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Modulatory effects of paraquat-tolerant cyanobacteria on herbicide toxicity in rice seedlings

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ABSTRACT

The application of herbicides in rice fields necessitates an understanding of their effects on non-target organisms, particularly cyanobacteria, which serve as primary producers in the ecosystem. This study examined the impact of commercial paraquat and butachlor formulations on the paraquat-tolerant cyanobacterium, *Nostoc* sp. N1 and its intricate interactions during the growth of rice seedlings. The research evaluated the acute toxic response of *Nostoc* sp. N1 to herbicides, subsequently conducting 7 day germination and 21 day pot experiments to determine the mitigating or synergistic effects of *Nostoc* sp. N1 in simultaneously exposed rice seedlings. Initially, the study evaluated baseline toxicity, demonstrating that paraquat (median inhibitory concentrations EC_{50}= 0.241 mg L^{-1}) exhibited significantly higher toxicity to *Nostoc* sp. N1 than butachlor (EC_{50}= 2.074 mg L^{-1}). Subsequent physiological assays revealed two significant and opposing results. First, *Nostoc* sp. N1 responded differently to the herbicides; paraquat caused dose-dependent inhibition of growth, chlorophyll-*a*, and phycocyanin, whereas butachlor improved biomass accumulation and chlorophyll-*a* production, while only concurrently reducing the phycocyanin content at high doses. Second, differential responses resulted in contrasting findings in rice seedlings. Specifically, *Nostoc* sp. N1 demonstrated efficacy as a mitigating agent against paraquat, showing dose-dependent protection in the 7 day germination assay and providing complete defense in the 21 day experiment by restoring various physical and biochemical parameters. In contrast, *Nostoc* sp. N1 demonstrated a significant synergistic negative effect with butachlor in the 7 day assay, significantly enhancing its toxicity at all concentrations. This developed in the 21 day experiment into only partial mitigation, in which *Nostoc* sp. N1 restored biochemical stress indicators (such as amino acids) but did not recover physical growth. Based on these results, cyanobacterial bio-inoculants might not be uniformly advantageous and their use should be herbicide-specific. Although *Nostoc* sp. N1 is a potential agent for paraquat bioremediation, its incompatible interaction with butachlor could inadvertently accelerate crop damage.

Keywords: herbicide toxicity, *Nostoc* sp., bioremediation, synergistic toxicity, *Oryza sativa*

INTRODUCTION

The cultivation of rice (*Oryza sativa* L.) is essential for the global food security of more than one-half of the world's population, as it is the primary staple diet. In conventional rice cultivation systems, chemical herbicides are essential for the management of weeds, because ignoring them can lead to substantial production losses (Brar et al., 2025). Non-selective contact herbicides like paraquat and selective pre-emergence herbicide such as butachlor herbicide have been extensively used in rice plantations throughout Asia, including Thailand, because of their cost-effectiveness

and high efficiency. Nevertheless, the extensive and prolonged use of these chemicals has prompted substantial environmental and ecological concerns, particularly in relation to their effects on non-target organisms that are essential to the health of the agroecosystem (Huang et al., 2024)

The environmental persistence of these herbicides is a critical concern. For example, the use of paraquat was prohibited in Thailand from the end of 2020; however, it has shown remarkable soil persistence, primarily due to its rapid and powerful adsorption to clay minerals and organic matter, substantially restricting its mobility and microbial degradation (Salvestrini et al., 2024). As a result, the accumulation of paraquat residues in upper soil layers could be a long-term hazard to the soil biosphere (Huang et al., 2019). Similarly, butachlor, although still in legal use, can remain in soil and aquatic systems, where its fate and transportation are influenced by environmental conditions and soil properties (Kaur et al., 2017). A complex chemical stress environment is established in rice paddies as a result of the coexistence of residues from both these herbicides.

In the rice paddy ecosystem, diazotrophic cyanobacteria (nitrogen fixation), particularly heterocystous forms such as *Nostoc* sp., play an important ecological role. These microorganisms make a substantial contribution to soil fertility by converting atmospheric nitrogen (N_2) into ammonia (NH_3), a form that is readily available to rice plants, through biological nitrogen fixation (Lalmuanzeli et al., 2025). This natural bio-fertilization has the potential to decrease the dependence on synthetic nitrogen fertilizers, thus promoting a more sustainable agricultural practice (Chittora et al., 2020). Nevertheless, it is widely recognized that these beneficial microorganisms are extremely susceptible to herbicide contamination. The detrimental effects of these herbicides on cyanobacteria have been documented in a variety of studies. For example, the active constituent of paraquat has been demonstrated to induce oxidative stress by generating reactive oxygen species. This process damages cellular components, inhibits photosynthetic activity, and suppresses nitrogenase, a key enzyme involved in nitrogen fixation (da Silva et al., 2024). Similarly, butachlor has been reported to inhibit the growth, synthesis of pigments, and assimilation of nutrients of a variety of cyanobacterial species such as *Nostoc* (Sheeba and Prasad, 2020). Nevertheless, a critical review of the current literature has revealed major study gaps. First, most toxicological studies have focused on the pure active constituents of herbicides. Commercial formulations include adjuvants (such as surfactants and emulsifiers) to enhance herbicide efficacy, which could pose hazards. These additions can affect the bioavailability of the herbicide and exhibit toxicity, resulting in synergistic effects that make the formulation more hazardous to non-target organisms than the active ingredient alone (Nagy et al., 2019). Secondly, previous ecotoxicological research frequently utilized standard laboratory model organisms that may not be native species in agricultural ecosystems. Sometimes, native taxa can display different sensitivity to contaminants than non-native test species, highlighting the importance of including local strains for more accurate risk assessments (Gunnarsson and Castillo, 2018; Daam and van den Brink, 2010). However, in addition, it is important to recognize that in agroecosystems with continuous or intermittent herbicide exposure, microbial populations are subjected to strong selective pressure that can drive rapid adaptive responses (Hanson et al., 2013; Spurgeon et al., 2020). This can result in the emergence of tolerant strains that exhibit adaptive mechanisms such as enhanced antioxidant defenses, modifications of cell wall composition, or herbicide-degrading enzymes (Gaines et al., 2020; Singh et al., 2013). Thus, focusing only on sensitive laboratory strains for toxicological evaluations can result in an inadequate assessment of ecological risk. The response of these adapted and environmentally relevant strains is essential for conducting a realistic and accurate ecological risk assessment.

