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In Vitro Cyanobacterial Isolates Growth and their Effect on Rice Crop Cultivated in Pesticide-Treated Fields

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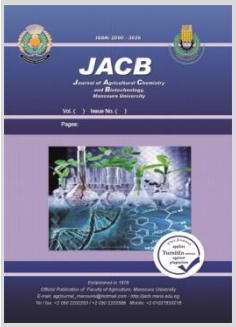

ABSTRACT

An experiment was carried out in Sakha Research Station, Kafrelsheikh governorate, Egypt during the 2018-2019 seasons to isolate the cyanobacteria dominated in the soil samples of rice fields polluted by two pesticides (carbofuran and penoxsulam). These cyanobacterial isolates were identified and the best isolates, regarding growth activities and nitrogen (N_2) fixation capacities, were chosen and further applied to the rice field to study their effect on rice yield under different urea concentrations (0, 35 and 69 kg N_2/fed). Data revealed that nine cyanobacterial isolates were successfully obtained and purified. A growth curve experiment was conducted on these isolates and revealed that three isolates (*Nostoc muscorum*, *Anabaena cylindrica*, and *Anabaena variabilis*) had the best growth activities and capacities for N_2 fixation. Within limit, both pesticides showed stimulation of both cell growth and N_2 fixation. With higher concentrations, these values tended to decline due to the possible inhibitory or toxic effect of these pesticides. Generally, cyanobacterial isolates were less tolerant to penoxsulam and were inhibited at lower concentrations compared to carbofuran. With carbofuran application on rice field (Egyptian hybrid rice 1 variety), the interaction between cyanobacteria and urea resulted in the highest grain yield with the recommended dose of urea (69 kg N_2/fed), while the highest 1000-grain weight was obtained with half the recommended dose of urea (35 kg N_2/fed). Moreover, the use of a mixture of cyanobacterial species rather than a single one resulted in more satisfactory results.

Keywords: Cyanobacteria, Nitrogen fixation, Nostoc, Anabaena, Pesticides, Rice crop.

INTRODUCTION

Inorganic fertilizers are chemical substances composed of known quantities of nitrogen, phosphorus, and potassium, and their excessive use resulted in air and groundwater pollution (Youssef and Eissa, 2014). So, efforts have been introduced to encourage the use of biofertilizers either solely or in combination with inorganic fertilizers (Raja, 2013).

Biofertilizers are the natural microflora of the soil including all kinds of bacteria and fungi. They can be selectively added to the soil to enhance its content of micro- and macro-nutrients via N_2 fixation, phosphate, and potassium solubilization or mineralization, secreting plant growth regulators, and exhibiting biocontrol abilities (Sinha *et al.*, 2014). Also, a consortium of microorganisms can be considered effective for remediation of the pesticide-polluted ecosystems. Moreover, pesticides can provide a source of energy for microorganisms, thereby increasing the overall potential for degradation (Abdel-Razek *et al.*, 2019).

Cyanobacteria are a group of photosynthetic prokaryotes. The heterocystous species are capable of fixing atmospheric nitrogen. However, several non-heterocystous species can fix atmospheric nitrogen under micro-aerophilic conditions (Prasanna and Kaushik, 2006). Also, some cyanobacteria are known to secrete growth promoters (auxin, gibberellins, and cytokinin). So, many of them have been used as biofertilizers in agriculture (Tantawy and Atef, 2010).

This work aims to study the effect of combined use of some cyanobacterial isolates (biofertilizer) and urea (a traditional inorganic fertilizer) on the growth of rice crop in pesticides (carbofuran or penoxsulam) -treated soil regarding reducing the use of chemical fertilizers, the overall cost.

MATERIALS AND METHODS

A) Study Design and Setting

1. Isolation, purification, and identification were conducted at the Agriculture Botany (Microbiology) Department laboratories, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.
2. In vitro experiment: using batch culture method to study the effect of different pesticides (carbofuran or penoxsulam) concentrations on N2-fixation and cell dry weight of cyanobacterial isolates. This experiment was carried out at the Agricultural Microbiology Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt.
3. Field experiment: using split-plot design with three replicates to study the effect of different combinations of fertilizers (cyanobacteria and urea) with pesticides (carbofuran or penoxsulam) on the growth parameters of rice crop, in addition to cyanobacterial count in the soil. This experiment was carried out during the 2019 summer season at Sakha Research Station, Kafrelsheikh, Egypt.

B) Study Supplies:

1. Rice variety: The used variety was Egyptian hybrid rice one (EHR1) purchased from Sakha Research Station, Kafrelsheikh, Egypt.

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2.Pesticides: Two pesticides were used: carbofuran (Furadan), a nematocide, [(2,2-Dimethyl-3H-1-benzofuran-7-yl) N-methylcarbamate] purchased from Kafrelzayat company, Egypt; and penoxsulam (Granite), a herbicide [: 2-(2,2-difluoroethoxy)-N- (5,8-dimethoxy [1,2,4] triazolo [1,5c] pyrimidin-2-yl)-6-(trifluoromethyl) benzenesulfonamide] purchased from Dow AgroSciences local supplier, Egypt.

3.Urea fertilizer: (46.5% nitrogen content) was supplied from the local market.

C) Isolation and Purification:

Soil samples were collected from rice fields polluted with the two pesticides (carbofuran or penoxsulam) from the rhizosphere area of rice at depth of 0-30 cm from the soil surface during the 2018 summer season from Sakha Research Station, Kafrelsheikh, Egypt. The samples were air-dried then ground to pass through a 2 mm sieve, then, well-mixed. The procedure of preparation and measurements of the soil extract was made according to The Oxford Manual of Culture Media (Manual, 1990).

A sterilized 7% agarized Z medium (Abdel-Razek *et al.*, 2019) was poured into Petri dishes (10 cm in diameter). Few milligrams of soil samples were spread in the form of a strip (1cm in broad) in each Petri dish (Black *et al.*, 1965) and the dishes were then incubated at 30ºC under continuous light of 120 cm long white fluorescent lamps with the light intensity of 3000 Lux.

