Journal of Applied Biology & Biotechnology Vol. 9(04), pp. 10-18, July, 2021
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2021.9402

Toxicological effect of pendimethalin on some physiological parameters of the diazotrophic cyanobacterium *Desmonostoc muscorum* PUPCCC 405.10

Manzoor Ahmad Bhat[1], Davinder Pal Singh[1*], Jasvirinder Singh Khattar[1], Ram Sarup Singh[2]

[1]Department of Botany, Punjabi University, Patiala, India
[2]Department of Biotechnology, Punjabi University, Patiala, India

ARTICLE INFO

Article history:
Received on: January 22, 2021
Accepted on: March 12, 2021
Available online: July 10, 2021

Key words:
Cyanobacteria, *Desmonostoc*, pendimethalin, carbon metabolism, nitrogen assimilation

ABSTRACT

Application of herbicides for the control of weeds in agri-ecosystem poses a great threat to natural inhabiting beneficial microbes. Toxicological impact of herbicides on the survivability of cyanobacteria has recently gained much attention. Thus, the present study was undertaken to understand the mode of action of pendimethalin on assimilation of carbon and nitrogen in diazotrophic cyanobacterium *Desmonostoc muscorum* PUPCCC 405.10. The lethal dose of pendimethalin for the test organism was 20 mg/l. Pendimethalin (4–12 mg/l) exhibits negative effect on the growth, photosynthetic pigments (8%–85%), photosynthesis (29%–58%), photochemical activities (16%–64%), and respiration (25%–62%). Nitrogen assimilation parameters such as nitrate (39%–65%), nitrite (37%–83%), and ammonium (11%–37%) uptake and its enzymes such as nitrate reductase (27%–40%), nitrite reductase (3%–18%), and glutamine synthetase (15%–39%) were also negatively affected by herbicide. Kinetic studies further revealed that the affinity of nitrate and nitrite uptake was unaffected by the herbicide, but the affinity of ammonium uptake was affected. Pendimethalin interacted non-competitively with activities of nitrogen assimilation enzymes.

1. INTRODUCTION

The present global human population of 7.7 billion is expected to cross over 9 billion by 2050. Feeding this burgeoning population will require 70%–100% increase in food grain production globally in future [1]. Amongst several biotic and abiotic constraints to food crop production, weeds are one of the most important biotic factors causing nearly 13% reduced food crop yields globally [2]. More than 11 billion dollars of Indian agricultural produce was lost due to weeds [3]. Despite many limitations, the uses of herbicides to eradicate weeds in agri-ecosystems allow to grow crop plants at a competitive advantage is one of the important strategies [4]. However, the use of herbicides on large scale in crops affects non-target beneficial microbial flora including cyanobacteria for which use is not intended [5–7]. Cyanobacteria are most primitive Gram negative photoautotrophic unicellular to multicellular organisms exhibiting a great structural and distributional diversity [8,9]. The interdependence of carbon and nitrogen assimilation in cyanobacteria makes these organisms to grow and tolerate different adverse environmental conditions. This feature of cyanobacteria makes this microbial group a ubiquitous in distribution and makes considerable contribution in productivity of agri-ecosystem [4,10]. Cyanobacteria are considered to be a well-known natural biofertilizer, since these organisms build up soil fertility by enhancing soil nitrogen and liberating growth promoting substances such as amino acids, vitamins, and hormones, increases phosphate solubilization, water holding capacity, and also decreases soil salinity [11–13].

Many studies analyzed the harmful effects of herbicides in single or mixed form on cyanobacteria [11,14–16]. Among various herbicides, the inhibitory effect of herbicide 2, 4-D on the growth, development, and physiology of cyanobacteria has been widely investigated [10,14,17,18]. The impact of other herbicides such as bromacil and thiobendazole on cyanobacterial mat [19], glyphosate on *Microcystis aeruginosa, Anabaena variabilis, Chroococcus*

*Corresponding Author
Davinder Pal Singh, Department of Botany, Punjabi University, Patiala, India. E-mail: dp.khokhar@rediffmail.com

minutus, Fischerella sp., and *Nostoc muscorum* [20,21], 2,4 D on *Scytonema geitleri, A. variabilis*, and *Hapalosiphon* sp. [22,23], anilofos on *Synechocystis* sp. PUPCCC 64 and *Anabaena torulosa* [24,25], pretilachlor on *Anabaena* sp., *N. muscorum,* and *Desmonostoc muscorum* [15,26,27] has been reported.