Although the direct toxicity of herbicides in rice plants has been reported extensively, their indirect effects, which occur through the disruption of beneficial soil microbes, are less understood. A reduction in the population and activity of nitrogen-fixing cyanobacteria resulting from herbicide stress could affect the natural nitrogen supply to rice plants, thus impacting their growth and development in the long term. Therefore, to address these critical gaps and simulate a realistic ecological scenario, this study focused on a paraquat-tolerant strain, *Nostoc* sp. N1, isolated from a rice field. The specific objectives were to: (1) assess the influence of low-dose, environmentally relevant concentrations of commercial paraquat and butachlor formulations on the growth and physiological parameters of *Nostoc* sp. N1;

and (2) evaluate the individual and combined effects of *Nostoc* sp. N1 and these herbicides on the germination and early development of a local rice variety, *Oryza sativa* L. cv. San-Pah-Tawng 1. The study should provide important information about herbicide-specific interactions between rice and beneficial cyanobacteria, and support that toxicological outcomes – ranging from mitigation to synergistic toxicity – are dependent on the specific chemical residues present.

MATERIALS AND METHODS

Rice and herbicide

Oryza sativa L. cv. San-Pah-Tawng 1 rice was obtained from the Phayao Rice Seed Center, Phayao, Thailand. Two commercial herbicide formulations were obtained from different companies: 1) paraquat (27.6% 1,1'-dimethyl-4,4'-bipyridinium dichloride (w/v) SL) was purchased from Syngenta, Thailand; and 2) butachlor (N-butoxymethyl-2-chloro-2',6'-diethylacetanilide (w/v) EC) was purchased from Thai Herbicide, Thailand. The herbicide concentrations used in the experiments were prepared by diluting with de-ionized (DI) water based on the percentage of their active ingredient in the formulations.

Screening of cyanobacteria

The cyanobacterial isolates were obtained from paddy field soils in Phayao Province, Thailand. The soil suspensions were spread on three agar media: BG-11; nitrogen-free BG-11; and BG-11 supplemented with 0.1 mg L^{-1} paraquat. Paraquat was used as a selective agent because it is more toxic to cyanobacteria than butachlor, thus enriching strains with herbicide tolerance. The plates were incubated under controlled conditions (3,000–5,000 lux, continuous light, 28–30 °C) for 2–3 weeks. After incubation, colonies were counted and reported as colony-forming units (CFU) g^{-1} soil. Individual colonies were purified by cross-streaking to obtain axenic isolates and subsequently maintained in liquid BG-11. Taxonomic identification was performed using light microscopy following standard morphological criteria (cell/filament shape and size, sheath and pigment characteristics) according to Desikachary (1959) and Komárek and Anagnostidis (1989).

Toxicity of paraquat and butachlor in *Nostoc* sp.

Acute toxicity test

From our previous study, *Nostoc* sp. N1, based on its chlorophyll-*a* content, reached the stationary phase 12 days after cultivation (Tansay et al. 2021). Therefore, 12 days of cultivation of *Nostoc* sp. N1 was used as an inoculum for these experiments. According to the modified method of Bérard et al. (1999), median inhibitory concentrations (EC_{50}) were used to determine the acute toxicity of paraquat and butachlor. *Nostoc* sp. N1 cells from a stock culture were added to various dilutions of paraquat and butachlor. After 96 h of exposure to herbicides, the chlorophyll-*a* content of the cells was measured. Using a linear regression equation, the estimated value of EC_{50} was determined by plotting a dose-response curve between those concentrations of chemical herbicides and the *Nostoc* sp. N1 chlorophyll-*a* content.

Toxicity of paraquat and butachlor in dry weight, chlorophyll, and phycocyanin content of Nostoc sp. N1

Cells were grown in a 250 mL flask containing N-free BG-11 medium. Two different concentrations of each paraquat and butachlor were added to the medium, consisting of 0.01 and 0.05 mg L^{-1} for paraquat and 0.03 and 0.06 mg L^{-1} for butachlor. Each flask was kept at room temperature, while the cells were exposed to fluorescent bulbs for 12 h, following a light-dark photoperiod of 4000 lux. The paraquat and butachlor concentrations used in this study were selected to reflect the range of environmentally relevant concentrations reported in water (Supplementary Table 1).

To determine cellular development, dry weight measurements of cell biomass were taken every 3 days for 12 days or until the cells entered their stationary phase. After being filtered using a Whatman GF/C glass microfiber filter (GE Healthcare; UK), the *Nostoc* sp. N1 cultures were dried overnight at 80°C in an oven before being weighed. The chlorophyll-*a* concentration was determined using the technique described by Wintermans and deMost (1965). An aliquot of 10 mL of *Nostoc* sp. N1 was filtered through filter paper (GF/C) and subsequently extracted with 10 mL of 90% methanol at 20°C. After centrifugation of the extract at 5,000 rpm for 10 min, the

absorbance was determined using a GENESYS 10S UV-vis spectrophotometer (Thermo Scientific; USA) at 630, 645, 665, and 750 nm. The calculation of the chlorophyll-*a* concentration was performed utilizing the equation:

$$\text{Chlorophyll-}a = \frac{\begin{array}{c}[11.6(A665-A750) - \\ -1.31(A645-A750) - \\ -0.14(A630-A750) \times \\ \times \text{Volume of methanol (mL)}]\end{array}}{\begin{array}{c}[\text{Volume of filtered water (L)} \times \\ \times 1/\text{ Cuvette width}]\end{array}} \quad (1)$$

where: A630, A645, A665 and A750 are the absorbance values at 630, 645, 665 and 750 nm, respectively.