Most cyanobacteria are usually associated with other microorganisms; hence, they must be purified from any contaminants. Standard cyanobacterial isolation and purification techniques described by previous studies were applied (Desikachary, 1959, Black *et al.*, 1965 and El-Ayouty and Ayyad, 1972). Washing and mercuric chloride treatments were the most effective method for obtaining cyanobacteria cultures free from bacteria.

D) Selection of the most efficient N_2-fixing cyanobacterial isolates:

A growth curve experiment was conducted for the isolated cyanobacterial strains in three replicates. 500 ml Erlenmeyer, flasks each containing 250 ml of Modified Watanabe liquid medium (Staub, 1961) inoculated with 1 ml of the cell suspension of a 10-day-old culture of each cyanobacterial isolate. Inoculated flasks were incubated at 28-30ºC under continuous illumination (3000 lux) for 42 days and 10-ml samples (for N_2 fixation and cell dry weight, one sample for each) were taken at weekly intervals. N_2 fixation was assessed to choose the most efficient isolates. While cell dry weight was assessed over time to determine the interval required for reaching maximal growth (at the end of log phase). Both were used for determining the conditions of the further laboratory experiments.

The mean of total nitrogen in the samples was determined using the micro-Kjeldahl method (Holt, 1994) and the results were expressed as mg N_2/100 ml culture. Also, cells of the growing cyanobacteria in the samples were filtered using Whatman No.1 filter paper. The mean weight of the cyanobacterial growth was recorded after oven dryness at 70ºC for 24hr and subtracted from the cell-free medium sample (control). Data of the cyanobacterial growth were calculated as gram dry weight/100 ml culture. Then, the isolates were sub cultured monthly on nutrient agar medium for maintenance (Watanabe *et al.*, 1951).

E) Identification of cyanobacterial isolates:

For characterization and identification of the purified cyanobacteria isolates, 500 ml Erlenmeyer flasks each containing 250 ml of Modified Watanabe liquid medium and also plates of agarized Modified Watanabe medium (Watanabe *et al.*, 1951) were inoculated with a loop full of the 10-day-old culture of each cyanobacterial isolates. Inoculated flasks and plates were incubated at 28-30ºC under continuous illumination (3000 lux) for 10 days. For identification of cyanobacteria cells, they were examined for a cultural appearance on both solid and liquid media as well as the characteristics of the trichome, sheath, vegetative cells, and the heterocysts produced by each isolate. Diameters and lengths of 100 vegetative cells, 100 heterocysts, and 100 akinetes were measured. Also, the type and the presence of branching were examined.

F) In vitro experiment:

The experiment was done in 3 replicates. 500-ml Erlenmeyer flasks each containing 100 ml of Modified Watanabe liquid medium (Watanabe *et al.*, 1951) with both carbon and nitrogen sources replaced with 4 different concentrations (0, 40, 80, and 120 ppm) prepared from two pesticides, carbofuran or penoxsulam (each alone). Flasks were inoculated with 1 ml of the cell suspension of each isolate (containing 9.38 $x10^7$ cfu), then incubated under continuous illumination (3000 lux) at 28-30ºC for the duration determined from the previous experiment. The mean total nitrogen and mean cell dry weight were evaluated as previously described at 10-day intervals.

G) Field experiment:

The physical and chemical properties of the experimental soil in 2019 summer season were clay (56.1 %), silt (31.3 %), sand (12.6 %), organic matter (1.4 %), total nitrogen (450 mg/Kg), available phosphorus (13 mg/Kg), pH (8.3 with 1:2.5 W/V soil suspension), and electrical conductivity (2 ds/m in soil paste). While, soluble cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) were (5.1, 2.1, 0.4, and 12 meq/L) respectively, soluble anions (Co^{3-}, HCO^{3-}, CL^-, and SO^{4-}) were (0.00, 3.5, 14.8, and 1.30 meq/L) respectively, and available micronutrients (F^{2+}, Zn^{2+}, and Mn^{2+}) were (6.05, 0.89, and 3.37 ppm) respectively.

The studied treatments were carried out on plots of 3 × 4 m (12 m^2) in three replicates. Each main plot contained cyanobacterial isolates (either nil, individual, or a mixture), each subplot contained different urea concentrations [0, 0.5 recommended dose(35 kg N_2/fed), or the whole recommended dose(69 kg N_2/fed)], and each sub- subplot contained the recommended dose of each pesticide [carbofuran(0.5 kg/fed) or penoxsulam(300 ml/fed)] was sprayed.

Filtrate of cyanobacterial 1000-ml cultures (grown on Modified Watanabe liquid medium under the same previous incubation conditions) was mixed with about 1 kg sand (as a carrier) and was added to each plot 7 days after rice transplanting. While urea was added in the form of 1/3 of the total quantity before irrigation and the rest 30 days after rice transplanting. Finally, carbofuran was added with rice transplanting and penoxsulam was added 3 days after rice transplanting.

The evaluated means of rice crop parameters were measured either 100 days after transplanting [plant height (cm) and the number of panicles/hill] or after harvesting [1000-grain weight (g) and grain yield (tons/fed)]. Also, the

mean cyanobacterial count in the soil (cfu) was done using the plate count method on agarized Modified Watanabe medium (Petri dishes, 10 cm in diameter) after a 21-day incubation period under the same previous conditions.

Statistical analysis

All data collected were subjected to standard statistical analysis. Data obtained throughout this study were analyzed by computer-assisted one-way ANOVA, using the software package stat graphics version 5.0 (costat). Least significance differences (LSDs) were calculated at level of significance $P < 0.05$ (Ing, 1987).