Pendimethalin (N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine, an orange yellow color chemical compound belong to class dinitroaniline, is the third after paraquat and glyphosate, most often used herbicide worldwide. The estimated annual consumption of pendimethalin in 2014 was about 10 million pounds [18]. This pre- and post-emergent herbicide commonly used for the eradication of broadleaf weeds of cotton, soybeans, wheat, rice, maize, and annual grasses [28,29]. Pendimethalin kills weeds through inhibition of cell elongation and division. Compared to other dinitroaniline herbicides, pendimethalin has low volatility which makes it persistent in soil for a longer period of 10–92 days and its residual amount has been frequently detected in soil, ground, and surface water [30,31]. Commercially, pendimethalin is available as both granular and emulsifiable concentrate, under the trade names of Prowl™, Stomp™, Herbadox™, and Pay-off™. As a matter of fact, there is no substantial amount of literature available on the toxic effect of pendimethalin on the growth and development of cyanobacteria. The assimilation of carbon and nitrogen is an important interdependent growth and development regulatory metabolic pathway of cyanobacteria. These two pathways in cyanobacteria are the sources of all cellular components such as photo-pigments, different proteins, genetic materials, and energy rich compounds such as adinosine triphosphate (ATP) and nicotine adenine dinucleotide hydride (NADH)/nicotine adenine dinucleotide hydrogen phosphate (NADPH). Nitrogen assimilation in cyanobacteria depends upon the carbon skeleton for biosynthesis of compounds thus the limitation or oversupply of one of these elements strongly affects the metabolism of the other [32]. Thus, this present study was undertaken to understand the mechanism of pendimethalin interaction with carbon and nitrogen assimilation by the abundantly growing paddy field cyanobacterium *D. muscorum* PUPCCC 405.10.

2. MATERIALS AND METHODS

2.1. Microorganism and Culture Conditions

The microalga *D. muscorum* PUPCCC 405.10 employed in the present study was isolated from rice fields of Punjab, India by our laboratory [26] and grown photoautotrophically in slight modified Chu-10 medium [33] supplemented with micronutrients of Allen and Arnon [34]. The stock and experimental cultures were maintained in algal culture room at 28°C ± 2°C illuminated with LED tubes giving a light intensity of 44.5 µmol photons flux/m^2/s (µE) on the surface of culture flasks for 14 hours every day. The cultures were maintained in homogenous state by shaking manually 4–6 times daily and kept in actively dividing stage by transferring into fresh medium after every 6–8 days. Six-day-old exponentially growing cultures were used throughout the study and each experiment was repeated three times.

2.2. Tolerance Limit Determination

The tolerance limit of the *D. muscorum* towards pendimethalin was determined by measuring its growth in terms of increase in absorbance with time in Chu-10 medium containing 2.0 to 20 mg/l pendimethalin as per the protocol described by Swatch *et al*. [15]. The experiment was conducted in 250 ml Erlenmeyer flasks. The log phase stock cultures condensed by centrifugation (5,000 g, 10 minutes) were washed thrice with sterilized double distilled water and were inoculated in flasks containing 150 ml Chu-10 medium supplemented with different doses of pendimethalin to get an initial absorbance of 0.1 at 720 nm. Ten ml of the cultures were withdrawn at intervals of 2 days, up to 12 days and the absorbance of cultures was noted at a wavelength of 720 nm using UV-Visible spectrophotometer (Shimadzu 1280, Kyoto, Japan). Percent inhibition in growth was calculated using growth data of 2–8 days. The generation time was calculated according to Singh *et al*. [35]. Folin-phenol reagent was used to determine protein content of experimental cultures [36].

2.3. Photosynthetic Pigments Estimation

Aliquots of 10 ml pendimethalin treated and untreated experimental cultures were centrifuged at 5,000 g for 5 minutes. The obtained pellet, washed thrice with double distilled water was resuspended in 5 ml of 80% acetone. After shaking vigorously, the mixture was kept at 4°C overnight to release the pigments from cells and centrifuged. The absorbance of the supernatant was noted at 660, 645, and 450 nm and the amount of chlorophyll *a* and carotenoids was quantified according to Holm [37] and Myers and Kratz [38], respectively. Phycobiliproteins such as phycocyanin, allophycocyanin, and phycoerythrin were extracted in water from the biomass using freeze-thaw method [39]. A known volume of herbicide treated and untreated culture was centrifuged at 5,000 g for 10 minutes and the pellet obtained was resuspended in double distilled water and subjected to freeze-thaw cycles till all the pigments from the cells were released in water. The solution was then centrifuged at 5,000 g for 10 minutes and absorbance of the supernatant was noted at 562,615 and 652 nm using UV visible spectrophotometer. The phycobiliproteins were calculated using the equation of Bennett and Bogorad [40].

2.4. Photosynthesis, Photochemical Activities, and Respiration

The rate of photosynthesis, photochemical and respiratory activities were determined according to Chen *et al*. [5]. Ten ml of each 12 hours pendimethalin treated and untreated control cultures were centrifuged at 5,000 g for 10 minutes and made a thick biomass suspension equivalent to 10 µg chlorophyll *a*/ml in 3 ml of 25 mM bis tris propane buffer (BTP) of pH 7.8. This mixture was kept in a reaction chamber fitted with an oxygen electrode of dissolved oxygen analyzer (Model 5300A, YSI Bioanalytical Products, Yellow Springs, OH) and stirred with magnetic stirrer. The surface of reaction chamber was illuminated with 225 µE light intensity and the rate of increase in dissolved oxygen was followed for 10 minutes. The rate of photosynthesis was expressed as nmol O_2 evolved/µg chlorophyll *a*.minute. The rate of respiration was measured in terms of uptake of dissolved oxygen with time in dark and expressed as nmol O_2 consumed/µg chlorophyll *a*.minute.