Furthermore, a quantification of the phycocyanin concentration was carried out. By filtering through GF/C filter paper, the biomass of *Nostoc* sp. N1 was obtained, resulting in the production of a 100 mg cell pellet. Subsequently, the pellet was homogenized in 1 mL of a phosphate buffer solution with a concentration of 0.2 M and a pH of 7. After conducting a series of five freeze-thaw cycles, the resulting mixture was subjected to 15 min of centrifugation at 6000 rpm and 4 °C using a TD5 centrifuge (Yingtai Instrument Co., Ltd.; China). The supernatant was carefully decanted and its absorbance was recorded at 615 and 652 nm using a GENESYSTM 10S UV-vis spectrophotometer (Thermo Scientific; Germany). Then, the concentration of phycocyanin (PC) was determined using the formula established by Bennett and Bogorad (1973):

$$\text{PC (mg mL}^{-1}) = \frac{A_{615} - 0.474\,(A_{652})}{5.34} \quad (2)$$

where: A615 and A652 are the absorbance values at 615 and 652 nm, respectively

Impact of paraquat, butachlor, and cyanobacteria on rice seedling germination

Rice seeds were sterilized with 10% sodium hypochlorite for 10 min and then rinsed three times with distilled water. The seeds were placed in sterile Petri dishes lined with filter paper; then, 10 mL of each test solution were poured into each Petri dishes. The concentration of *Nostoc* sp. N1 was selected based on preliminary experiments (Supplementary Tables 2 and 3), determining the optimal concentrations of 5.0 g L^{-1} and 10.0 g L^{-1}, which provided the highest seedling vigor index. The following treatments were tested: (1) *Nostoc* sp. N1 cells applied at concentrations of 5.0 and 10.0 g L^{-1}; (2) herbicides applied at various concentrations (0.05, 0.1, 0.5, 1.0 mg L^{-1} for paraquat; 0.1, 0.5, 1.0 , 2.0 mg L^{-1} for butachlor); (3) a combination of *Nostoc* sp. N1 cells with the those herbicides applied; and (4) no cyanobacteria cells nor herbicides applied (control). Germination was monitored every 24 h for 7 days following seeding, and the seedling vigor index (SVI) was calculated (Abdul-Baki and Anderson, 1973). In addition, the physical characteristics of the seedlings (root and shoot lengths and fresh and dry weights) were measured 7 days after seeding (Supplementary Table 4 and 5). The method described by Tansay et al. (2021) was used to calculate the phytotoxicity index, defined as the average of the phytotoxicity classes derived based on the percentage decrease in growth and physiological parameters (Supplementary Table 6).

Impact of paraquat and cyanobacteria on the growth of 21-day-old rice seedlings

Our earlier research revealed that the most efficient method for soil treatment with cyanobacteria is mixing cyanobacteria cells into the soil, resulting in a higher number of all rice growth parameters than from applying cyanobacteria cells to the surface of the soil (Tansay et al. 2021). Thus, for the 21 day rice seedling experiment, the technique of mixing cyanobacteria cells into the soil was used. The rice seeds were soaked in DI water overnight before being covered by a moist fabric sheet and left to germinate in the dark. Subsequently, 10 germinated seeds were planted in plastic pots with prepared soil and watered every 2 days with 20 mL of DI water. The experimental soil was excavated from the top 20 cm of an agricultural field in Phayao Province, then air-dried, crushed, and sieved through a mesh of 6.0 mm. Then, 500 g of air-dried and sieved topsoil were placed in each plastic container. Similarly, the concentrations selected for the soil experiment (paraquat 7 and 70 mg kg^{-1}; butachlor 0.15 and 1.5 mg kg^{-1}) were chosen to represent the residue levels reported in the soil (Supplementary Table 1). The sieved and air-dried soil were combined with different amounts of paraquat, butachlor, and fresh *Nostoc* sp. N1 cells (10 g kg^{-1}). Rice was grown under fluorescent lights with a light-dark photoperiod of 12:12 h using 4,000 lux. The physical parameters of the rice seedlings were examined after 21 days of treatment. The chlorophyll-*a*

content was determined as described previously. The total sugar content was evaluated using the phenol-sulfuric acid technique (Dubois et al., 1956) and the total free amino acid content was determined using the ninhydrin carbon dioxide method (Hamilton and Van Slyke, 1943), with leucine serving as a standard.

Statistical analysis

Each experiment was performed in triplicate. The mean value was presented with the standard deviation (SD). A statistical analysis of variance was performed on all data and then Duncan's multiple range test (DMRT) was used to determine significant differences between treatments at a significance level of $p \leq 0.05$.

RESULTS

Screening of cyanobacteria

In total, 48 cyanobacterial isolates were identified, consisting of 11 on BG-11, 16 on nitrogen-free BG-11, and 21 on BG-11 supplemented with 0.1 mg L^{-1} paraquat. Five genera – *Cylindrospermopsis* (n=2), *Nostoc* (n=3), *Oscillatoria* (n=3), *Planktolyngbya* (n=1) and *Pseudanabaena* (n=2) – were observed on BG-11. Only diazotrophic genera – *Anabaena* (n=5) and *Nostoc* (n=11; 68.8%) – were obtained in nitrogen-free BG-11. *Nostoc* (n=16) and *Anabaena* (n=5) were further enriched by the addition of paraquat, with *Nostoc* accounting for 76.2% of the isolates in this condition (Table 1). In total, *Nostoc* constituted 62.5% (30/48) of all isolates, indicating that traits linked to N_2 fixation and oxidative-stress tolerance likely favored *Nostoc* under N-limitation and low-level paraquat exposure.

From the paraquat-supplemented BG-11, five representative isolates – two *Anabaena* (A1, A2) and three *Nostoc* (N1–N3) – were purified and then evaluated in a spot test on 0.0–6.4 mg L^{-1} paraquat. The highest tolerance to paraquat was demonstrated by *Nostoc* sp. N1, which maintained high biomass throughout the entire gradient (~18% decline), as illustrated in Fig. 1. In contrast, *Anabaena* A1, *Anabaena* A2, *Nostoc* N2, and *Nostoc* N3 exhibited significant dose-dependent reductions, particularly above 0.6–0.8 mg L^{-1}, approaching ≤~1–1.5 mg $spot^{-1}$ at 6.4 mg L^{-1}(Fig. 1).

Toxicity of paraquat and butachlor on *Nostoc* sp.

The acute toxic study of commercial paraquat and butachlor formulations on *Nostoc* sp. N1 demonstrated that paraquat was more toxic than butachlor, with EC_{50} values of 0.241 and 2.074 mg L^{-1}, respectively (Table 2). The data indicated that *Nostoc* sp. N1 was approximately 8.6 times more toxic than butachlor when in contact with paraquat.