RESULTS AND DISCUSSION

Results

Isolation, growth curve experiment, and identification:

Nine cyanobacterial isolates were successfully obtained as bacteria-free cyanobacteria. After incorporation in the growth curve experiment, results in Fig. 1 showed gradual increases in cyanobacteria cell dry weight as the experiment proceeded, where the highest weights were recorded with all isolates at the log phase between 14 and 35 days of the incubation period. Three isolates showed the highest means of cyanobacterial cell dry weight (0.318, 0.268, and 0.199 gm / 100 ml culture) compared to other isolates (from 0.187 until 0.064 gm / 100 ml culture) at the end of the experiment.

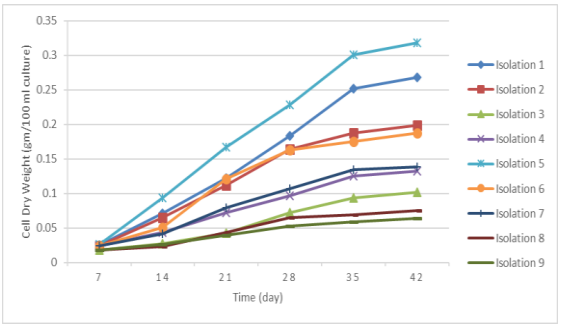

Fig. 1. Growth curve experiment - Dry weight of cyanobacterial isolates.

On the other hand, N_2-fixation showed a gradual increase with proceeding into the experiment and the same three isolates showed relatively higher mean total nitrogen (17.6, 16.8, 16.11 mg N_2 / 100 ml culture) compared to other isolates (from 12.8 until 5 mg N_2/100 ml culture) as shown in Fig. 2.

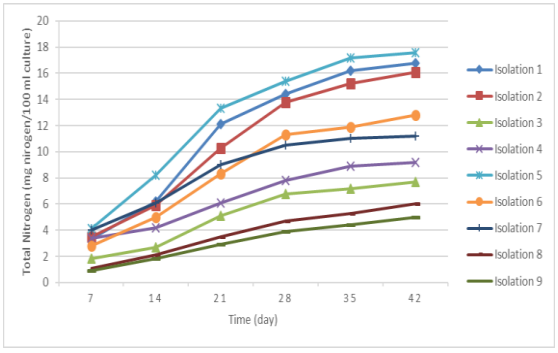

Fig. 2. Growth curve experiment - Nitrogen fixation by cyanobacterial isolates.

These three isolates were further used in the following experiments and were subsequently identified. The highest N_2-fixing capacity was noted up to about 4 weeks, so that, in vitro experiment was conducted for 30 days.

Growth features of the most efficient three isolates:

The most efficient cyanobacterial isolates were examined for their morphological and cultural characteristic (Holt, 1994):

1) Isolate 1: culture showed dark green trichomes with no ramifications. They were uniseriate, single, aggregated, and showed neither polarity nor tapering. No sheath was formed. Trichomes were composed of three sizes and shapes of cells; a) barrel cells (5-6 x 5.5-7µ); b) granular, ellipsoidal cells (5 x 5.5-7.5µm); c) yellowish-brown rounded cells of 8.9µ in diameter. Few heterocysts were observed. They were of single occurrence with 2 positions, intercalary and terminal. There for, this isolate was found to be *Nostoc muscorum*.
2) Isolates 2 and 3: gave a localized growth with a fibrous appearance on the agar surface. Growth was opaque and green in color without coloration of the medium. The liquid medium showed green homogenous growth.

- Isolate 2: Microscopic examination revealed that trichomes were not-ramified, uniseriate, singly arranged wavy without tapering, and were not enveloped within a sheath. Vegetative cells were short angular (4-4.3 x 2-3.3 µm). Most apical cells were pointed and heterocysts were of single occurrence and mostly produced intercalary. They were of barrel shape (5-5.5 x 6.5-7.1µm). No spores were observed. Hormogonia have the same width as the filaments. There for, this isolate was found to be *Anabaena cylindrica*.
- Isolate 3: olivaceous green flexuous trichomes flexuous, blue-green, loosely arranged, constricted at the cross-walls, cylindrical, generally elongated cells 3.6-4.1µ in width, and 4.1-5 (-8)µ in length, with rounded apical cell. Heterocysts were terminal or intercalary singles and spherical, 4.2-5 (-6.1)µ in width and 4.2-5.4 (-7.2)µ in length. Akinete showed long chains away from heterocysts, ellipsoidal or oblong, with 5.0-6.3 (-7.2)µ in width and 5-8.1(-12)µ in length, thin endospores, thick exospores, and were separated from endospores. There for, this isolate was found to be *Anabaena variabilis*.

In vitro experiment:

a) Effect of carbofuran as a sole carbon and nitrogen source on cyanobacteria:

Results in Tables 1 and 2 showed that there is an obvious increase of both cell dry weight and N_2 fixation with increasing the incubation period. On the other hand, at the same incubation period, there was an increase in both cell dry weight and N_2 fixation with increasing carbofuran concentration from 0 until 80 ppm, then there was a decline at the concentration of 120 ppm. The highest cell dry weight and total nitrogen were noted with *Nostoc muscorum* (alone) at all carbofuran concentrations and all incubation periods (0.176 g / 100 ml culture, 13.53 mg N_2/100 ml culture at carbofuran 80 ppm and 30-day incubation period, respectively). These results were only preceded with culturing a mixture of the three cyanobacterial isolates together (0.195 g / 100 ml culture, 14.9 mg N_2/100 ml culture at carbofuran 80 ppm, and 30-day incubation period, respectively). It was noted that, there were significant differences between cyanobacterial isolates.