The activity of photosystem (PS)-I was measured in terms of O_2 uptake in presence of light by thick cell suspension of pendimethalin treated and untreated control culture prepared as described above in 25 mM BTP buffer supplemented with electron donor (0.1

mM 2, 6-dichlorophenol indophenols; DCPIP), 5 mM ascorbic acid for reducing DCPIP to $DCPIPH_2$, electron acceptor (0.1 mM methyl viologen; MV), inhibitor of respiration (1 mM NaN_3), and PS-Π system inhibitor [10 µM 3-(3,4- dichlorophenyl)-1,1-dimethylurea (DCMU)]. The activity of PS-II was measured in terms of O_2 evolved in 5 mM BTP buffer (pH 7.8) containing 1 mM p-benzoquinone (electron acceptor) using water as the source of electron donor. The whole chain photosynthetic electron transport (PET) activity was measured as uptake of dissolved oxygen in presence of light using water as the electron donor in BTP (25 mM, pH 7.8) buffer containing 0.1 mM MV as electron acceptor and 1 mM NaN_3 as respiratory inhibitor.

2.5. Nitrogen Source Uptake and Enzyme Assay

Potassium nitrate, sodium nitrite, and ammonium chloride were employed as nitrate, nitrite, and ammonium source, respectively for uptake studies. Nitrogen uptake by the test microorganism was measured in terms of its depletion with time from the medium. A known volume of untreated and pre-treated with pendimethalin for 12 hours was harvested, washed with double distilled water, and inoculated in separate 100 ml Erlenmeyer flasks containing 50 ml Chu-10 medium supplemented with 100 µM nitrate, nitrite, or ammonium. The flasks were kept at 28°C ± 2°C in the culture room and illuminated with LED lights providing a light intensity of 44.5 µE on the surface of vessels. At desired time, the biomass was separated by centrifugation (5,000 g, 10 minutes) and the supernatant was used for the estimation of residual amount of nitrate, nitrite, or ammonium. The protocol developed by Robinson *et al*. [41] and Nicholas and Nason [42] was used for the estimation of nitrate and nitrite, respectively. The method of Solarzano [43] was used for the estimation of ammonium. The kinetics of nitrate, nitrite, and ammonium uptake was studied by measuring the respective nitrogen uptake by the pendimethalin treated and untreated cell suspension kept in different concentrations of individual nitrogen source (10–100 µM). The K_m and V_{max} values for each substrate were calculated by drawing Lineweaver–Burk double reciprocal plots.

The biomass pellet obtained above was used for enzyme assay. The protocol of Herrero *et al*. [44] was used to measure the activity of nitrate reductase (NR) and nitrite reductase (NiR) as described earlier in detail [26]. The enzyme activity in term of enzyme units (U) is expressed as µmol nitrite formed or reduced/mg protein.minute under standard assay conditions. The activity of whole cell glutamine synthetase (GS) was measured according to the method of Shapiro and Stadtman [45]. One unit of GS enzyme is expressed as µmol of L-glutamic acid ɤ-monohydroxamate formed/mg protein.minute under standard assay conditions.

2.6. Chemicals

The chemicals used in the present study for the preparation of nutrient medium and assay of enzymes were procured from SD Fine Chemical Limited, India, Merck, India and Sigma Aldrich, USA. Commercial grade pendimethalin (Dhanutop 30%) manufactured by Dhanuka Agritech Limited, Gurgaon, Haryana, India was purchased from the local market.

2.7. Statistical Analysis

The average data of three independently performed experiments ± standard deviation (SD was used throughout the current study. The whole data were analyzed statistically by employing one-way analysis of variance and Tukey's post hoc significance difference test.

All statistical analyses were tested at 5% level of significance against probability value at 95% confidence level ($p < 0.05$) using software Graph Pad Prism 6.0 version 6.0 (http://www.graphpad.com).

3. RESULTS

3.1. Tolerance Limit

Percent inhibition in growth of *D. muscorum* in terms of absorbance at 720 nm is given in Figure 1. The test organism showed a reduction in growth by 16%, 25%, 36%, 50%, 66%, 75%, 82%, and 93% in presence of 2, 4, 6, 8, 10, 12, 14, 16, and 18 mg pendimethalin/l, respectively. The test organism failed to grow in 20 mg pendimethalin/l. The cultures grown in different concentrations of herbicide were also examined microscopically. The cultures in 4 mg pendimethalin/l showed slight change in color from bluish green to yellowish. Fragmentation of filaments started after 6 mg/l pendimethalin containing culture and severe fragmentation occurred in 12 mg/l herbicide supplemented cultures. Microscopic examination of culture containing 20 mg/l herbicide revealed that above 97% of cells were lysed to release of pigments in the medium (Fig. 2). The growth data was further analyzed by calculating the generation time. The results revealed that generation time increased to 32–85 hours in the presence of 4–12 mg pendimethalin/l compared to 25.2 hours doubling time of control culture (Fig. 1 inset). Based on percent inhibition in

