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Impact of Environmentally Relevant Concentrations of Glyphosate and 2,4-D Commercial Formulations on *Nostoc* sp. N1 and *Oryza sativa* L. Rice Seedlings

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Wide applications of glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) in rice paddy fields could lead to their residues in environment, posing adverse effects on rice growth and primary producers in the rice ecosystem. This research aims to determine the effects of environmentally relevant concentrations of glyphosate and 2,4-D commercial formulations on *Nostoc* sp. N1 and rice seedlings. The effects of herbicides on *Nostoc* sp. N1 were measured from the growth and acute toxicity. The germination and growth were used to determine the effects of herbicides on rice seedlings by measuring their physical and biochemical characteristics. Results showed that while glyphosate had higher toxicity than 2,4-D, both herbicides could stimulate the growth of *Nostoc* sp. N1 as indicated by their increase in biomass and chlorophyll a content. In Petri dish experiments, *Nostoc* sp. N1 cells not only promoted the germination of rice seedlings when added alone, but they also alleviated the toxicity of both herbicides to the rice seedlings. In pot experiments, the addition of *Nostoc* sp. N1 cells combined with herbicides promoted the biochemical characteristics of the rice seedlings by increasing the total chlorophyll, carotenoid and total amino acid content. Our results suggested that environmentally relevant concentrations of glyphosate and 2,4-D formulations should not pose any adverse effects on *Nostoc* sp. N1. Also, with their toxicity-mitigating and growth-promoting effects on rice seedlings, *Nostoc* sp. N1 cells could be applied in the alleviation of herbicide residue toxicity in paddy fields.

Keywords: herbicide toxicity, bio-fertilizers, paddy field cyanobacteria, plant growth-stimulating effects, pesticide residues

INTRODUCTION

The massive application of herbicides in agricultural systems led to their detection in various environmental matrices of surface water, groundwater, sediment and soil (Aparicio et al., 2013; Xu et al., 2017). In 2050, worldwide pesticide production is predicted to be 2.7 times higher than that in 2000 (Oberemok et al., 2015). In Thailand, there was an increasing trend of top 10 pesticides imported from 100,405 tons in 2012 to ∼115,720 tons in 2018, with herbicides taking the highest proportion of 62–79% (Department of Agriculture, 2020). Among the imported herbicides, glyphosate and 2,4-D are commonly applied in massive quantities to agricultural fields in Thailand. While glyphosate is a non-selective systemic herbicide formulated to control both broadleaf and grassy weeds, 2,4-D is a selective post-emergence herbicide that selectively kills many terrestrial and aquatic broadleaf weeds. Both herbicides have regularly been used to control weeds in rice cultivation areas, leading to their residues remaining in the field.

Different amounts of glyphosate ranging from 0.0 to 9.9 mg/kg soil have been reported worldwide (Paipard et al., 2014; Silva et al., 2018; Hagner et al., 2019; Helander et al., 2019). Residues of 2,4-D in soil and sediment were detected at much lower concentrations, which are ranging from <0.652 mg/kg of soil (Ismail et al., 2011; Baumgartner et al., 2017) to <10 µg/kg of soil (Haynes et al., 2000). However, these small amounts of herbicide residues can adversely affect living organisms in the rice ecosystem where there are more than 700 animal species per hectare presented (Clay, 2004). Moreover, they may accumulate in non-target rice fields inhabiting organisms such as the rice frog (*Fejervarya limnocharis*) and menwig frog (*Physalaemus albonotatus*) (Jantawongsri et al., 2015; Curi et al., 2019).

On the first level of the rice field tropic chain, cyanobacteria are one of the most important susceptible species that can be affected by the toxicity of pesticides (Dash et al., 2017). Filamentous cyanobacteria widely distributed in wetland rice ecosystems, especially the heterocystous filamentous cyanobacteria such as *Nostoc* spp. and *Anabaena* spp., are able to maintain the field fertility through their nitrogen fixation (Kim and Lee, 2006). Additionally, various strains of cyanobacteria can produce extracellular polymeric substances (EPSs) comprising mainly polysaccharides, which can increase soil aggregation and improve soil quality (Costa et al., 2018). Therefore, cyanobacteria have been considered as a promising alternative for reducing agrochemical uses and their adverse impacts. A previous study showed that applying cyanobacteria together with chemical fertilizers can reduce agrochemical costs and improve rice yields (Chittapun et al., 2017). It was also found that the toxicity of glyphosate and 2,4-D could be reduced by addition of the cyanobacteria. *Nostoc punctiforme* could take up and use glyphosate as a phosphorus source, exhibiting the high potential of its cells to metabolize phosphonate (Forlani et al., 2008). In the case of 2,4-D, *Nostoc hatei* and *Anabaena lutea* were shown to reduce 2,4-D contamination of water from rice paddy fields by 39.73% after 14 days of application (Kophimail and Bunnag, 2014).