To investigate further, the effects of paraquat and butachlor toxicity on *Nostoc* sp. N1 were determined by measuring changes in the dry weight, chlorophyll-*a*, and phycocyanin contents during 12 days (Fig. 2). The dry weight of *Nostoc* sp. N1

Table 1. Genus identification and number of isolate of cyanobacteria

Medium	Genus	Isolate count	% of medium total
BG-11	*Cylindrospermopsis*	2	18.2
	Nostoc	3	27.3
	Oscillatoria	3	27.3
	Planktolyngbya	1	9.1
	Pseudanabaena	2	18.2
Total		11	100
N-free BG-11	*Anabeana*	5	31.2
	Nostoc	11	68.8
Total		16	100
BG-11 + paraquat 0.1 mg L^{-1}	*Anabeana*	5	23.8
	Nostoc	16	76.2
Total		21	100
Overall total		48	

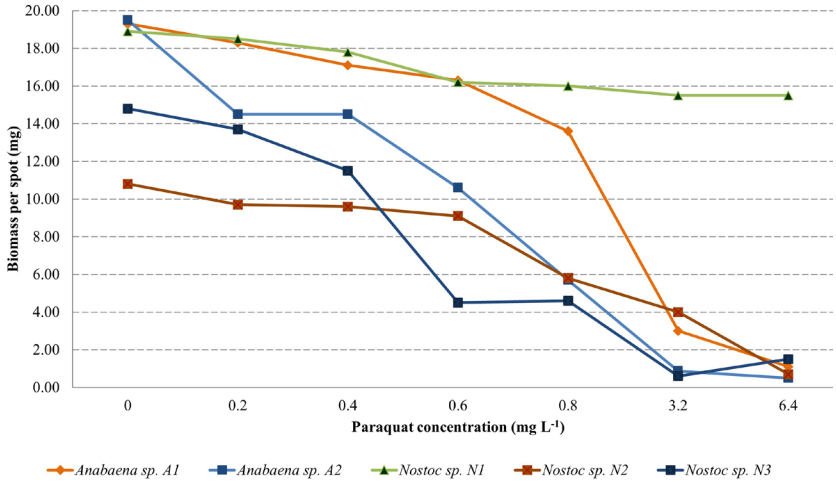

Figure 1. Dose-responses of cyanobacterial biomass to paraquat based on spot testing

Table 2 Acute toxicity of herbicides on *Nostoc* sp. N1

Herbicide	Linear regression	R^2	EC_{50} (mg L^{-1})
Paraquat	Y = -10.3+ (-0.93×X)	0.947	0.241
Butachlor	Y = 1.02 + (-3.19×X)	0.999	2.074

Note: Linear regression – the equation derived from the dose-response relationship between herbicide concentration (X) and chlorophyll-*a* content reduction (Y); R^2 – coefficient of determination; EC_{50} – median effective concentration causing a 50% reduction in chlorophyll-*a* content after 96 h of exposure.

was affected by paraquat and butachlor. Paraquat (Fig. 2a) exhibited clear dose-dependent toxicity at a concentration of 0.05 mg L^{-1}. However, the 0.01 mg L^{-1} concentration exhibited a hormetic effect (stimulation at low doses and inhibition at high doses). Notably, butachlor was not toxic at the tested concentrations of 0.03 and 0.06 mg L^{-1}. However, it seemed to have a stimulating effect on biomass accumulation. (Fig. 2b). The effects of the herbicides on the chlorophyll-*a* content are shown in Figs 2c and 2d. A clear dose-dependent inhibitory effect was observed for paraquat (Fig. 2c). The chlorophyll-*a* content in the control culture reached its maximum on day 6, followed by a slight decrease. The lower concentration of paraquat (P 0.01 mg L^{-1}) reached its maximum on day 3 and subsequently exhibited a steady decline, concluding significantly below the control on days 6–12. The high concentration (P 0.05 mg L^{-1}) exhibited significant toxicity; after an initial increase, the chlorophyll-*a* content experienced a considerable decline after day 6, reaching the lowest recorded level on day 12. In contrast, butachlor (Fig. 2d) demonstrated a stimulating effect on pigment production. The chlorophyll-a level of the control group decreased after day 6, while both the butachlor treatment groups of 0.03 and 0.06 mg L^{-1} exhibited continued increases. On day 12, the butachlor-treated cultures exhibited a significantly elevated chlorophyll-*a* level, in contrast to the declining control group.

The impact of herbicides was evaluated on the accessory pigment phycocyanin (Figs. 2e and 2f). A significant inhibitory effect was observed for exposure to paraquat, especially at high concentrations. The lower dose (P 0.01 mg L^{-1}) caused elevated levels of phycocyanin that peaked on day 3 and remained similar to the control for the duration of the experiment. In contrast, the higher dose (P 0.05 mg L^{-1}) resulted in a significant and progressive decrease in the phycocyanin content after day 3. A different pattern was observed with butachlor (Fig. 2f). All cultures, including the control, exhibited the highest phycocyanin content on day 3. The lower concentration (B 0.03 mg L^{-1}) exhibited a minor effect, with levels closely aligned with the control during the 12 days of the experiment. The higher concentration (B 0.06 mg L^{-1}) resulted in a significant reduction in phycocyanin content from day 6, with markedly lower levels observed on days 9 and 12 compared to the control and lower-dose treatments.