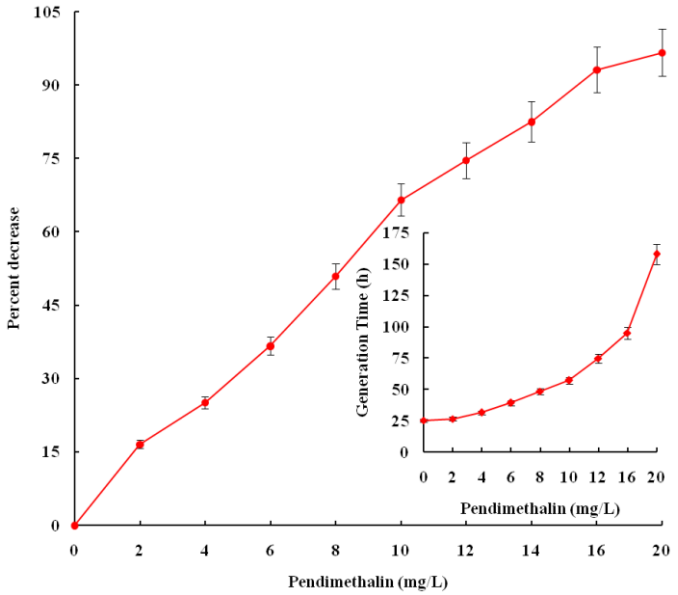

Figure 1: Percent inhibition in growth (A_{720}) of *D. muscorum* in the presence of pendimethalin. Inset figure: Generation time. The growth data of day 2–8 was taken to calculate the generation time. All data in the figure are different from each other at 5% level of significance ($p < 0.05$).

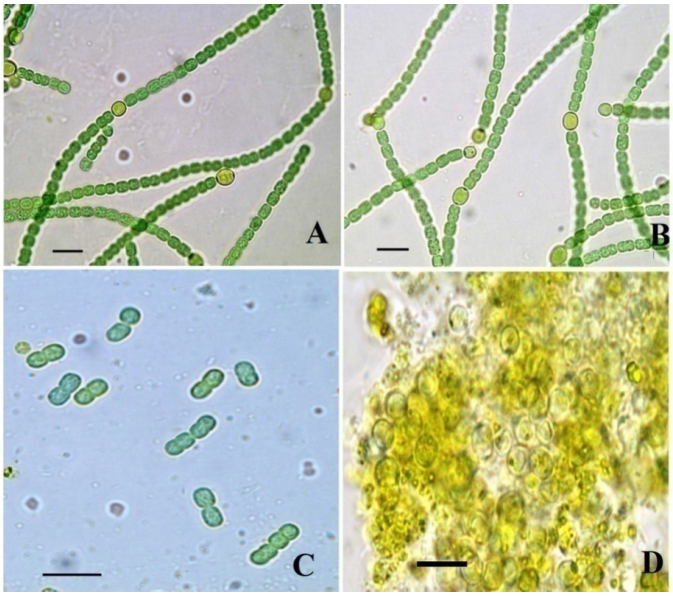

Figure 2: Microphotographs of *D. muscorum* in different concentrations of pendimethalin on day 4 (Scale bar 10 µm). (A) Control (0 mg/l), (B) 04 mg/l, (C) 12 mg/l, and (D) 20 mg/l.

growth, three concentrations of pendimethalin, viz. 4, 8, and 12 mg/l equivalent to inhibitory concentration (IC) IC_{25}, IC_{50}, and IC_{75} were selected for further studies.

3.2. Effect of Pendimethalin on Growth Parameters

The results related to the growth parameters obtained in this study exhibited a dose-dependent effect of pendimethalin on dry biomass and protein content of *D. muscorum*. On day 6, the test organism registered a decrease of 24%, 41%, and 58% in dry biomass in presence of 4, 8, and 12 mg pendimethalin/l, respectively. The protein content of test organism recorded a decrease of 23%, 42%, and 59% in presence of respective selected doses of pendimethalin (Fig. 3).

3.3. Effect of Pendimethalin on Photosynthetic Pigments, Photosynthesis, Photosynthetic and Respiratory Activities

The inhibitory effect of pendimethalin on chlorophyll *a*, carotenoids, phycocyanin, allophycocyanin, and phycoerythrin of the test microorganism was also concentration dependent (Figures 4 and 5). Chlorophyll *a* and carotenoid contents were decreased in the range of 13%–86% and 8%–45%, respectively (Fig. 4), while phycocyanin, allophycocyanin, and phycoerythrin contents were decreased by 25%–84%, 21%–48%, and 24%–60%, respectively (Fig. 5). Pendimethalin deleteriously affected the performance of photosynthetic as well as respiratory activity of the test organism (Table 1). Treatment with 4–12 mg pendimethalin/l caused 29%–58% and 25%–61% reduction in rate of photosynthesis and respiration, respectively. The results of photochemical activity of test organism revealed that pendimethalin decreased the activity of PS-I and PS-II by 33%–64% and 24%–62%, respectively. The whole PET activity exhibited a decrease of 16%–41% in presence 4–12 mg pendimethalin/l (Table 1).

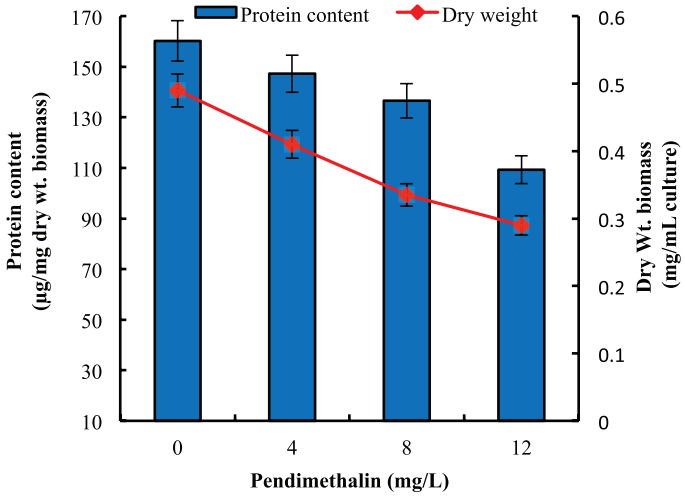

Figure 3: Effect of pendimethalin (mg/l) on dry biomass and total protein content of *D. muscorum* on 6 days. Growth data of each parameter in different concentrations of pendimethalin as well as control are different from each other at 5% level of significance ($p < 0.05$).