The residue of herbicides possibly poses both direct and indirect adverse side effects on rice and cyanobacteria in the rice field. The toxicity of herbicides to paddy field cyanobacteria and rice has been previously investigated; however, most studies have been performed separately on cyanobacteria or rice (Chen et al., 2007; Sheeba et al., 2011; El-Nahhal et al., 2015) with the focus only on the active ingredients of herbicides. It has been proven that the presence of pure glyphosate [N-(phosphonomethyl)glycine] had a stimulating effect on the growth of several cyanobacteria including *Nostoc muscorum* (Drzyzga and Lipok, 2018). In contrast, it was found that glyphosate at 97% purity induced oxidative stress, cell apoptosis, and toxin release in cyanobacteria *Microcystis aeruginosa* (Wu et al., 2016). Similarly, the stress from a high concentration of 2,4-D at 98% purity could activate a harmful effect evidently as chlorosis occurrence in *Nostoc hatei* strain TISTR 8405 (Pimda and Bunnag, 2017). Nevertheless, the organic solvents and surfactants present in some of the herbicide formulations could alter the toxicity of those herbicides on cyanobacteria. Pereira et al. (2009) indicated higher antialgal toxicity of Spasors formulation than that of sole glyphosate, its active ingredient. Therefore, the study on the toxicity of glyphosate and 2,4-D should be conducted using the commercial formula rather than the pure chemicals. Moreover, there are relatively few data reported for the herbicide's toxicity at very low concentrations relative to the residue levels.

In this study, the effects of low dose exposure of commercial herbicide formulations of glyphosate and 2,4-D on the growth of *Nostoc* sp. N1 and *Oryza sativa* L. cv. San-Pah-Tawng 1 seedlings were investigated. *Nostoc* sp. N1 was chosen as a model organism based on its habitat and rice growth-promoting characteristics. By using the environmentally relevant concentrations of the commercial herbicide formulations of glyphosate and 2,4-D obtained from previous studies, the findings could be useful for the assessment of actual herbicide toxicity on rice and the rice-field cyanobacteria.

MATERIALS AND METHODS

Herbicides, Cyanobacteria, and Rice

Two commercial herbicide formulations were obtained from different companies: (1) Roundup [(*N*-(phosphonomethyl) 48% glycine (w/v) SL)] was purchased from Monsanto, USA, and (2) Keystone [84% 2-(2,4-dichlorophenoxy) acetic acid (w/v) SL] was purchased from Amada, 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. *Nostoc* sp. N1 was isolated from paddy fields in Phayao Province (Tansai et al., 2018) and taxonomically identified as *Nostoc* sp. based on the morphological characteristics presented in the standard literatures (Desikachary, 1959; Komárek and Anagnostidis, 1989) (**Supplementary Figure 1**). *Oryza sativa* L. cv. San-Pah-Tawng 1 rice was obtained from the Phayao Rice Seed Center, Phayao, Thailand.

TABLE 1 | Environmentally relevant concentrations of herbicides used in this study.

Herbicide	Half-life[a] (days)	Herbicide residues (mg/L)			MIC* (mg/L)	Selected concentration (mg/L)	References
		Min[b]	Av	Max[c]			
In water							
Glyphosate	7–14	0.001	0.165	0.700	0.5	0.1 and 0.5	Giesy et al., 2000[a] Kjaer et al., 2004[b] Peruzzo et al., 2018[c]
2,4-D	14	0.00005	0.079	0.954	0.1	0.05 and 0.1	Kollman and Segawa, 1995[a] Félix–Cañedo et al., 2013[b] Fisher et al., 2007[c]
In soil							
Glyphosate	4–19	0.016	1.651	9.99	–	1.0 and 10.0	Al-Rajab and Schiavon, 2010[a] Laitinen et al., 2007[b] Paipard et al., 2014[c]
2,4-D	6.2	Nd	0.153	1.100	–	0.1 and 1.0	U. S., Environmental Protection Agency, 2005[a] Baumgartner et al., 2017[b] Zhichkina et al., 2020[c]

*Nd, not detected; Min, Minimum residue value; Max, Maximum residue value; Av, Average residue values obtained from other studies (**Supplementary Table 1**); *MIC, Minimum inhibitory concentration of herbicide on the growth of rice seedling obtained from our preliminary test (**Supplementary Table 2**). [a–c]References in each row were used for the values of half-life, minimum residue, and maximum residue, respectively.*

Growth of *Nostoc* sp. N1

The growth of *Nostoc* sp. N1 was determined based on the level of chlorophyll a. The chlorophyll a content was determined according to the method of Wintermans and De Mots (1965). For each treatment, *Nostoc* sp. N1 cells were grown in nitrogen-free BG-11 liquid medium before incubation at room temperature and illumination under fluorescent lamps with a 12:12 h light-dark photoperiod of 4,000 lux. The growth was then determined by measuring the chlorophyll a content every 3 days until the end of the experiment (30 days).