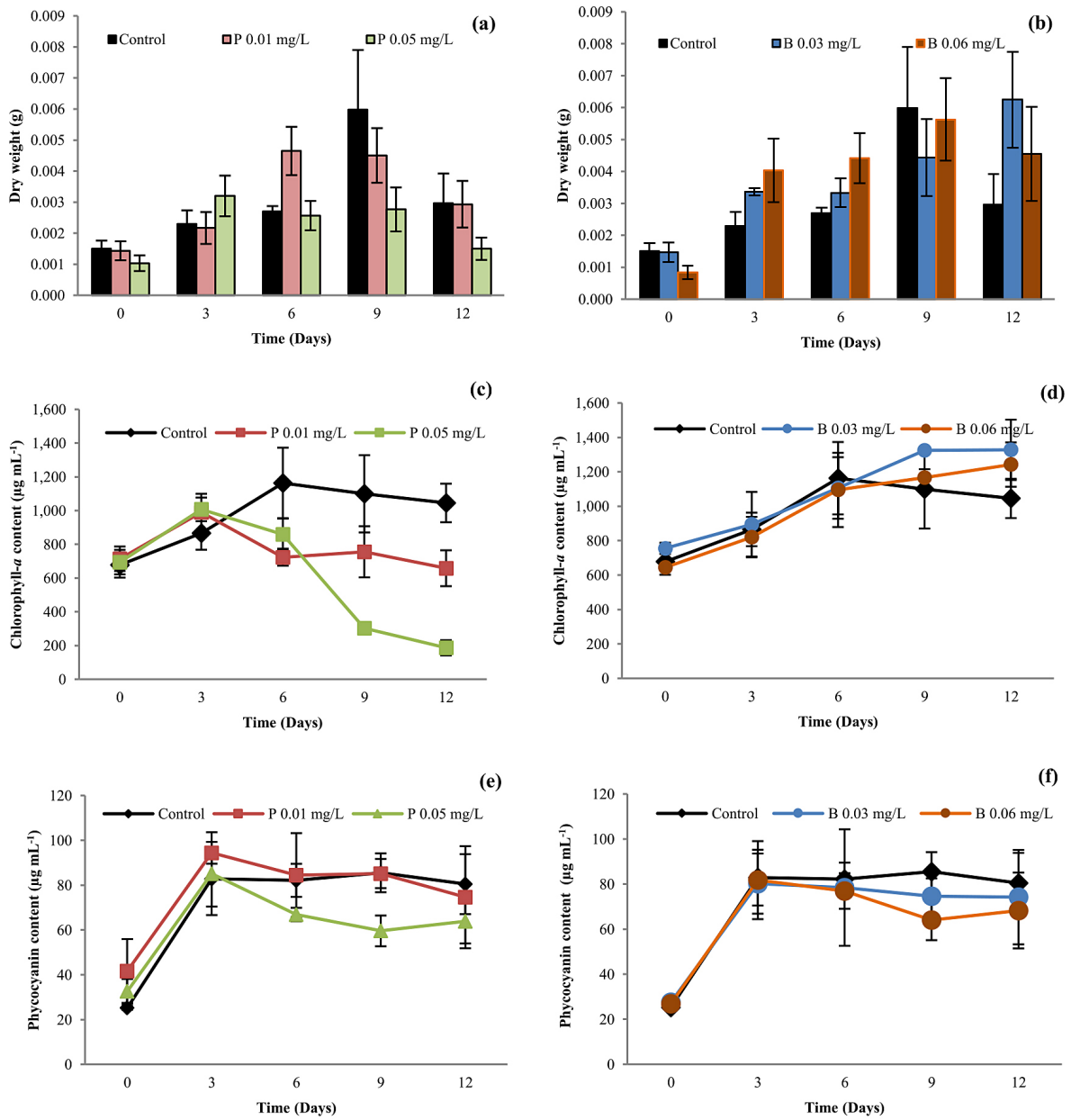

Figure 2. Toxicity of paraquat and butachlor on dry weight, chlorophyll, and phycocyanin contents of *Nostoc* sp. N1: (a) Dry weight in response to paraquat, (b) Dry weight in response to butachlor, (c) Chlorophyll-a content in response to paraquat, (d) Chlorophyll-a content in response to butachlor, (e) Phycocyanin content in response to paraquat, and (f) Phycocyanin content in response to butachlor. Treatments: Control (no herbicide); P 0.01 (0.01 mg L^{-1} paraquat); P 0.05 (0.05 mg L^{-1} paraquat); B 0.03 (0.03 mg L^{-1} butachlor); B 0.06 (0.06 mg L^{-1} butachlor). Values represent mean ± standard deviation (n=3)

The ratio of phycocyanin-to-chlorophyll-*a* (PC/Chl-*a*), an important stress indicator, was significantly affected by paraquat, while butachlor had minimal impact (Fig. 3). Exposure to higher paraquat concentrations (P 0.05 mg L^{-1}) led to an exponential increase in the PC/Chl-*a* ratio over time, as evidenced by a strong correlation (R^2 = 0.9644). This result did not suggest an enhancement in phycocyanin synthesis and demonstrated the preferential degradation of chlorophyll-*a*, as illustrated in Fig 2c, compared to the more stable phycocyanin pigment shown in Fig 2e. This indicated that paraquat-induced oxidative stress led to a more accelerated degradation of the chlorophyll-*a*-containing core reaction centers compared to the phycobilisome antenna complex. The PC/Chl-*a* ratios in both cultures treated with butachlor (B 0.03 and B 0.06 mg L^{-1}) and the low-dose paraquat treatment (P 0.01 mg L^{-1}) exhibited relative stability, closely comparable to the

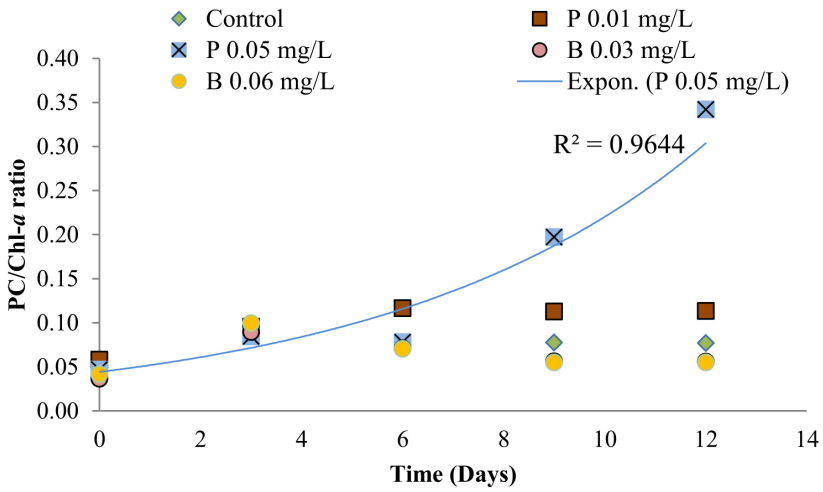

Figure 3. Phycocyanin-to-chlorophyll-*a* (PC/Chl-*a*) ratio in *Nostoc* sp. N1 exposed to paraquat (P) and butachlor (B) over 12 days, showing exponential (Expon.) trendline for P 0.05 mg L^{-1} treatment (R^2 = 0.9644), where points represent mean values (n=3)

control group, suggesting that butachlor did not significantly affect pigment balance in the photosynthesis system of *Nostoc* sp. N1.