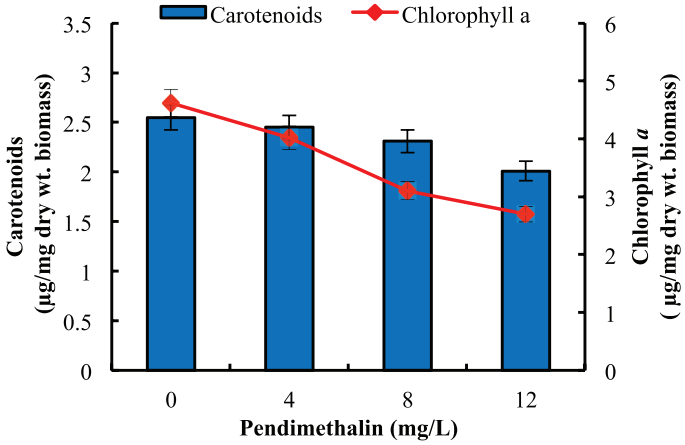

Figure 4: Effect of pendimethalin (mg/l) on Chlorophyll *a* and carotenoid content of *D. muscorum* on 6 days. Data presented in figure of each pigment in presence of different concentrations of pendimethalin as well as of control are different from each other at 5% level of significance ($p < 0.05$).

3.4. Effect of Pendimethalin on Nitrate and Nitrite Uptake and Its Assimilation

The incubation of untreated control cultures of *D. muscorum* in 100 µM nitrate and 50 µM nitrite for 6 hours took up 0.84 and 1.2 µmol of nitrate and nitrite/mg protein. The culture treated with pendimethalin (4–12 mg/l) showed an uptake of nitrate and nitrite in the range of 0.29–0.51 and 0.20–0.75 µmol/mg protein, respectively (Table 2). A Lineweaver–Burk double reciprocal plot for kinetics of nitrate uptake showed a V_{max} value of 26.6 and 47.6 µmol nitrate taken/mg protein.hour and same K_m values of 0.59 mmol/l for pendimethalin treated and untreated control cultures, respectively. The trend of effect of herbicide on kinetics of nitrite uptake was similar to nitrate uptake. The kinetics of nitrite uptake showed a V_{max} value of 1.17 and 2 µmol nitrite taken/mg protein. hour and same K_m values of 50 µmol/l for pendimethalin treated and untreated control cultures, respectively (Table 3). The effect of

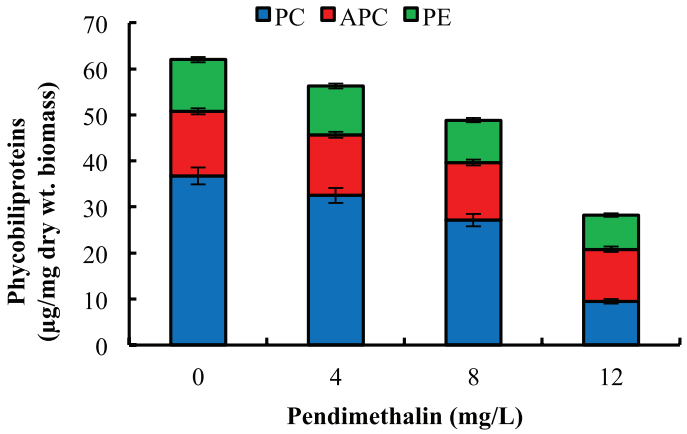

Figure 5: Effect of pendimethalin (mg/l) on phycobiliprotein content of *D. muscorum* on 6 days. Data presented in figure of each pigment in presence of different concentrations of pendimethalin as well as of control are different from each other at 5% level of significance ($p < 0.05$).

pendimethalin on NR and NiR activities was also dose-dependent. The test organism showed a 32.6–39.2 U of NR and 52–62 U of NiR with treatment of 4–12 mg pendimethalin/l (Table 2). The kinetics of NR activity showed a V_{max} value of 25 and 33 nmol nitrite formed/mg protein.minute and same K_m value of 3.3 mmol/l for pendimethalin treated and untreated control cultures (Table 3). The V_{max} value for NiR activity was 166 and 125 nmol nitrite decreased/mg protein.minute and the K_m value of 40 mmol/l for both control and pendimethalin treated cultures remained same (Table 3).