Effect of Herbicides on *Nostoc* sp. N1

Twelve-day cultivated *Nostoc* sp. N1 was used as an inoculum. The cells were grown in a 250 mL flask containing N-free BG-11 media supplemented with different concentrations of glyphosate (0.1 and 0.5 mg/L) and 2,4-D herbicides (0.05 and 0.1 mg/L) at room temperature and illuminated under fluorescent lamps with a 12:12 h light-dark photoperiod of 4,000 lux. The concentrations of herbicides used in this experiment were selected based on their environmentally relevant concentrations in water reported by other studies (**Table 1** and **Supplementary Table 1**), and their minimum inhibitory concentration (MIC) on the growth of rice seedling was obtained from our preliminary test (**Supplementary Table 2**). Cell biomass (on a dry weight basis) was determined every 3 days for 12 days until the stationary phase was reached. *Nostoc* sp. N1 cultures were harvested by filtering through a Whatman GF/C glass microfiber filter (GE Healthcare, Leicestershire, UK), then oven-dried at 80°C overnight and weighed. The chlorophyll a content was determined as described above. The phycocyanin content was also investigated. Briefly, The *Nostoc* sp. N1 suspension was filtered with a GF/C filter paper to obtain cell pellet of 100 mg. Pellet cells were homogenized in 1 mL of 0.2 M phosphate buffer solution (pH 7). Five freeze–thaw cycles were carried out and then centrifuged (TD5, Yingtai Instrument, China) at 6,000 rpm and 4°C for 15 min. The supernatant was carefully separated from the sediment. The absorbance was measured at 615 and 652 nm (GENESYS™ 10S UV-Vis Spectrophotometer, Thermo Scientific™, Germany), and the phycocyanin concentration was calculated according to Bennett and Bogorad (1973).

Acute toxicity of the herbicides was determined from the median inhibitory concentrations (EC_{50}) according to the modified method of Bérard et al. (1999). *Nostoc* sp. N1 cells in the log phase from stock culture were added to different dilutions of the herbicides. After the cells were exposed to the herbicides for 96 h, their chlorophyll a content was measured. The estimated EC_{50} value was calculated by plotting a dose-response curve between the herbicide concentrations and *Nostoc* sp. N1 chlorophyll a content using a linear regression equation.

Effects of Herbicides and *Nostoc* sp. N1 on the Germination of Rice Seedlings

Healthy rice seeds were surface sterilized with 10% sodium hypochlorite for 10 min and then washed in distilled water three times. The seeds were sown in sterile Petri dishes filled with filter paper; 10 mL of the test solutions was then added to each Petri dish. The different treatments were as follows: (1) *Nostoc* sp. N1 cells applied at concentrations of 5 and 10 g/L; (2) glyphosate and 2,4-D herbicides applied at different concentrations (0.5, 1.0, 2.5, and 5 mg/L glyphosate; 0.01, 0.1, 1.0, and 5.0 mg/L 2,4-D); (3) a mixture of *Nostoc* sp. N1 cells and the glyphosate or 2,4-D herbicides applied; and (4) neither *Nostoc* sp. N1 cells or herbicides was applied (control) (**Supplementary Table 3**). Germination was recorded at the interval of 24 h until 7 days after sowing, and then the seedling