These findings indicated that paraquat exhibited a significant dose-dependent inhibitory effect on the growth (dry weight), chlorophyll-*a*, and phycocyanin contents of *Nostoc* sp. N1, thus confirming its high toxic risk to this cyanobacterium. In contrast, butachlor demonstrated a more complex and differential effect, since it neither inhibited nor limited biomass accumulation and chlorophyll-*a* synthesis compared to the control; rather, it stimulated these processes. However, it simultaneously resulted in a decrease in phycocyanin content at a high concentration. Thus, the use of paraquat threatens cyanobacterial populations. Butachlor, considered safer for *Nostoc* growth, could increase its growth relative to other organisms, altering the aquatic microbial balance.

Impact of paraquat, butachlor, and cyanobacteria on rice seedling germination

The effects of the paraquat, butachlor herbicides and *Nostoc* sp. N1 cells on the germination and growth parameters of rice seedlings aged 7 days are presented in Table 3. *Nostoc* sp. N1, when applied alone, exhibited a significant growth-promoting effect, enhancing the SVI by 52.07% at a concentration of 5.0 g L^{-1} (percentage increase compared to the control calculated based on the SVI, PIC = 52.07, phytotoxic index = -1.75) in the paraquat experiment. In contrast, each herbicide applied individually demonstrated significant, dose-dependent phytotoxicity. Paraquat at 1.00 mg L^{-1} and butachlor at 2.0 mg L^{-1} resulted in a reduction of the PIC to -41.32% and -19.72%, respectively, accompanied by high phytotoxicity indices of 2.50 and 3.00, respectively. *Nostoc* sp. N1 exhibited a significant, dose-dependent protective effect against paraquat toxicity. The paraquat concentration at 0.50 mg L^{-1} demonstrated a significant improvement in the PIC, increasing from -25.62% (toxic) to 0.00% (fully mitigated) with the addition of N 10.0 g L^{-1}. The ameliorating effect was consistent across the range in the phytotoxicity index, which declined from 2.50 to 1.50 in the same treatments. *Nostoc* sp. N1 significantly increased the toxicity of butachlor. For example, butachlor applied at 2.0 mg L^{-1} exhibited a PIC of -19.72%. However, when combined with *Nostoc* at concentrations of 5.0 g L^{-1} or 10.0 g L^{-1}, the PIC significantly decreased to -53.93% and -52.01%, respectively, showing a notable synergistic negative effect on seedling growth – a pattern that was consistent across all tested butachlor concentrations.

Impact of paraquat and cyanobacteria on the growth of 21-day-old rice seedlings

Figure 4 demonstrates the dual effect of *Nostoc* sp. N1 in promoting growth and alleviating herbicide stress in rice seedlings aged 21 days. When *Nostoc* sp. N1 was applied at a concentration of 10 g kg^{-1} (N10) alone to assess its bio-stimulant properties, it did not produce a statistically

Table 3. Effects of paraquat, butachlor herbicides and *Nostoc* sp. N1 cells on germination and growth parameters of rice seedlings aged 7 days

Treatment	Germination and growth parameters			Treatment	Germination and growth parameters		
	SVI	PIC (%)	Phytotoxic index		SVI	PIC (%)	Phytotoxic index
Control	1210.0	0	0.00	Control	5730.0	0	0.00
N 5.0 g L^{-1}	1840.0	52.07	-1.75	N 5.0 g L^{-1}	5780.0	0.87	-0.75
N 10.0 g L^{-1}	1370.0	13.22	-1.00	N 10.0 g L^{-1}	5400.0	-5.76	0.50
P 0.05 mg L^{-1}	1710.0	41.32	0.00	B 0.1 mg L^{-1}	5650.0	-1.40	0.00
P 0.10 mg L^{-1}	1270.0	4.96	0.75	B 0.5 mg L^{-1}	3290.0	-42.58	2.25
P 0.50 mg L^{-1}	900.0	-25.62	2.50	B 1.0 mg L^{-1}	3300.0	-50.09	2.50
P 1.00 mg L^{-1}	710.0	-41.32	2.50	B 2.0 mg L^{-1}	2860.0	-19.72	3.00
N 5.0+P 0.05	1411.38	16.64	0.50	N 5.0+B 0.1	4600.0	-25.65	0.75
N 5.0+P 0.10	1300.0	7.44	0.25	N 5.0+B 0.5	4260.0	-25.65	1.25
N 5.0+P 0.50	1140.71	-5.73	1.50	N 5.0+B 1.0	2780.0	-51.48	2.75
N 5.0+P 1.00	750.0	-38.02	2.00	N 5.0+B 2.0	2640.0	-53.93	3.00
N 10.0+P 0.05	1170.0	-3.31	1.50	N 10.0+B 0.1	4860.0	-15.18	0.75
N 10.0+ P 0.10	1520.0	25.62	1.75	N 10.0+B 0.5	4600.0	-19.72	0.50
N 10.0+P 0.50	1210.0	0.00	1.50	N 10.0+B1.0	3180.0	-44.50	2.25
N 10.0+P 1.00	870.0	-28.10	2.00	N 10.0+B 2.0	2750.0	-52.01	3.00

Notes: N – *Nostoc* sp. N1; P – paraquat herbicide; B – butachlor herbicide; SVI – seedling vigor index (calculated based on seedling dry weight in mg); PIC – percentage increase compared to the control calculated based on the SVI. Phytotoxic index was classified according to the percentage reduction in different growth parameters (supplementary Table 5).

significant difference compared to the untreated control group in most growth parameters. (Figs 4a, 4b, 4c, 4d), indicating that during the 21 days of these soil experimental conditions, the direct growth-promoting effect of *Nostoc* sp. N1 alone was not significant.

The herbicides exhibited a distinct dose-dependent phytotoxicity. The low concentrations (7 mg kg^{-1} of paraquat and 0.15 mg kg^{-1} of butachlor) did not exhibit a significant adverse effect on most growth parameters compared to the untreated control. However, high concentrations (P70 and B1.5) tended to exhibit toxicity, resulting in a marked decrease in the length of the shoot, the biomass (Figs 4a and 4b), and the photosynthetic pigments (Fig. 4c). The co-application of *Nostoc* (N10) demonstrated a significant, though selective, reduction in herbicide toxicity, as summarized in Table 4. This table categorizes the mitigation potential, highlighting where *Nostoc* sp. N1 provided statistically significant alleviation (++) versus partial or no effect (-).