Table 1. Effect of carbofuran concentration (ppm) on cell dry weight (g/100 ml culture) of cyanobacterial isolates

Incubation Period(day)	Carbofuran conc. (ppm)	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
10	0	0.025^{ef}	0.021^{g}	0.022^{h}	0.032^{f}
	40	0.056^{c}	0.036^{e}	0.042e	0.074d
	80	0.065^{c}	0.043^{d}	0.044e	0.078^{d}
	120	0.022^{f}	0.021^{g}	0.021^{h}	0.029^{f}
20	0	0.039^{d}	0.035^{e}	0.037^{f}	0.067^{e}
	40	0.090^{b}	0.054^{b}	0.070^{c}	0.107c
	80	0.094^{b}	0.055^{b}	0.073^{bc}	0.132b
	120	0.033^{de}	0.026^{f}	0.029^{g}	0.065^{e}
30	0	0.061^{c}	0.049^{c}	0.054^{d}	0.082^{d}
	40	0.171^{a}	0.074^{a}	0.076^{b}	0.017^{g}
	80	0.176^{a}	0.075^{a}	0.085^{a}	0.195^{a}
	120	0.059^{c}	0.048^{c}	0.053^{d}	0.079^{d}
LSD 0.05 =		0.008	0.004	0.004	0.006

Table 2. Effect of carbofuran concentration (ppm) on nitrogen fixation (mg N_2/100 ml culture) by cyanobacterial isolates

Incubation Period(day)	Carbofuran conc. (ppm)	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
10	0	2.82^{j}	1.92^{i}	2.51^{g}	3.22^{j}
	40	5.56^{i}	2.4^{h}	3.32^{f}	5.2^{i}
	80	5.98^{h}	3.84^{g}	4.87^{e}	6.22^{h}
	120	2.81^{j}	1.62^{j}	2.43^{g}	3.1^{j}
20	0	9.1^{f}	4.22^{f}	6.43^{d}	9.54^{f}
	40	10.12^{e}	5.12^{e}	7.74^{c}	10.32^{e}
	80	10.68^{d}	6.33^{d}	8.21^{c}	11.2^{d}
	120	8.13^{g}	3.98^{fg}	5.98^{d}	9.22^{g}
30	0	11.2^{c}	7.24^{c}	8.98^{b}	12.83^{c}
	40	12.38^{b}	8.11^{b}	9.84^{a}	13.22^{b}
	80	13.53^{a}	9.7^{a}	10.23^{a}	14.9^{a}
	120	10.54^{d}	6.34^{d}	8.16^{c}	10.33^{e}
LSD 0.05 =		0.371	0.270	0.600	0.231

b) Effect of penoxsulam as a sole carbon and nitrogen source on cyanobacteria:

Results in Tables 3 and 4 showed that there is an obvious increase of both cell dry weight and N_2 fixation with increasing incubation period. On the other hand, at the same incubation period, there was an increase in both cell dry weight and N_2 fixation with increasing penoxsulam concentration from 0 until 40 ppm then declines at 80 ppm with both *Nostoc muscorum* and the mixture. While Anabaena species showed a progressive decrease with increasing penoxsulam concentrations. Worthy to mention that penoxsulam at 120 ppm concentration resulted in a complete absence of cyanobacterial growth. Consequently, there was an absence of N_2 fixation at all incubation periods. The highest cell dry weight and total nitrogen were noted with *Nostoc muscorum* (alone) at penoxsulam concentrations (0-80 ppm) and all incubation periods (0.064 g / 100 culture, 6 mg N_2/100 ml culture at penoxsulam 40 ppm, and 30-day incubation period). These results were only preceded with culturing a mixture of the three cyanobacterial isolates together (0.094 g / 100 ml culture, 8.64 mg N_2 /100 ml culture at penoxsulam 40 ppm, and 30-day incubation period). It was noted that, there were significant differences between cyanobacterial isolates.

Table 3. Effect of penoxsulam concentration (ppm) on cell dry weight (g/100 ml culture) of cyanobacterial isolates

Incubation Period(day)	Penoxsulam conc. (ppm)	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
10	0	0.025^{fg}	0.021^{fg}	0.022^{e}	0.032^{e}
	40	0.026^{f}	0.019^{g}	0.021^{e}	0.034^{e}
	80	0.023^{g}	0.012^{h}	0.019^{e}	0.027^{e}
	120	—	—	—	—
20	0	0.039^{d}	0.035^{d}	0.037^{c}	0.067^{c}
	40	0.041^{d}	0.028^{e}	0.031^{d}	0.071^{c}
	80	0.034^{e}	0.024^{f}	0.028^{d}	0.049^{d}
	120	—	—	—	—
30	0	0.061^{b}	0.049^{a}	0.054^{a}	0.082^{b}
	40	0.064^{a}	0.045^{b}	0.049^{b}	0.094^{a}
	80	0.056^{c}	0.041^{c}	0.046^{b}	0.067^{c}
	120	—	—	—	—
LSD 0.05 =		0.002	0.003	0.003	0.005

Table 4. Effect of penoxsulam concentration (ppm) on nitrogen fixation (mg N_2/100 ml culture) by cyanobacterial isolates

Incubation Period(day)	Penoxsulam conc. (ppm)	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
10	0	1.82^{e}	1.72^{f}	1.79^{e}	1.92^{f}
	40	1.85^{e}	1.24^{g}	1.72^{e}	2.32^{e}
	80	1.79^{e}	0.98^{h}	1.22^{f}	1.85^{f}
	120	—	—	—	—
20	0	2.86^{c}	2.3^{c}	2.55^{c}	5.98^{c}
	40	2.91^{c}	2.11^{d}	2.17^{d}	6.88^{b}
	80	2.36^{d}	1.78^{e}	1.8^{e}	3.78^{d}
	120	—	—	—	—
30	0	5.94^{a}	4.89^{a}	5.21^{a}	7.52^{a}
	40	6.0^{a}	4.43^{a}	5.0^{a}	8.64^{a}
	80	5.33^{b}	3.91^{b}	4.66^{b}	5.98^{c}
	120	—	—	—	—
LSD 0.05 =		0.066	0.056	0.066	0.110

Field experiment:

a) Effect of cyanobacterial inoculation on rice plant height and number of panicles per hill under different nitrogen levels and different pesticides:

Results in Tables 5 and 6 showed that both plant height and the number of panicles/hill tended to increase with increasing urea level from zero until 69 kg N_2/fed in the absence or presence of pesticides and cyanobacterial isolates. Generally, results of plant height and number of panicles/hill were the highest with using carbofuran.

