nitrate reductase, glutamine synthetase, and succinate dehydrogenase were used to suppress the activities of nitrogen-fixing, ammonia-assimilating, and respiratory enzymes, respectively, for *Westiellopsis prolifica* Janet. Moreover, the highest doses of fungicide were more suppressive to the activities of the three enzymes. Nitrate assimilation is the major process of nitrogen acquisition in cyanobacteria [5]. It is transported into the cells by an active transport system and reduced to ammonium

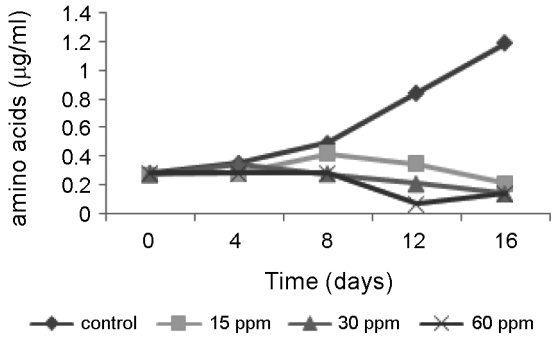

Fig. 9. Concentration (µg/ml) of amino acid content in *W. prolifica* at different doses of tebuconazole.

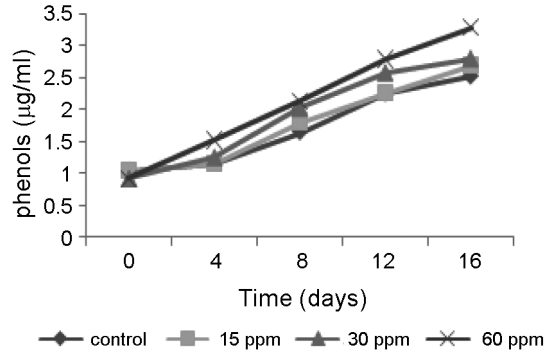

Fig. 10. Concentration (µg/ml) of phenol content in *W. prolifica* at different doses of tebuconazole.

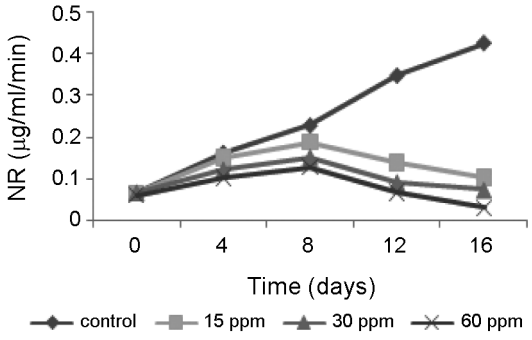

Fig. 11. Nitrate reductase enzyme activity (µg/ml/min) in *W. prolifica* at different doses of tebuconazole.

by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR) prior to fixation into amino acids through the glutamine synthetase (GS) pathway. The nitrate reductase activity of *Westiellopsis prolifica* Janet was reduced by 90% when treated with 60 ppm fungicide concentration (Fig. 11). Prasad *et al.* [22] studied the biological effects of a fungicide on *Nostoc muscorum* and quoted similar results. The decreased inhibitory effect of tebuconazole on ammonium assimilation after 16 days of treatment explained the harmful effects of the fungicide

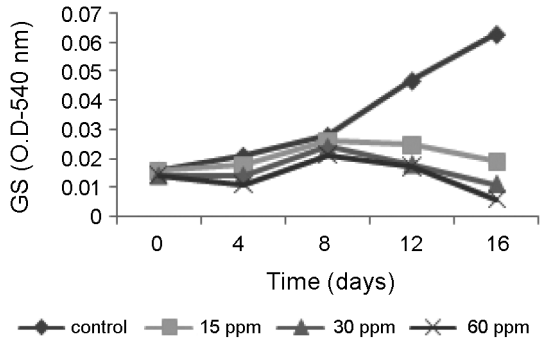

Fig. 12. Glutamine synthetase enzyme activity (O.D-540 nm) in *W. prolifica* at different doses of tebuconazole.

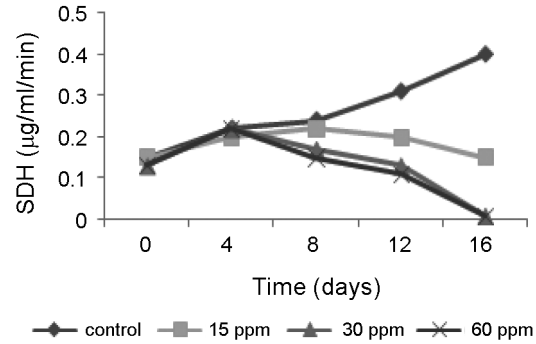

Fig. 13. Succinate dehydrogenase enzyme activity (µg/ml/min) in *W. prolifica* at different doses of tebuconazole.

on the cyanobacterium. A field study conducted showed that residues of tebuconazole lasted up to 63 days in the upper soil layer [1]. Glutamine synthetase, an important ammonia-assimilating enzyme, displayed a significant inhibition upon the fungicide treatment, leading to a 90% reduction in enzyme activity when compared with their control (Fig. 12). This observation has also been further supported by findings of a remarkable decrease in GS activity resulting from different pesticides [23]. Succinate dehydrogenase activity was severely diminished by 98% when treated with different doses of tebuconazole (Fig. 13). Moreover, it has been reported that the inhibition of succinate dehydrogenase in the fungi *Rhizoctania solani* resulted from treatment with thiazole carboxanilide fungicides [20].

Experiments were conducted with a view to determining the chronic toxic effects of the fungicide ((*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl) pentan-3-ol), or tebuconazole, on the growth, metabolites, and nitrogen-fixing and respiratory enzymes of the cyanobacterium *Westiellopsis prolifica* Janet, found abundantly in paddy field soil. The isolate under study was tested for its ability to grow in the presence of varying concentrations of the fungicide. This study has revealed that tebuconazole treatment adversely affects the growth of the isolate, even

at lower concentrations of 15 ppm. The release of metabolites such as carbohydrates, proteins, amino acids, and phenols as well as enzymes such as nitrate reductase, glutamine synthetase, and succinate dehydrogenase were all unfavorably affected by increasing concentrations of fungicide treatments. For a more thorough understanding of the environmental impact of fungicides on natural nitrogen-fixing cyanobacteria, more detailed field studies are needed.

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