For paraquat, in the P70 co-treatment (N10+P70), *Nostoc* sp. N1 provided a broad spectrum of protection (Table 4), significantly restoring physical characteristics (shoot length, fresh weight, dry weight) and biochemical markers (amino acids) to levels statistically comparable to the untreated control (Figs 4a, 4b, 4d). Only the sugar content (Fig 4d) showed a partial response (+) and remained significantly lower than the control. In contrast, the mitigation of butachlor (B1.5) was significantly more restricted and specific. As shown in Table 4, *Nostoc* sp. N1 failed to alleviate toxicity regarding physical dimensions, with the shoot and root lengths showing no recovery (-). However, a significant alleviating effect (++) was observed in biomass (dry weight) and amino acid accumulation. Although the N10+B1.5 treatment significantly improved these parameters compared to B1.5 alone, the recovery was not complete.

Based on these results, *Nostoc* sp. N1 utilized different mitigation strategies in response to the two herbicides. It provided complete defense against high-dose paraquat toxicity, restoring a broad range of physical and biochemical parameters. In contrast, its protective effect against butachlor was partial, focusing mainly on metabolic stress relief (amino acids and dry mass accumulation) rather than the complete restoration of physical growth structure.

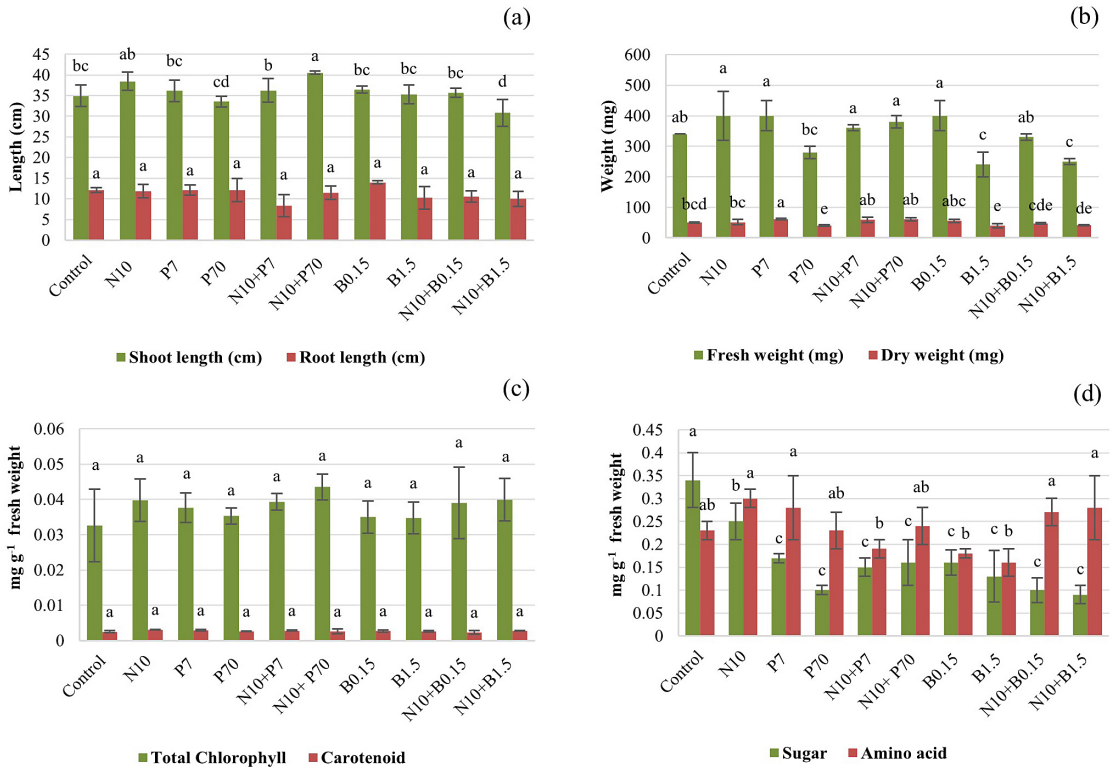

Figure 4. Effects of *Nostoc* sp. N1 (N), paraquat (P), and butachlor (B) on the physical and biochemical parameters of rice seedlings aged 21 days: (a) shoot length and root length, (b) fresh weight and dry weight, (c) total chlorophyll and carotenoid contents, (d) sugar and amino acid contents. Treatments: Control (no herbicide, no *Nostoc*); N10 (*Nostoc* sp. N1 at 10 g kg^{-1}); P7 (paraquat at 7 mg kg^{-1}); P70 (paraquat at 70 mg kg^{-1}); N10+P7 (combination); N10+P70 (combination); B0.15 (butachlor at 0.15 mg kg^{-1}); B1.5 (butachlor at 1.5 mg kg^{-1}); N10+B0.15 (combination); N10+B1.5 (combination). Values represent mean ± standard deviation (n=3). For each parameter, bars sharing the same lowercase letter are not significantly different ($p \leq 0.05$, DMRT)

DISCUSSION

Complex and differential response of *Nostoc* sp. N1 exposure to herbicides

Based on the current results, there were two contrasting and differential interactions between the tested herbicides and *Nostoc* sp. N1. Paraquat demonstrated evident, dose-dependent toxicity, markedly reducing growth and pigment content. In contrast, butachlor improved biomass accumulation and chlorophyll-*a* production, while decreasing phycocyanin levels only at high concentrations.

The reported toxicity of paraquat aligns closely with its known mechanism of action in promoting the formation of superoxide radicals (O_2^-) by acting as an alternative electron acceptor and producing reactive oxygen species (ROS) that cause oxidative damage (Dragolova et al. 2002). This oxidative stress mechanism elucidated the reduction in biomass and degradation of pigments seen in *Nostoc* sp. N1. The decrease in biomass and pigment aligned with ROS-induced lipid peroxidation of cell membranes and a reduction in electron transport, which combined to limit carbon assimilation and growth (Bai et al. 2019). The exponential increase in the PC/Chl-*a* ratio was a clear indicator of this stress. This did not indicate an increase in the production of phycocyanin, but rather reflected the selective degradation of chlorophyll-*a* (the major component of the reaction center) compared to the more stable phycobiliproteins (the antenna complex). Rapid destruction of the photosystem and subsequent pigment degradation are a typical consequence of exposure to paraquats, often occurring before prolonged growth failure (Zhang et al. 2014).