Table 5. Effect of cyanobacteria inoculation on rice (EHR1) plant height (cm) under different nitrogen levels and different pesticides

Pesticide	Urea (kg nitrogen /fed)	Nil	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
Nil	0	77.30^{g}	83.17^{f}	81.15^{h}	81.50^{h}	84.25^{e}
	35	81.20^{e}	85.00^{e}	84.15^{f}	83.90^{f}	86.17^{d}
	69	84.12^{c}	88.39^{d}	86.05^{d}	86.19^{e}	90.02^{c}
Carbofuran	0	83.10^{d}	88.15^{d}	85.17^{e}	86.21^{e}	88.92^{c}
	35	85.15^{b}	92.19^{c}	88.25^{c}	88.72^{c}	93.20^{b}
	69	86.21^{a}	97.92^{a}	90.48^{a}	94.55^{a}	99.12^{a}
Penoxsulam	0	80.35^{f}	84.85^{e}	82.25^{g}	83.14^{g}	85.36^{d}
	35	83.15^{d}	88.80^{d}	86.00^{d}	86.85^{d}	88.98^{c}
	69	86.19^{a}	97.03^{b}	90.12^{b}	94.17^{b}	98.53^{a}
LSD 0.05 =		0.171	0.641	0.149	0.198	1.069

Table 6. Effect of cyanobacteria inoculation on rice (EHR1) number of panicles / hill under different nitrogen levels and different pesticides

Pesticide	Urea(kg nitrogen /fed)	Nil	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
Nil	0	12^{c}	16^{a}	14^{b}	15^{a}	18.5^{c}
	35	13.5^{abc}	16.5^{a}	14.5^{ab}	16^{a}	19.5^{bc}
	69	14^{abc}	17^{a}	15.5^{ab}	17^{a}	21^{abc}
Carbofuran	0	13^{bc}	18^{a}	14.5^{ab}	15.5^{a}	19^{c}
	35	14^{abc}	19^{a}	15.5^{ab}	17^{a}	20.5^{abc}
	69	15.5^{a}	19.5^{a}	17^{a}	17.5^{a}	22^{ab}
Penoxsulam	0	13.2^{abc}	16.7^{a}	14^{b}	15.3^{a}	20^{abc}
	35	14^{abc}	17^{a}	15^{ab}	16.5^{a}	22^{ab}
	69	15^{ab}	18^{a}	16^{ab}	17.5^{a}	22.5^{a}
LSD 0.05 =		1.514	2.277	1.762	1.693	1.715

A mixture of the three cyanobacterial isolates resulted in the highest value of plant height (99.12 cm), while *Nostoc muscorum* resulted in the highest value (97.92 cm) as a single isolate when using the recommended dose of urea and application of carbofuran. Also, a mixture of the three cyanobacterial isolates resulted in the highest number of panicles/hill (22.5), while *Nostoc muscorum* resulted in the highest value (19.5) as a single isolate when using the recommended dose of urea and application of carbofuran. It was noted that, there were significant differences between cyanobacterial isolates.

b) Effect of cyanobacterial inoculation on rice 1000-grain weight under different nitrogen levels and different pesticides:

Results in Table 7 showed that values of rice 1000-grain weight were the least in absence of both urea and pesticides.

Table 7. Effect of cyanobacteria inoculation on rice (EHR1) 1000-grain weight (g) under different nitrogen levels and different pesticides

Pesticide	Urea(kg nitrogen /fed)	Nil	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
Nil	0	22.3^{d}	26.58^{b}	25.22^{d}	26^{a}	27^{c}
	35	26.85^{ab}	27.72^{ab}	26.86^{b}	27^{a}	27.88^{a}
	69	27.28^{a}	27.87^{a}	27.3^{a}	27.7^{a}	27.9^{a}
Carbofuran	0	26.25^{b}	26.81^{ab}	26.69^{b}	26.72^{a}	27.2^{c}
	35	27.25^{a}	27.8^{a}	27.3^{a}	27.5^{a}	27.8^{a}
	69	26.8^{ab}	27.05^{ab}	26.82^{b}	26.95^{a}	27.3^{bc}
Penoxsulam	0	24.12^{c}	27.59^{ab}	27.2^{a}	27.38^{a}	27.63^{ab}
	35	27.15^{a}	27.72^{ab}	27.22^{a}	27.42^{a}	27.68^{ab}
	69	26.25^{b}	26.78^{ab}	26.29^{c}	26.32^{a}	27.09^{c}
LSD 0.05 =		0.464	0.751	0.282	2.343	0.313

These values changed to be the highest with rising urea concentration from zero until 35 kg N_2/fed before decreasing with rising urea concentration up to 69 kg N_2/fed, in presence of pesticides. With the application of pesticides with urea, the values tended to be lower compared to values with urea alone (in the same concentrations). Also, the application of pesticides in absence of urea tended to increase the values of 1000-grain weight compared to nil pesticides. The mixture of the three cyanobacterial isolates resulted in the highest value of 1000-grain weight (27.9 g), while *Nostoc muscorum* resulted in the highest value (27.87 g) as a single isolate when using half the recommended dose of urea and absence of pesticides. On the other hand, the lack of any cyanobacteria supplementation showed the lowest results. It was noted that, there were significant differences between cyanobacterial isolates.

c) Effect of cyanobacterial inoculation on rice grain yield under different nitrogen levels and different pesticides:

Results in Table 8 showed that the values of rice grain yield tended to increase with rising urea concentration from zero until 69 kg N_2/fed. Generally, the highest results were noted with the application of carbofuran, while nil pesticides resulted in the lowest values. It was noticed that *Nostoc muscorum* alone showed the highest values in absence of urea, while the mixture of the three cyanobacterial isolates showed the highest values in the presence of urea. The mixture of the three cyanobacterial isolates resulted in the highest value of grain yield (5.8 ton/fed), while *Nostoc muscorum* resulted in the highest value (5.61 ton/fed) as a single isolate when using the recommended dose of urea and application of carbofuran, while the lack of any cyanobacteria supplementation showed the lowest results. It was noted that, there were significant differences between cyanobacterial isolates.