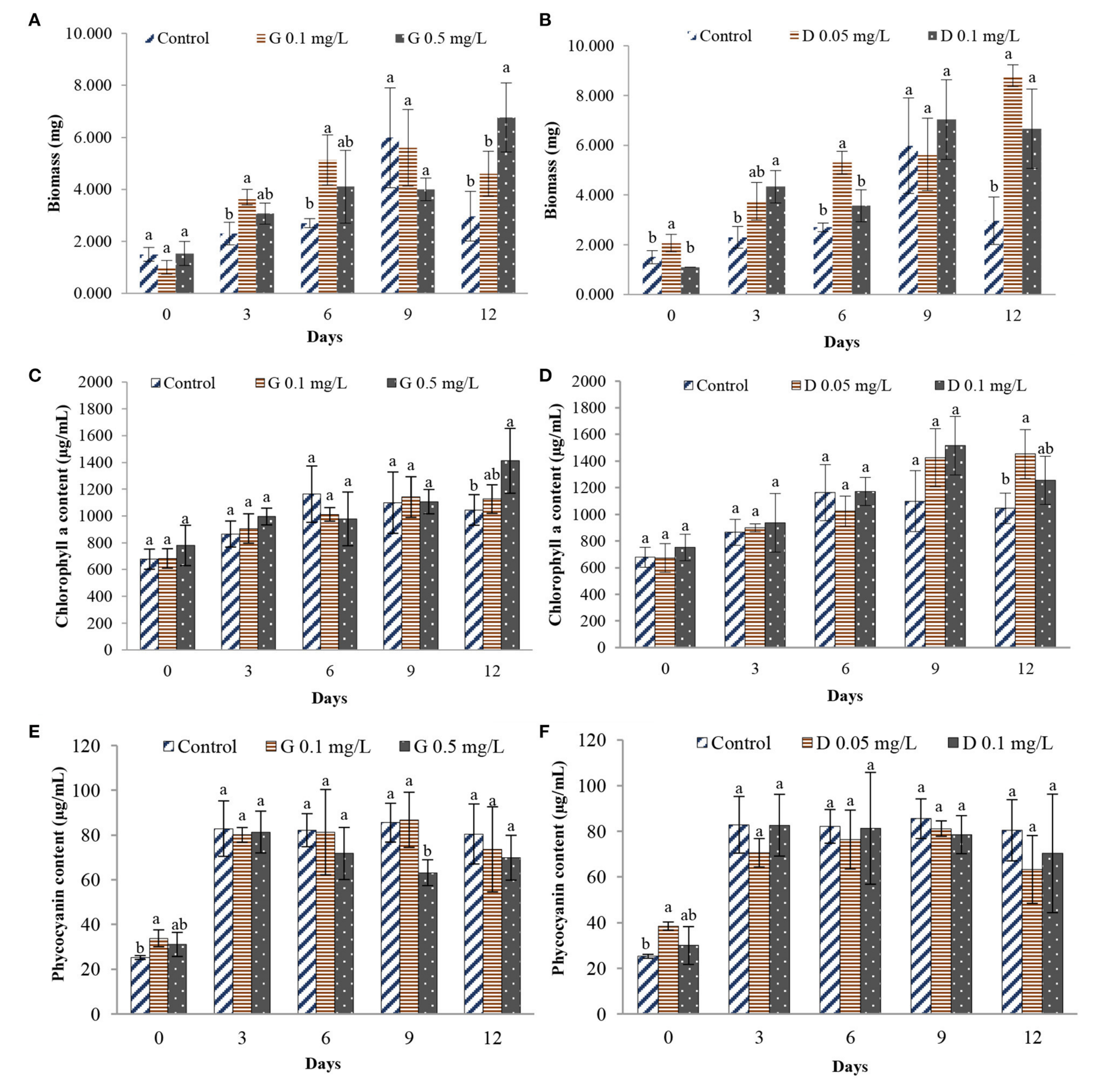

FIGURE 1 | Effects of glyphosate and 2,4-D herbicides on the biomass and pigment contents of *Nostoc* sp. N1. **(A)** Effect of glyphosate herbicide on the biomass **(B)** Effect of 2,4-D herbicide on the biomass **(C)** Effect of glyphosate herbicide on the chlorophyll a content **(D)** Effect of 2,4-D herbicide on the chlorophyll a content **(E)** Effect of glyphosate herbicide on the phycocyanin content **(F)** Effect of 2,4-D herbicide on the phycocyanin content. G, Glyphosate herbicide; D, 2,4-D herbicide. The same letter are not significantly different at $P = 0.05$.

vigor index (SVI) was determined (Abdul-Baki and Anderson, 1973). Moreover, the physical parameters, which are root and shoot lengths and fresh and dry weights of the seedlings, were measured 7 days after sowing. The phytotoxicity index, which is the average of the phytotoxicity classes calculated based on the percent reduction in growth and physiological parameters, was calculated according to the method described by Aziz et al. (2015) (**Supplementary Table 4**).

Determination of Suitable Media and Application Methods for a Pot Experiment

To evaluate the suitability of culture media and the application method of *Nostoc* sp. N1 to the soil, BG-11 media and nitrogen-free BG-11 (N-free BG-11) media were separately used for culturing of *Nostoc* sp. N1. Experimental soil was excavated from the surface of a paddy field in Phayao Province (0–20 cm depth), after which it was air-dried, ground and sieved through a

6.00 mm sieve to remove rock and plant fragments. Plastic pots, each containing 500 g of the sieved and air-dried topsoil, were then prepared. Two application methods of *Nostoc* sp. N1 were compared: (1) mixing of *Nostoc* sp. N1 cells into the soil, and (2) applying *Nostoc* sp. N1 cells to the surface of the soil. The concentration of 10 g/kg of *Nostoc* sp. N1 was mixed into the soil or poured onto the surface of the soil.

Healthy rice seeds were surface sterilized with 10% sodium hypochlorite for 10 min, then rinsed 3 times with distilled water. The rice seeds were soaked in DI water overnight, then wrapped in a wet sheet cloth and incubated in the dark until germination. Consequently, ten germinated seeds were sown in the plastic pots containing the prepared soil and watered every 2 days with 20 mL of distilled water. Rice seedlings were grown under fluorescent lamps with a 12:12 h light-dark photoperiod of 4,000 lux. The experiment was arranged in a randomized complete block design with three replicates. The physical characteristics, i.e., root and shoot lengths along with fresh and dry weights, were measured after 15 days of application. The best application method was selected for subsequent experiments.

Effects of Herbicides and *Nostoc* sp. N1 on the Growth of 21-Day-Old Rice Seedlings

The germinated rice seeds and soil were prepared as previously described. The concentrations of glyphosate and 2,4-D herbicides used in this experiment were selected based on their environmentally relevant concentrations in soil described by other studies (**Table 1**). Sieved and air-dried soil was mixed with selected concentrations of glyphosate herbicide (1.0 and 10.0 mg/kg), 2,4-D herbicide (0.1 and 1.0 mg/kg) and fresh *Nostoc* sp. N1 (10 g/kg) for the pot experiments. Rice was grown under fluorescent lamps with a 12:12 h light-dark photoperiod of 4,000 lux. After 21 days of application, the physical characteristics of rice seedlings were determined. The chlorophyll a content was analyzed as described previously. The total sugar content was quantified according to the phenol-sulfuric acid method (Dubois et al., 1956) and the total free amino acid content was determined according to the ninhydrin carbon dioxide method (Hamilton and Van-Slyke, 1943), using leucine as a standard.