3.4.2. Effect of Pendimethalin on Ammonium Uptake and Its Assimilation

Pendimethalin (4–12 mg/l) treated cells incubated in 200 µM ammonium for 6 hours decreased the uptake of ammonium to 0.65–0.92 µmol/mg protein compared to 1.02 µmol uptake by control culture (Table 2). A Lineweaver–Burk double reciprocal plot for kinetics of ammonium uptake showed a V_{max} value of 0.33 and 0.8 µmol ammonium taken up mg/protein.minute and K_m value of 11.1 and 33.3 mmol ammonium/l for pendimethalin treated and untreated control cultures, respectively (Table 3). Pendimethalin decreased the GS activity of test organism to 7–10 U (Table 3). The kinetic studies of GS showed V_{max} of 9.09 and 16.6 µmol ɣ glutamate hydroxamate formed/mg protein.minute and same K_m value of 50 mmol/l for pendimethalin treated and untreated control cultures, respectively (Table 3).

4. DISCUSSION

Diazotrophic cyanobacteria are well-known natural nitrogen fixing microbes enormously contributing to the fertility of the soil ecosystem. This necessitates to examine the effect of agricultural pollutants such as pesticides including herbicides on their growth, development, and nitrogen assimilation [4,11]. The

Table 3: Pendimethalin effect on nitrogen source uptake kinetics.

Parameters	V_{max}		K_m	
	Control	8 mg/l	Control	8 mg/l
Nitrate uptake	47.6[a]	26.6[a]	0.59[b]	0.59[b]
Nitrite uptake	2[a]	1.17[a]	50[g]	50[g]
Ammonium uptake	0.33[f]	0.8[f]	33.3[b]	11.1[b]
NR	33.5[c]	25[c]	3.33[b]	3.33[b]
NiR	166[d]	125[d]	40[b]	40[b]
GS	16.6[e]	9.09[e]	50[b]	50[b]

[a]µmol nitrate or nitrite taken/mg protein.hour.
[b]mmol/l.
[c]nmol nitrite formed/mg protein.minute
[d]nmol nitrite decreased/mg protein.minute.
[e]µmol ɣ glutamate hydroxamate formed/mg protein.minute
[f]µmol ammonium taken/mg protein.hour.
[g]µmol/l.

Table 1: Effect of pendimethalin on rate of photosynthesis, photochemical activities, and respiration of *D. muscorum* after 12 hours treatment.

Treatments (mg/l)	Photosynthesis(nmol O_2 evolved/µg chlorophyll *a*.minute)	Photosystem I (nmol oxygen consumed/µg chlorophyll *a*.minute)	Photosystem II (nmol oxygen evolved/µg chlorophyll *a*.minute)	PET activity(nmol oxygen evolved/µg chlorophyll *a*.minute)	Respiration(nmol oxygen consumed/µg chlorophyll *a*.minute)
0	185.4 ± 9.27	83.4 ± 4.1	212.5 ± 10.6	76.3 ± 3.8	85.7 ± 4.2
4	130.9 ± 6.5(29.39)	55.5 ± 2.7(33.4)	160.5 ± 8.0(24.4)	63.9 ± 3.1(16.25)	63.9 ± 3.1(25.44)
8	96.6 ± 4.83(47.89)	38.9 ± 1.9(53.2)	119.5 ± 5.9(43.7)	57.6 ± 2.8(24.5)	45.2 ± 2.26(47.25)
12	77.9 ± 3.89(57.98)	29.6 ± 1.4(64.5)	80.2 ± 4.0(62.25)	45.2 ± 2.2(40.7)	32.7 ± 1.63(61.84)

Data given in parenthesis indicate percent decrease over control.
All the data presented in table is the mean values of three independent performed experiments ± SD.
The data of each parameters of pendimethalin treated and untreated control (C) culture are significantly different from each other at 95% confidence level ($p < 0.05$).

Table 2: Pendimethalin effect on nitrogen source uptake and assimilation enzymes of *D. muscorum* after 12 hours treatment.

Treatments (mg/)	Nitrate(µmol of nitrate taken/mg protein)	Nitrite (µmol of nitrite taken/mg protein)	Ammonium (µmol of ammonium taken/mg protein)	NR activity(nmol nitrite formed/mg protein. minute)	NiR activity(nmol nitrite reduced/mg protein.minute)	GS activity(µmol ɣ- glutamate hydroxamate formed/mg protein. minute)
0	0.84 ± 0.04	1.2 ± 0.06	1.02 ± 0.05	45.08 ± 2.25	64 ± 3.2	12.26 ± 0.63
4	0.51 ± 0.02(39.2)	0.75 ± 0.03(37.5)	0.91 ± 0.04(11.3)	39.2 ± 1.96(27.54)	62 ± 3.1(3.12)	10.4 ± 0.52(15.17)
8	0.41 ± 0.02(51.1)	0.49 ± 0.02(59.16)	0.78 ± 0.039 (23.9)	35 ± 1.75 (35.3)	57.7 ± 2.88 (9.84)	9.6 ± 0.48 (21.6)
12	0.29 ± 0.01(65.4)	0.20 ± 0.01(83.33)	0.65 ± 0.032(36.6)	32.6 ± 1.63(39.74)	52.73 ± 2.63(17.60)	7.43 ± 0.37(39.39)

Data given in parenthesis is percent decrease of enzymes compared to control.
All data are the mean values of three independent experiments ± SD.
Data of pendimethalin treated and untreated control (C) cultures are significantly different from each other at 95% confidence level ($p < 0.05$).

cyanobacterium *D. muscorum* employed during the present study is commonly occurring in the paddy fields of Punjab, India [26]. Pendimethalin selected for this study is employed by the farmers on a large scale in agri-ecosystems such as rice, cotton, soya bean, and tobacco to kill annual grasses and many broadleaf weeds [46–48]. A decrease in growth of the organism was observed with increase in the herbicide as evidenced from percent inhibition in growth and increased generation time (Fig. 1). The test microorganism failed to grow in 20 mg pendimethalin/l and the culture showed above 97% cell lyses as reported in other studies [25,27,49].