The stimulatory effect of butachlor provided a novel and intriguing discovery, suggesting various possibilities. First, *Nostoc* sp. N1, a paraquat-tolerant strain isolated from an agricultural setting, may have the metabolic pathways

Table 4. Potential of *Nostoc* sp. N1 cells alleviating herbicide toxicity in rice seedling growth at age 21 days

Herbicide	Concentration (mg kg^{-1})	Potential of *Nostoc* sp. N1 cells in mediating toxicity							
		Physical characteristic			Biochemical characteristic				
		SHL	RL	FW	DW	T Chl	Car	Sugar	Amino acids
Paraquat	7	-	-	-	-	+	+	-	-
	70	++	-	++	++	+	-	+	++
Butachlor	0.15	-	-	-	-	+	-	-	++
	1.5	-	-	+	++	+	+	-	++

Note: +: Reduces toxicity but not significantly different at $p \leq 0.05$, ++: reduces toxicity and significantly different at $p \leq 0.05$, -: no effect, SHL – shoot length, RL – shoot length, FW – fresh weight, DW – dry weight, T Chl – total chlorophyll content, Car – carotenoid content, Sugar – sugar content, Amino – amino acid content.

necessary to break-down butachlor, possibly using it as a carbon or nitrogen source. This would directly explain the observed increase in biomass. Numerous studies have reported the biodegradation of herbicides by cyanobacteria (Singh et al. 2013; Moirangthem et al. 2014; Yadav et al. 2025). Secondly, this study used a commercial formulation rather than a pure active substance. As mentioned in the introduction, these formulations contain adjuvants (surfactants, emulsifiers). It is quite possible that the stimulatory effect originated not from the butachlor itself but from the additives that *Nostoc* sp. N1 may metabolize as an available nutrient source (Singh and Datta, 2006). Finally, the different pigment response – an increase in chlorophyll-*a* with a reduction in phycocyanin – indicated an effective reallocation of cellular resources. In response to the mild stress or modified nutrient conditions induced by the butachlor formulation, the cell can breakdown its accessory light-harvesting antennae (phycobilisomes, containing PC) to conserve resources (Chen et al. 2012), while simultaneously reallocating those resources to its primary photosynthetic apparatus (chlorophyll-*a*) to exploit the new source of nutrients.

Opposing protective versus synergistic effects on rice seedlings

The most significant findings in our investigation were the contrasting interactions of *Nostoc* sp. N1 with the two herbicides throughout the development of the rice seedlings. *Nostoc* sp. N1 served as an effective mitigating agent for paraquat. The observed protective effect, which restored a wide range of physical and biochemical parameters during the 21 days of the experiment, was likely related to a combination of mechanisms. The primary mechanism proposed is biosorption, in which the cationic paraquat herbicide attaches to negatively charged surfaces of cyanobacterial cells and extracellular polymeric substances, thus immobilizing the herbicide and decreasing its bioavailability to rice roots (De Philippis et al. 2011; Jindakaraked et al. 2023). The strain's bio-stimulant properties probably improved the ability to recover the rice seedlings (Saeed et al. 2025; Tansay et al. 2021), as indicated by the recovery of stress indicators such as amino acids.

The interaction with butachlor was complex and demonstrated time-dependent characteristics. In the 7 day germination assay, *Nostoc* sp. N1 exhibited an acute synergistic negative effect, significantly increasing butachlor toxicity. The observed effect was likely associated with the partial metabolism of butachlor by *Nostoc*. The metabolic activation of a parent compound into a more toxic intermediate is a common occurrence in environmental toxicology, exemplified by cyanobacteria's ability to convert herbicides into varying degrees of toxicity. Although there are no known published reports detailing the effects of these metabolites on rice germination, the toxicological principle has been supported by comparable evidence. Research on *Nostoc muscorum* indicated that butachlor toxicity occurred from both the parent compound and its degradation products, including dialkylquinoneimine (Anees et al. 2014). The conversion to more toxic metabolites has been suggested as a mechanism for ongoing mortality in other organisms, including juvenile crabs (Wu et al. 2024).

In the 21 day experiment, this synergistic toxicity was not observed. *Nostoc* performed partial mitigation, mainly by decreasing biochemical stress indicators (amino acids), indicating that

over an extended duration, *Nostoc* sp. N1 may further degrade the toxic intermediate into less harmful compounds. This concept of a complex, multi-step degradation pathway is well-supported; butachlor metabolism is known to produce numerous derivatives and metabolites in rice itself (Li et al. 2022), and metabolic studies on the related *Nostoc muscorum* have confirmed its ability to complete this detoxification, identifying the final degradation products as phenols and benzene dicarboxylic acid (Anees et al. 2014). The bio-stimulant effects of cyanobacteria likely provided adaptability to the developed seedlings, permitting them to tolerate residual chemical stress, despite incomplete recovery of physical growth.

A notable observation from the current study is that *Nostoc* sp. N1 is not only a bio-fertilizer, but an active biological agent that exhibits highly specific and antagonistic interactions with conventional herbicides. Although it demonstrated significant potential for alleviating paraquat toxicity, its immediately apparent synergistic adverse interaction with butachlor was an important finding, highlighting the considerable risk of using cyanobacterial inoculants in agroecosystems without adequate understanding of their specific metabolic interactions with existing chemical residues. Our current findings should support a system-level, herbicide-specific, bioremediation strategy, since an unsuitable combination could unintentionally accelerate, rather than mitigate, environmental toxicity.

CONCLUSIONS

This research effectively identified a paraquat-tolerant cyanobacterium, *Nostoc* sp. N1, and revealed its intricate, differential, and herbicide-specific interactions with paraquat and butachlor. The physiological response of *Nostoc* sp. N1 varied significantly between the two herbicides. *Nostoc* sp. N1 served as a potent bioremediation agent for paraquat, providing dose-dependent protection and restoring physical and biochemical parameters in rice seedlings. However, *Nostoc* sp. N1 demonstrated an acute synergistic negative effect with butachlor, significantly enhancing phytotoxicity in developing seedlings. These findings challenge the "one-size-fits-all" approach to bio-fertilization and highlight that cyanobacterial inoculants are not universally advantageous. Therefore, we propose a herbicide-specific application strategy: while *Nostoc*-based bio-fertilizers are highly compatible and beneficial for paraquat-contaminated soils, their application must be avoided or delayed in fields treated with butachlor to prevent synergistic toxicity during early germination. This distinction is crucial to ensure that bio-inoculation fosters sustainable agriculture rather than inadvertently exacerbating crop damage.

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