Statistical Analysis

All experiments were performed in triplicate. The results are described in terms of the average ± standard deviation (SD). All the data were analyzed statistically by the analysis of variance followed by Duncan's multiple range test (DMRT) to verify the significant differences between the treatments at $p \leq 0.05$.

RESULTS

Effects of Herbicides on *Nostoc* sp. N1

The growth of *Nostoc* sp. N1, estimated from chlorophyll a content, was in the exponential phase from day 3 to day 12 before reaching the stationary phase and decreasing continuously after that (**Supplementary Figure 2**). Therefore, 12 days were chosen as the experimental time for subsequent experiments. After 12 days of exposure to the environmentally relevant concentrations of glyphosate and 2,4-D herbicides, the growth of *Nostoc* sp. N1 was significantly stimulated compared with the control condition ($p < 0.05$), and it was increased when the concentrations of the herbicides increased (**Figure 1**). The highest biomass and the maximum chlorophyll a content were observed at 0.5 mg/L of glyphosate and 0.05 mg/L of 2,4-D herbicides (**Figures 1A–D**). For the effects of herbicides on the content of phycocyanin, no difference was observed in the treatments compared with the control except at day 9 of the experiment where the amount of phycocyanin was significantly reduced by 0.5 mg/L of glyphosate (**Figures 1E,F**).

TABLE 2 | Acute toxicity of herbicide formulations on *Nostoc* sp. N1 (96 h).

Herbicides	EC_{50} Values (mg/L)
Glyphosate	0.617
2,4-D	209.434

The median inhibitory concentrations (EC_{50}) of glyphosate and 2,4-D herbicides were monitored for their acute toxicity to *Nostoc* sp. N1 cells. According to the results (**Table 2**), glyphosate was more toxic to *Nostoc* sp. N1 cells than 2,4-D with the EC_{50} values of 0.617 and 209.434 mg/L, respectively.

Effects of Herbicides and *Nostoc* sp. N1 on the Germination and Growth of Rice Seedlings

The effects of herbicides and *Nostoc* sp. N1 cells on the germination and growth of 7-day old rice seedlings were observed both separately and concurrently, and the results are shown in **Table 3**. The growth of rice seedlings was determined from the SVI and their physical characteristics. It was found that the addition of only *Nostoc* sp. N1 at 10 g/L promoted rice seedling growth with the highest SVI of 1,014.67 and 790 for both experiments conducted. Moreover, the growth-promoting efficiency of *Nostoc* sp. N1 could also be indicated by the positive value of percent increase of the treatment compared with that of the control (PIC). On the other hand, the addition of the herbicides tended to reduce the germination and growth of the rice seedlings (**Supplementary Tables 5, 6**). As the concentration of glyphosate herbicide increased from 0.5 to 5.0 mg/L, the PIC decreased from −10.44 to −79.20, respectively. Similar inhibitory effect was also found for 2,4-D herbicide at a concentration of 0.1–5.0 mg/L; however, a stimulatory effect was observed at the lowest concentration of 0.01 mg/L.

However, the addition of the *Nostoc* sp. N1 cells could alleviate the toxic effects of the herbicides on rice seedlings. When combined with either 5.0 and 10.0 g/L of *Nostoc* sp. N1, the treatments with glyphosate herbicide exhibited the increased PICs in all glyphosate concentrations (**Table 3**). Highest PICs were obtained at the lowest concentration of glyphosate herbicide (0.5 mg/L). Similarly, the addition of 2,4-D herbicide combined with *Nostoc* sp. N1 slightly increased the SVIs compared with those from the herbicide 2,4-D treatment alone.

TABLE 3 | Effects of glyphosate, 2,4-D herbicides and *Nostoc* sp. N1 cells on the germination and growth parameters of 7-day-old rice seedlings.