Table 8. Effect of cyanobacteria inoculation on rice (EHR1) grain yield (ton / fed) under different nitrogen levels and different pesticides

Pesticide	Urea(kg nitrogen /fed)	Nil	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
Nil	0	2.1^{e}	3.2^{i}	2.51^{f}	3.10^{f}	3.09^{g}
	35	3.01^{d}	4.1^{g}	3.17^{e}	3.82^{d}	3.98^{f}
	69	3.75^{b}	4.51^{e}	3.8^{c}	4.00^{c}	4.60^{e}
Carbofuran	0	2.25^{e}	4.27^{f}	3.54^{d}	3.5^{e}	4.01^{f}
	35	3.4^{c}	5.45^{b}	4.34^{b}	4.55^{b}	5.59^{b}
	69	4.6^{a}	5.61^{a}	4.68^{a}	4.85^{a}	5.80^{a}
Penoxsulam	0	2.11^{e}	3.78^{h}	3.00^{e}	3.22^{f}	3.18^{g}
	35	3.35^{c}	4.74^{d}	4.22^{b}	4.45^{b}	4.89^{d}
	69	4.5^{a}	5.02^{c}	4.53^{a}	4.81^{a}	5.23^{c}
LSD 0.05 =		0.164	0.117	0.184	0.174	0.140

d) Effect of duration on the cyanobacterial most probable number in a rice field under the influence of cyanobacteria supplementation, urea concentration, and pesticide application:

Results in Table 9 showed that the values of cyanobacterial most probable number in the rice field with the application of pesticides tended to increase with rising urea concentration from zero until 35 kg N_2/fed, then decreased, while these values tended to increase with increasing urea concentration up to 69 kg N_2/fed in absence of pesticides. The mixture of the three cyanobacterial isolates showed the highest values, while *Nostoc muscorum* showed the highest values as a single isolate, with a urea concentration of 35 kg N_2/fed. Moreover, the values of cyanobacterial most probable number tended to increase up to 80 days, then they started to decline, in all treatments, except for *Nostoc muscorum*, *Anabaena variabilis*, and the mixture of the three cyanobacterial isolates, with carbofuran application, which increased up to 40 days only, then they started to decline.

Table 9. Effect of duration on cyanobacterial most probable number (CFUx10^3/g) in rice field influenced by cyanobacteria inoculation, urea concentration, and pesticide application.

Pesticide	*Cyano-bacterial Isolates*	Nil	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture	Nil	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture	Nil	*Nostoc moscorum*	*Anabeana cylindrica*	*Anabeana variabilis*	Mixture
	Duration after inoculation(day)	Urea (kg nitrogen / fed)														
		0					35					69				
Nil	0	0.018^{e}	0.039^{e}	0.032^{g}	0.033^{e}	0.041^{f}	0.016^{h}	0.041^{e}	0.033^{i}	0.035^{f}	0.044^{i}	0.019^{h}	0.038^{h}	0.036^{h}	0.031^{h}	0.04^{f}
	40	0.066^{ab}	0.156^{bc}	0.123^{c}	0.136^{b}	0.19^{b}	0.087^{c}	0.168^{cd}	0.142^{e}	0.149^{cd}	0.204^{fg}	0.144^{b}	0.203^{bc}	0.183^{b}	0.193^{b}	0.253^{c}
	80	0.068^{ab}	0.16^{abc}	0.13^{bc}	0.14^{b}	0.203^{b}	0.092^{c}	0.175^{cd}	0.151^{d}	0.157^{c}	0.216^{ef}	0.15^{a}	0.233^{a}	0.193^{a}	0.203^{a}	0.333^{a}
	120	0.047^{d}	0.145^{bc}	0.098^{d}	0.103^{c}	0.165^{c}	0.063^{e}	0.154^{d}	0.112^{f}	0.126^{de}	0.185^{h}	0.119^{c}	0.209^{b}	0.163^{c}	0.171^{c}	0.241^{c}
Carbofuran	0	0.017^{e}	0.035^{e}	0.033^{g}	0.034^{e}	0.043^{f}	0.018^{h}	0.041^{e}	0.035^{i}	0.033^{f}	0.044^{i}	0.02^{h}	0.039^{h}	0.031^{h}	0.032^{h}	0.045^{f}
	40	0.062^{b}	0.176^{a}	0.136^{b}	0.156^{a}	0.283^{a}	0.08^{d}	0.254^{a}	0.193^{b}	0.23^{a}	0.41^{a}	0.069^{ef}	0.194^{bc}	0.147^{d}	0.171^{c}	0.298^{b}
	80	0.072^{a}	0.163^{ab}	0.17^{a}	0.13^{b}	0.17^{c}	0.17^{a}	0.237^{a}	0.203^{a}	0.218^{a}	0.333^{b}	0.081^{d}	0.182^{cd}	0.192^{a}	0.158^{d}	0.277^{b}
	120	0.049^{cd}	0.151^{bc}	0.134^{bc}	0.105^{c}	0.167^{c}	0.123^{b}	0.214^{b}	0.178^{c}	0.191^{b}	0.274^{c}	0.073^{e}	0.165^{de}	0.166^{c}	0.131^{e}	0.234^{c}
Penoxsulam	0	0.018^{e}	0.037^{e}	0.033^{g}	0.035^{e}	0.04^{f}	0.021^{h}	0.037^{e}	0.03^{i}	0.035^{f}	0.045^{i}	0.019^{h}	0.041^{h}	0.034^{h}	0.036^{h}	0.043^{f}
	40	0.023^{e}	0.111^{d}	0.068^{f}	0.099^{c}	0.143^{d}	0.051^{f}	0.156^{d}	0.099^{g}	0.13^{de}	0.226^{e}	0.037^{g}	0.125^{g}	0.078^{g}	0.112^{f}	0.174^{de}
	80	0.054^{c}	0.14^{c}	0.13^{bc}	0.11^{c}	0.15^{cd}	0.076^{d}	0.19^{c}	0.176^{c}	0.163^{c}	0.257^{d}	0.067^{f}	0.152^{ef}	0.137^{e}	0.125^{e}	0.185^{d}
	120	0.021^{e}	0.123^{d}	0.081^{e}	0.072^{d}	0.119^{e}	0.042^{g}	0.176^{cd}	0.084^{h}	0.112^{e}	0.195^{gh}	0.034^{g}	0.14^{fg}	0.101^{f}	0.097^{g}	0.159^{e}
LSD 0.05 =		0.005	0.014	0.008	0.014	0.014	0.005	0.018	0.005	0.019	0.015	0.004	0.017	0.005	0.008	0.021