The tolerance level and growth performance of any microorganism are the manifestation of prevailing environmental conditions. The inhibitory effect of herbicide on growth of cyanobacteria rely on the nature of microorganism and herbicide used [14–16,27]. Pendimethalin exhibited a dose-dependent effect on cellular activities which ultimately led to decrease in dry biomass and protein content. Treatment with pendimethalin (4–12 mg/l) decreased dry biomass and protein content by 23%–59% (Fig. 3). Similar types of reports on other cyanobacteria with other herbicides are available in the literature [21,22,27]. The inhibition in growth of the cyanobacterium in presence of different concentrations of pendimethalin may be due to its interaction with cells structural and functional membranous proteins or other component of photosynthetic machinery such as photo pigments, PSs or interaction with nitrogen assimilation [15,50–52].

Since most of the earlier studies were targeted either towards carbon or nitrogen assimilation, the exact mechanism by which pendimethalin exhibits its toxic effect on cyanobacteria is lacking. Thus, the effect of pendimethalin on individual components of carbon and nitrogen parameters was targeted in this study. Pendimethalin caused a dose-dependent decrease in chlorophyll *a*, carotenoid, phycocyanin, allophycocyanin, and phycoerythrin content of the test cyanobacterium. Chlorophyll *a* and carotenoid contents were decreased by 8%–86% (Fig. 4). The contents of phycocyanin, allophycocyanin, and phycoerythrin were decreased in range 25%–84%, 21%–48%, and 24%–60%, respectively (Fig. 5). The reduction in phycobiliproteins with pendimethalin treatment might be due to their direct exposure to herbicide because of their extrinsic localization on the thylakoid membrane [27]. The decrease in carotenoid content in the presence of pendimethalin could be attributed to its protective role against stress caused by the herbicide [53]. The reduction in growth of *D. muscorum* in presence of pendimethalin may also be as a result of degradation of photosynthetic pigments by pendimethalin or due to its interference in synthesis of these pigments.

The role of photosynthesis and respiration is extremely crucial in microbes and plants due to generation of reducing power NADPH/NADH and ATP. The rates of photosynthesis and respiration in this cyanobacterium were clearly compromised upon exposure to pendimethalin (Table 1). Treatment of the organism with 4–12 mg pendimethalin/l caused dose dependent reduction in rate of photosynthesis by 29%–58%. Pendimethalin may block energy transfer from light harvesting phycobiliproteins to PS II due to the blockage of PET, thereby reducing photophosphorylation and hence the photosynthetic oxygen evolution as studied in other cyanobacteria with other herbicides [15,54]. To substantiate this, the interaction of pendimethalin with PSs and whole PET activity of test microorganism was studied. The results of photochemical activity of test organism revealed that pendimethalin decreased the activity of PS-I, PS-II, and whole PET in the range 33%–64%, 24%–62%, and 16%–41%, respectively (Table 1). These results confirmed that pendimethalin inhibits the energy transfer from phycobiliproteins to PS-II by blocking of PET at or near PS-I resulting in a low rate of photosynthesis in test organism. Similar to our observations, herbicides such as ioxynil, DCMU, and terbutryn have also been shown to affect PS-II in other photoautotrophic cyanobacteria [55–57]. Herbicides block electrons from sites Q_A to Q_B by binding with the D_1 protein subunit of Q_B of PS-II subunits of cyanobacteria [56,57].

The inhibitory effect of pendimethalin on respiration of the test organism was also observed to be dose-dependent. The decrease in the rate of respiration of the test organism ranged between 25% and 62% with treatment of 4–12 mg pendimethalin/l (Table 1). These observations revealed that together with photosynthesis, respiration of the organism was also affected. Thus, additional studies are required to know the effect of herbicide on respiratory electron transport chain of cyanobacteria. Similar observations have been reported for other cyanobacteria with different herbicides [14]. Bensulfuron methyl, butachlor, and dimethoate have also been reported to decrease the rate of respiration in *Nostoc* sp. [5].

Cyanobacteria use nitrate, nitrite, and ammonium for their growth and development [58]. This necessitates to study the effect of pendimethalin on nitrogen uptake and its assimilating enzymes. Pendimethalin (4–12 mg/l) treated cultures exhibited a decrease in nitrate and nitrite uptake by 39%–65% and 37%–83%, respectively (Table 2). A Lineweaver–Burk double reciprocal plot for kinetics of nitrate and nitrite uptake showed different V_{max} and similar K_m values for pendimethalin treated and untreated control cultures (Table 3). The unchanged value of K_m for nitrate and nitrite uptake by pendimethalin treated and untreated control cultures of test microorganism gives an indication that herbicide did not have any inhibitory effect on the affinity of nitrate and nitrite uptake system. It is well known that nitrogen (nitrate and nitrite) uptake in photosynthetic cyanobacteria is an energetically light dependent process controlled by a set of nitrate/nitrite transporters [59,60]. The decrease in V_{max} for pendimethalin treated compared to control cultures points towards the interference of herbicide with photosynthetically generated energy. The results of this study corroborate to our earlier report on this organism with another herbicide, pretilachlor [26].