Treatment	Germination and growth parameters			Treatment	Germination and growth parameters		
	SVI	PIC (%)	Phytotoxic index		SVI	PIC (%)	Phytotoxic Index
Control	746.60[cd]	0	0.00	Control	621.22[abcd]	0	0.00
N 5.0 g/L	939.67[ab]	25.86	−1.5	N 5.0 g/L	686.67[abc]	10.54	−1.25
N 10.0 g/L	1,014.67[a]	35.91	−1.5	N 10.0 g/L	790.00[a]	27.17	−2.00
G 0.5 mg/L	668.67[d]	−10.44	0.50	D 0.01 mg/L	766.33[a]	23.36	−1.00
G 1.0 mg/L	563.78[e]	−24.49	1.25	D 0.1 mg/L	501.00[d]	−19.35	−0.25
G 2.5 mg/L	301.33[g]	−59.64	2.50	D 1.0 mg/L	480.67[d]	−22.62	−0.25
G 5.0 mg/L	155.33[h]	−79.20	2.75	D 5.0 mg/L	247.00[e]	−60.24	2.25
N 5.0 + G 0.5	907.33[b]	20.99	−1.25	N 5.0 + D 0.01	705.33[ab]	13.54	−1.25
N 5.0 + G 1.0	728.00[cd]	−2.49	−0.50	N 5.0 + D 0.1	539.00[bcd]	−13.24	0.25
N 5.0 + G 2.5	427.67[f]	−42.72	1.50	N 5.0 + D 1.0	520.67[cd]	−16.19	0.25
N 5.0 + G 5.0	192.33[h]	−74.24	2.75	N 5.0 + D 5.0	235.08[e]	−62.16	2.25
N 10.0 + G 0.5	782.80[c]	4.85	−0.50	N 10.0 + D 0.01	693.33[abc]	11.61	−0.75
N 10.0 + G 1.0	690.33[cd]	−7.54	−0.50	N 10.0 + D 0.1	575.00[bcd]	−7.44	−0.50
N 10.0 + G 2.5	473.67[ef]	−36.56	1.25	N 10.0 + D1.0	509.00[d]	−18.06	0.00
N 10.0 + G 5.0	223[gh]	−70.13	2.50	N 10.0 + D 5.0	261.55[e]	−57.90	2.25

*N, Nostoc sp. N1; G, Glyphosate herbicide; D, 2,4-D herbicide; SVI, Seedling vigor index (calculated based on seedling length); PIC, Percent increase compared with the control calculated based on the SVI; Phytotoxic Index were classified according to the reduction (%) in different growth parameters (**Supplementary Table 4**).*
[a–h]Means followed by the same letter are not significantly different at P = 0.05.

The phytotoxicity index ranged between 0.00 (control) and 2.75 (5.0 mg/L of glyphosate herbicide). The higher value of phytotoxicity index demonstrated an adverse effect of both herbicides on physical and biochemical characteristics of rice seedling. Comparing the phytotoxicity index between two herbicides, glyphosate herbicide revealed higher toxicity than did 2,4-D herbicide. However, toxicity induced by those herbicides on rice seedling decreased with *Nostoc* sp. N1 addition as indicated by minimum phytotoxicity values (**Table 3**).

Effects of Herbicides and *Nostoc* sp. N1 on the Growth of Rice: A Pot Experiment

The physical characteristics of 15-day-old rice seedlings in response to the different types of BG-11 media and *Nostoc* sp. N1 application methods are shown in **Table 4**. From the results, both BG-11 and N-free BG-11 media caused no significant difference in any growth parameter compared with the control ($p > 0.05$). However, considering the *Nostoc* sp. N1 application methods, it was found that the application method of mixing *Nostoc* sp. N1 into the soil yielded higher numbers of all parameters than the method of applying *Nostoc* sp. N1 to the surface of the soil. Thus, the method of mixing *Nostoc* sp. N1 into the soil was selected for 21-day-old rice seedlings experiment.

For 21-day-old rice seedlings test (**Table 5**), it was found that both concentrations of glyphosate herbicide had no adverse effects on the dry weight of the seedlings. In contrast, rice seedlings were more strongly affected by 2,4-D herbicide than by glyphosate herbicide. The significantly lower dry weight was observed on the seedlings treated with both concentrations of 2,4-D herbicide compared with the seedlings in the other treatments ($p < 0.05$).

Interestingly, *Nostoc* sp. N1 cells demonstrated the potential to relieve the toxicity of both herbicides at environmentally relevant concentrations. The low dry weight of the rice seedlings, either resulted from glyphosate or 2,4-D, could be rescued by the addition of 10 g/kg *Nostoc* sp. N1 cells (**Table 5**). Also, the addition of *Nostoc* sp. N1 cells alone or combined with the herbicides tended to increase the total chlorophyll and carotenoid contents of the seedlings. A significantly higher total amino acid content was also found in the seedlings treated with *Nostoc* sp. N1 cells ($p < 0.05$) than in the seedlings in the other treatments.

DISCUSSION

Stimulation of *Nostoc* sp. N1 Cell Growth by the Herbicides

From the results, the growth of *Nostoc* sp. N1 was significantly stimulated after being exposed to both glyphosate and 2,4-D herbicides. Also, the growth stimulation was positively related to the concentrations of the herbicides applied. At 0.5 mg/L glyphosate and 0.05 mg/L 2,4-D herbicides (**Figures 1A–D**), the maximum chlorophyll a content was found. This result was supported by Wong (2000), who reported that the photosynthesis and chlorophyll a synthesis in *Scenedesmus quadricauda* was increased at 0.02 mg/L of glyphosate and 0.02 or 0.2 mg/L of 2,4-D. Whereas, 2 mg/L of glyphosate and 20 mg/L of 2,4-D significantly decreased the chlorophyll-a contents. Saygideger and Okkay (2008) evidenced the same result in *Chlorella vulgaris* and *Spirulina plantensis* exposed to 2,4-D.

In this study, the phycocyanin content in both herbicide treatments was not significantly different from the controls

TABLE 4 | Effects of different BG-11 media and application methods on the physical characteristics of rice seedlings.