Discussion

Many cyanobacterial species live in the rice field ecosystem. They mainly belong to the Nostoc, Anabaena, Tolypothrix, Aulosira, Cylindrospermum, Scytonema, Westiellopsis, and several other genera (Nayak *et al.*, 2004). Cyanobacteria play an important role in maintaining nutrient availability, porosity, soil pH, and reduction in salinity of the soil. Also, rice fields are considered a natural habitat for cyanobacteria to perform different functions including N_2 fixation and photosynthesis, thus help in sustaining various physicochemical processes (Wilson *et al.*, 2006 and Singh, 2014). Moreover, cyanobacteria can remove the toxicity of pesticides and increase plant tolerance to stress. There are many detoxification mechanisms available in the microorganisms used in phytoremediation by different pathways, such as those acting on nitrile and halogen residues or degrading the aromatic ring (Tetard-Jones and Edwards, 2015).

Previous studies showed that only 0.1% of the tested pesticides reach their target, while the rest remains in the soil, thus affecting the microorganisms inhabiting the soil and affect their activities (Singh and Singh, 2005, Dash and Debnath, 2006 and Pal, 2006). At a certain level, cyanobacteria have pollutant-degrading potential. Under mild stress conditions, cyanobacteria can undergo short-term adaptation. While long-term stresses can alter the photosynthetic apparatus (Singh et al., 2018). So, in this experiment, we studied the effect of cyanobacteria supplementation on rice plant growth parameters and phytoremediation of the applied pesticides.

We designed our study to start with a growth curve experiment to determine two important points, the duration required to reach the highest N_2-fixing capacity (about 4 weeks) and the most effective strains in N_2 fixation (Nostoc and Anabaena). This step was important from the economic point of view to use the least resources and produce the highest results.

To study the effect of the pesticides (carbofuran and penoxsulam) on cyanobacterial growth, we designed an in vitro experiment where we replaced both carbon and nitrogen sources with different concentrations from the pesticides (individual) and observed the effect on cell growth, the fixed nitrogen, and the differential activity of different cyanobacterial isolates. Generally, *Nostoc muscorum* showed the highest results as a single isolate, while the mixture of the three isolates showed the highest results at all, probably due to the synergistic effect between the isolates.

Within limit, both pesticides showed stimulation of both cell growth and N_2 fixation. Beyond these concentrations, these values tended to decline due to possible inhibitory or toxic effects of these pesticides, except for Anabaena species which showed a decline with any penoxsulam concentration probably due to high sensitivity and intolerance to its effect. According to a previous study, the differences in the effect of the pesticides on cyanobacteria are dependent on the application dose and type in addition to the cyanobacterial species. For example, *Anabaena variabilis* tolerates the herbicides arozin, alachlor, butachlor, and 2,4-dichlorophenoxyacetate, but with increasing the herbicides dose *Nostoc punctiforme*, *Nostoc calcicola*, *Anabaena variabilis*, *Gloeocapsa sp.* and *Aphanocapsa sp.* showed a gradual decline of growth, while arozin was more toxic to cyanobacterial growth (Singh and Datta, 2005).

Also, previous studies on the effect of pesticide concentrations on cyanobacterial growth and activity showed that this effect is dose-dependent. The low doses increased the photosynthetic pigments, while the high doses reduced the cyanobacterial growth, photosynthetic pigments, and enzymatic activities as well as enhancing the oxidative stress in the cyanobacterial strain in both Nostoc and Anabaena genera (Kumar *et al.*, 2008 and Kumar *et al.*, 2012).

Worthy to mention that penoxsulam, a potent herbicide, showed inhibitory effect at lower concentrations compared to carbofuran (insecticide). This could be understood if we knew that cyanobacteria share many physiological properties with higher plants which form the target site of different herbicidal action, thus are more sensitive to herbicides (Whitton, 2000).

Then we performed a field experiment to study the effect of cyanobacteria supplementation, pesticide application, and different concentrations of urea on rice plant growth parameters and cyanobacteria most probable number over time starting from transplantation until harvesting. Generally, *Nostoc muscorum* showed the highest results as a single isolate and the mixture of the three isolates showed the highest results at all. These results are similar to those from the in vitro experiment. The use of the

mixture increased the grain yield by 22.7 – 26.1% in case of application of carbofuran and recommended dose of urea compared to the nil cyanobacteria (control). While these values ranged between 2-3.4% in favor of the mixture compared to *Nostoc muscorum* alone under the same conditions. The higher values with the mixture are in line with the results discussed in a previous study which reported that the use of blue-green algae mixture increased the grain yield by 23.5 and 16. 9 % over the uninoculated control during wet and dry seasons respectively (Das et al., 2015).