Pendimethalin exhibited dose-dependent decrease in NR and NiR activities by 27%–40% and 3%–18%, respectively (Table 2). The results of kinetics of NR activity showed similar K_m value of 3.33 mmol/l and different V_{max} value of 25 and 33 nmol nitrite formed/mg protein.minute for pendimethalin treated and untreated control cultures (Table 3). The same is true for NiR activity as well, where a value of 166 and 125 nmol nitrite decreased/mg protein.minute as V_{max} was observed for control and pendimethalin treated cultures, respectively. The K_m value of 40 mmol nitrate/l for both control and pendimethalin treated cultures remained same (Table 3). These results showed that pendimethalin interacted in non-competitive manner with both these enzymes in this microorganism. It is thus

clear that pendimethalin caused a reduction in uptake of nitrate and nitrite as well as the activities of NR and NiR. This is because of less available energy as result of pendimethalin inhibition in photosynthetically generated reduced ferredoxin that donates electrons for nitrogen assimilation in cyanobacteria [60,61]. This was confirmed from the low rate of photosynthesis, photochemical activities, and even low respiration of the test microorganism in presence of pendimethalin (Table 1). Many reports related to progressive decrease in activity of NR and NiR in cyanobacteria *Anabaena* sp, *Aulosira fertilissima*, *Fischerella muscicola*, *N. muscorum,* and *Westiellopsis prolifica* by pesticides carbaryl, endosulfan, malathion, pretilachlor, trichlorfon, and tebuconazole have also supported our observation [26,52,62].

The effect of pendimethalin on ammonium uptake revealed the same pattern as of nitrate and nitrite uptake. Pendimethalin (4–12 mg/l) treatment decreased the uptake of ammonium by 11%–37.6%. The results of ammonium uptake kinetic studies revealed different value of V_{max} (0.33 and 0.8 µmol ammonium taken up mg/protein.minute) as well as K_m (11.1 and 33.3 mmol ammonium/l) for pendimethalin treated and control culture, respectively (Table 2 and 3). These results point towards the interaction of pendimethalin with the affinity of ammonium uptake transporter system of the cyanobacterium. It has been reported that PII signalling regulates ammonium uptake in *Synechocystis* sp. strain PCC 6803 through Amt1 ammonium permease similar to the known in bacterium *Escherichia coli* [58]. Thus, it appears that pendimethalin interacted with ammonium uptake regulator permease in this organism which needs further investigation. Ammonium produced in cyanobacteria through the activity of NR and NiR or taken up directly from the environment is further incorporated into the carbon skeleton in the sequential action of regulatory enzyme GS through the glutamine synthetase-glutamine-2-oxo-glutarate aminotransferase cycle [63]. Pendimethalin also decreased the GS activity of test organism by 15%–39% (Table 2). Similar to our results, earlier studies also reported effect on the GS activity in cyanobacteria by bagalol, mancozeb, thiodan, and phorate [64]. The reduction in GS activity by pendimethalin is correlated with the less reduction of nitrate and nitrite by the NR or NiR within the cells. The kinetics of GS showed different V_{max} (9.09 and 16.6 µmol ɣ glutamate hydroxamate formed/mg protein. minute) and similar K_m (50 mmol/l) values for pendimethalin treated and untreated cultures (Table 3). These results indicate that pendimethalin directly interacted with GS in a non-competitive manner to lower down its activity. Our results were supported by other studies where other herbicides also inhibited the GS activity in other cyanobacteria [24,26,62].

5. CONCLUSION

The overall results assert beyond doubt that pendimethalin negatively affected both carbon and nitrogen assimilation of cyanobacterium *D. muscorum*. The toxicity caused by iterated use of the pendimethalin can pose more serious negative impacts on the growth and development of farmer's eco-friendly paddy field diazotrophic cyanobacteria. Thus, there is a need to be cautious during the application of such herbicide in agricultural fields to protect the benevolent microbes that are the key component in maintaining the integrity and fertility of soil for sustainable agriculture.

6. ACKNOWLEDGMENTS

The authors are grateful to Head and Coordinator, FIST of DST, DRS SAP-II of UGC, Department of Botany, Punjabi University, Patiala, for providing laboratory and other infrastructure facilities. Mr Manzoor Ahmad Bhat acknowledges UGC, New Delhi for financial assistance under MANF-SRF research scheme.

7. AUTHOR'S CONTRIBUTION

DPS and JISK: Conceived the idea and designed the experiments. MAB: Performed the experiments and wrote the manuscript. DPS, JISK and RSS: Corrected, edited, and finalized the MS. All the authors contributed equally in this MS and agree to submit it for publication.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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How to cite this article:
Bhat MA, Singh DP, Khattar JIS, Singh RS. Toxicological effect of pendimethalin on some physiological parameters of the diazotrophic cyanobacterium *Desmonostoc muscorum* PUPCCC 405.10. J Appl Biol Biotech 2021; 9(04):10–18.