Treatment (10 g/kg)	Application method	Physical characteristic (means ± SDs)			
		Shoot length (cm)	Root length (cm)	Fresh weight (mg)	Dry weight (mg)
Control	–	21.40 ± 1.20[ab]	8.72 ± 2.64[a]	335.30 ± 38.90[a]	68.90 ± 5.10[ab]
BG-11	Mixed into soil	23.25 ± 0.58[ab]	11.97 ± 0.33[a]	452.60 ± 32.50[a]	68.40 ± 12.90[ab]
	Applied to surface	21.78 ± 1.30[ab]	8.78 ± 1.48[a]	364.70 ± 38.80[a]	54.20 ± 1.90[b]
N-free BG-11	Mixed into soil	23.32 ± 0.00[a]	10.35 ± 0.39[a]	442.70 ± 51.40[a]	74.00 ± 5.70[a]
	Applied to surface	20.99 ± 0.32[b]	11.35 ± 0.64[a]	363.90 ± 66.40[a]	53.30 ± 2.50[b]

[a–b]Means ± SDs followed by the same letter are not significantly different at P = 0.05.

TABLE 5 | Effects of glyphosate, 2,4-D herbicides and *Nostoc* sp. N1 on the physical and biochemical characteristics of 21-day-old rice seedlings.

Treatment	Physical and biochemical characteristics (means ± SDs) (mg/g fresh weight)				
	Dry weight (mg)	Total Chl	Car	Sugar	Amino acid
Control	51.00 ± 1.80[ab]	3.26 ± 1.03[a]	0.25 ± 0.03[ab]	0.34 ± 0.06 [a]	0.23 ± 0.02 [bc]
N 10.0 g/kg	51.40 ± 8.80[ab]	3.97 ± 0.60[a]	0.31 ± 0.01[a]	0.24 ± 0.04[b]	0.30 ± 0.02[a]
G 1.0 mg/kg	55.00 ± 5.00[a]	3.76 ± 0.24[a]	0.29 ± 0.01[ab]	0.16 ± 0.02[cd]	0.22 ± 0.03[bc]
G 10.0 mg/kg	51.00 ± 5.10[ab]	3.74 ± 0.26[a]	0.25 ± 0.02[ab]	0.04 ± 0.00[e]	0.24 ± 0.01[bc]
N 10.0 g/kg + G1.0 mg/kg	53.40 ± 1.20[ab]	3.80 ± 0.10[a]	0.27 ± 0.02[ab]	0.13 ± 0.02[d]	0.22 ± 0.02[bc]
N 10.0 g/kg + G10.0 mg/kg	57.30 ± 1.90[a]	3.97 ± 0.13[a]	0.29 ± 0.05[a]	0.14 ± 0.00[cd]	0.24 ± 0.03[bc]
D 0.1 mg/kg	42.30 ± 2.20[c]	3.22 ± 0.91[a]	0.23 ± 0.03[bc]	0.07 ± 0.02[e]	0.20 ± 0.04[c]
D 1.0 mg/kg	39.50 ± 2.20[c]	3.66± 0.14 [a]	0.29 ± 0.01[ab]	0.19 ± 0.04[c]	0.22 ± 0.01[bc]
N 10.0 g/kg + D 0.1 mg/kg	45.90 ± 4.20[bc]	4.19 ± 1.05[a]	0.19 ± 0.07[c]	0.18 ± 0.03[cd]	0.26 ± 0.04[ab]
N 10.0 g/kg + D1.0 mg/kg	52.10 ± 3.40[ab]	3.82 ± 0.57[a]	0.25 ± 0.01[ab]	0.07 ± 0.02[d]	0.22 ± 0.02[bc]

N, Nostoc sp. N1; G, Glyphosate herbicide; D, 2,4-D herbicide; Total Chl, Total chlorophyll; Car, Carotenoid content; Sugar, Sugar estimation; Amino acid, Total amino acids.
[a–e]Means ± SDs followed by the same letter are not significantly different at P = 0.05.

(**Figures 1E,F**). In contrast, a previous study reported significantly higher concentrations of the light-harvesting pigments, phycobiliprotein, in the cyanobacteria exposed to 2,4-D (Pumas et al., 2011). This might be because of the low concentrations of glyphosate and 2,4-D used in this study and possibly the extracellular polysaccharides produced by *Nostoc* sp. N1 to enhance protection against these herbicides.

The EC_{50} of glyphosate and 2,4-D herbicides suggested that *Nostoc* sp. N1 was more tolerant to 2,4-D than to glyphosate herbicides (**Table 2**). Higher toxicity of glyphosate to *Nostoc* sp. N1 cells could be due to its mechanism to prevent the biosynthesis of amino acids by inhibiting 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase in the shikimate pathway (Smedbol et al., 2017) and inhibiting ammonium uptake (Singh et al., 1989). For 2,4-D, its plant growth regulator characteristics might stimulate the growth of *Nostoc* sp. N1, resulting in less toxicity. It was found that high concentrations of 2,4-D inhibited growth and photosynthesis in *Chlorella elipsoidea*, but low concentrations could stimulate the parameters (Chai and Chung, 1975), which could be the case in this study. It should be noted that this study demonstrated a low EC_{50} value of glyphosate (0.617 mg/L) while other studies have shown higher values up to 120 mg/L for *Nostoc* sp. and 20 mg/L for *Nostoc muscorum* (He et al., 2013; Anees et al., 2014). The variations of EC_{50} values depends on the species of *Nostoc* and the different market-available formulations of herbicides used in the experiments. For example, the presence of isopropylamine in commercial Roundup formulations is of vital importance with respect to the toxicity of this herbicide toward aquatic phytoplankton (Lipok et al., 2010).