The application of pesticides showed an added effect on the vegetative growth in the form of increasing plant height and the number of panicles/hills compared to the control, with carbofuran showing higher values. These results could be attributed to the effective insect and weed control which are in line with a previous study that concluded that the high efficiency of chemical weed control in controlling weeds gave the chance to the good optimum vegetative growth of rice resulting in increasing yield and its components (Singh *et al.*, 2006). Despite the lower results of penoxsulam compared to carbofuran, penoxsulam showed higher values compared to nil pesticides similar to the results of a previous study which reported increased rice dry weight, number of panicles/m^2, panicle weight, and grain yield of rice in the two growing seasons (Abdelgawad and Abd El-Naby, 2018).

Also, the higher results of carbofuran (nematocide) compared to penoxsulam (herbicide) are comparable to a previous study that showed a superior effect of insecticides over herbicides while a mixture of both still showing better results over the herbicide alone (Das *et al.*, 2015).

In presence of cyanobacteria supplementation and pesticide application, the grain weight tended to decrease with using the recommended dose of urea, possibly due to the decline in cyanobacterial most probable number resulting from the increased toxic effect of pesticides with increasing urea concentration. These results are similar to those reported in a previous study which concluded that low urea doses were growth stimulatory to cyanobacteria, while moderate to high concentrations (> 50 ppm) had toxicity enhancing effect along with carbofuran (Padhy *et al.*, 2014). On the other hand, the use of the recommended dose of urea gave the highest grain yield, possibly due to increasing the number of panicles/hill and consequently the number of grains at the expense of their weight. This could be the result of carbofuran exerting promoting effects on cyanobacterial growth and N_2 fixation (Das *et al.*, 2015). Also, the recommended dose of urea in a combination of the fixed nitrogen together enhanced the vegetative growth probably at the expense of the grain weight. It was reported that the amount of N_2 fixation by independent cyanobacteria can as high as 29 kg N_2/hectare/year (DeLuca *et al.*, 2013). Moreover, the variations in the amount of N_2 fixation in the rice fields depend upon various factors including cyanobacterial growth, climate, biological and non-biological factors as well as on the physicochemical properties of sampling sites (Singh *et al.*, 2018).

If using the recommended dose of urea resulted in the highest grain yield while half the recommended dose resulted in the highest grain weight, both in presence of pesticides, we suppose that the use of half the recommended dose of urea in presence of pesticides and compensating the decline of nitrogen content with increasing cyanobacteria population will result in the highest grain weight and yield together. This assumption can be supported by the results reported by a previous study which showed higher grain yield with moderate urea concentration (80 kg/hectare) in combination with cyanobacteria or azola in comparison to the same or higher concentrations alone or lower concentrations in combination with cyanobacteria or azola (Mandal et al., 2011).

Due to the enhancing effect of carbofuran on vegetative growth, the cyanobacteria were deprived from exposure to sunlight earlier, resulting in an earlier drop of the cyanobacterial count and function in most of the species as described by Ma et al. (2015) who reported the highest dry weights, dry matter, protein, and chlorophyll contents under 90 μmol m^{-2} s^{-1} than under other intensities (both higher and lower) (Ma *et al.*, 2015).

This study is limited by the lack of evaluation of the effect of cyanobacteria on the amount of fixed nitrogen in the soil, evaluation of pesticides consumptions and their byproducts in the soil, application of pesticides at different duration from rice transplantation, the use of different inoculum sizes of cyanobacteria, and the mixed-use of both carbofuran and penoxsulam.

CONCLUSION

Within limit, the use of pesticides plays an important role in controlling both weeds and insects in addition to enhancing cyanobacterial growth and activity resulting in better growth parameters of rice plant. Also, the use of lower doses of urea seemed to be more economic and effective in attaining better grain weight and lower the toxic effect of pesticides.

Conflict of interest

The authors declare that they have no conflict interests.

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نمو عزلات السيانوبكتيريا معملياً وتأثيرها على زراعة محصول الأرز فى الحقول المعاملة بالمبيدات

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في هذه الدراسة تم إجراء تجربة بمحطة بحوث سخا بمحافظة كفر الشيخ بمصر خلال موسم 2018-2019 لعزل السيانوبكتيريا السائدة فى عينات تربة مأخوذة من حقول الأرز الملوثة بمبيدى (كاربوفيوران وبينوكسولام). وتم الحصول علي تسع عزلات من السيانوبكتيريا وتنقيتها، وتم إختبار هذه العزلات لاختيار أفضلها من حيث النمو وكفاءة تثبيتها للنيتروجين ومن ثم استخدامها في حقول الأرز لدراسة تأثيرها على المحصول ومكوناته تحت تأثير معاملات مختلفة من اليوريا. أظهرت تجربة منحنى النمو على هذه العزلات أن ثلاثة منها كانت الأفضل من حيث النمو وكفاءة تثبيت النيتروجين ، وأظهرت نتائج تعريف هذه العزلات أنها (*Nostoc muscorum* و *Anabaena Cylindrica* و *Anabaena variabilis*). وفى تجربة دراسة تأثير تركيز المبيدات على العزلات معملياً، أظهر كلا المبيدين تأثيراً محفزاً لنمو الخلايا وتثبيت النيتروجين عند التركيزات المنخفضة. وتميل هذه القيم إلى الإنخفاض عند التركيزات الأعلي بسبب التأثير المثبط أو السام لهذه المبيدات. بشكل عام ، كانت عزلات السيانوبكتيريا أقل تحملاً للبينوكسولام وتم تثبيطها بتركيزات منخفضة منه مقارنة بالكاربوفيوران. في تجربة حقلية وفى حالة استخدام مبيد الكاربوفيوران، نتج أعلي محصول من الأرز عند استخدام عزلات السيانوبكتيريا مع الجرعة الموصى بها من اليوريا، بينما تم الحصول على أعلى وزن للـ1000 حبة بنصف الجرعة الموصى بها من اليوريا. علاوة على ذلك كانت أفضل النتائج عند استخدام خليط من أنواع السيانوبكتيريا بدلاً من استخدام نوع واحد.