Reduction of the Herbicide Toxic Effects to Rice Seedlings by *Nostoc* sp. N1

The addition of only *Nostoc* sp. N1 (10 g/L) promoted rice seedling growth as observed by SVI and PIC data; on the other hand, the addition of glyphosate or 2,4-D reduced the germination and growth of the rice seedlings (**Table 3**). However, a stimulatory effect of 2,4-D was found at its lowest concentration (0.01 mg/L). Since 2,4-D herbicide has a similar structure to indol-3-acetic acid (IAA), the herbicide at low concentrations might act as an auxin-like hormone to promote plant growth, while at high doses, it drives excessive overgrowth (Grossmann, 2000).

The toxic effects of both glyphosate and 2,4-D on the rice seedlings could be reduced by the combined addition with *Nostoc* sp. N1 as clearly observed via SVI (**Table 3**). This phenomenon might be explained by two reasons. Firstly, nitrogen-fixing cyanobacteria can produce phytohormones such as auxin, gibberellins and cytokinin that promote the growth of rice (Hashtroudi et al., 2013; Singh et al., 2014). Secondly, *Nostoc* application could enhance the organic carbon and nitrogen content of the surface soil and promote plant growth and iron uptake of plants (Obana et al., 2007). A previous study showed that *Nostoc carneum* and *N. commune* promoted Pathum Thani rice seedling growth and yield, resulting in a decrease in rice production costs without negatively affecting on the environment (Bhooshan et al., 2018).

According to the results from the pot experiments (21-day-old rice seedlings), glyphosate herbicide showed no adverse effects on the dry weight of the rice seedlings while 2,4-D herbicide significantly had a negative effect on this parameter (**Table 5**). Glyphosate tend to be bound tightly in most soils with a K_{oc} of 24,000 mL/g (Wauchope et al., 1992; Duke and Powles, 2008) compared with 2,4-D with a lower K_{oc} of 61.7 mL/g (U. S., Environmental Protection Agency, 2005). This could lead to more leaching of 2,4-D by water and more uptake by rice seedlings, resulting in more adverse effects. Moreover, glyphosate might benefit cyanobacteria by providing a source of nutritive carbon, nitrogen or phosphorus that is as beneficial as inorganic phosphate (Drzyzga and Lipok, 2018), leading to less toxicity than 2,4-D.

Similar to the Petri dish experiments, *Nostoc* sp. N1 cells could reduce the toxicity of both herbicides at environmentally relevant concentrations chosen in this study. Moreover, the biochemical parameters, i.e., total chlorophyll and carotenoid contents of the seedlings, were found to be increased by *Nostoc* sp. N1 cells. It has been known that *Nostoc* spp. has the ability to reduce the toxic stress of herbicides by producing polysaccharides to protect themselves from herbicide exposure or stress conditions (Arora et al., 2010). Cyanobacteria also play an essential role in the soil by fixing carbon and nitrogen, which increases the fertility of and water retention in the soil (Chamizo et al., 2018). Therefore, cyanobacteria in paddy fields like *Nostoc* spp. could potentially act as a biodegrading organism to eliminate various types of agrochemical residues, benefiting the ecosystem quality.

Glyphosate and 2,4-D account for a considerable herbicide market share worldwide. However, information concerning the effects of their residues at environmentally relevant concentrations on the growth of important cyanobacteria and rice seedlings is still limited. In this study, environmentally relevant concentrations of both glyphosate and 2,4-D herbicides did not significantly affect the growth of pigment contents of *Nostoc* sp. N1. Furthermore, *Nostoc* sp. N1 cells promoted plant germination and growth by increasing several physical and biochemical characteristics of the rice seedlings. The application of *Nostoc* sp. N1 combined with both herbicides in the soil did not cause adverse effects on the growth of rice. In contrast, it seemed to promote several growths and biochemical parameters of the rice seedlings. These findings suggested that the environmentally relevant concentration of herbicide formulations used in this work did not adversely affect either *Nostoc* sp. N1 or the rice seedlings. Thus, *Nostoc* spp. could play an important role in paddy fields in terms of primary production and could alleviate the toxicity of herbicides via growth-stimulating effects on rice seedlings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KI was primarily responsible for the project plan, experimental design, data analyses, and all manuscript drafts. ST, NN, OC, and PT designed and performed the experiments. ST contributed to the statistical data analyses and composing figures. This work was carried out in collaboration between all authors. All authors have read the manuscript and approved this submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.661634/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.