EFFECT OF SPRAYING BLUE GREEN ALGAE (CYANOBACTERIA) EXTRACTS ON HYBRID RICE SEED PRODUCTION

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ABSTRACT: Two isolates of cyanobacteria (*Anabaena oryzae*, *Nosto centophytum*) were isolated from agricultural wastewater from the experimental farm of Sakha Research Station, Sakha, Kafr EL Sheikh, Egypt for testing their ability to fix atmospheric nitrogen and produce hormones and its effect on hybrid rice seed production by spraying the isolates extract and their mixture on the rice plants. Isolates were varied in their capacity in the extracellular-nitrogen, Intracellular secreted, produce hormones and dry weight of the cyanobacteria isolates (mg/100 ml-culture) where the lowest content was for isolate *Anabaena oryzae* and the highest was for *Nostoc centophytum* and mixture. The aim of the present investigation is to identify the effect of cyanobacteria extract isolated from Egyptian waste water on hybrid rice seed production. The used cytoplasmic male sterile lines were IR69625A and G46A and the restorer line was G.178. Application of mixture between *Anabaena oryzae* and *Nostoc centophytum* produced the highest values in most studied characters such as seed yield in both seasons.

Key words: hybrid rice, CMS, cyanobacteria, Anabaena oryzae, Nostoc centophytum, seed yield

INTRODUCTION

Rice is an important food crop and the main food source for more than half of the global population. Rice is cultivated in Egypt over an area of about 660 thousand hectares, with an annual production of about 4.6 million tons of paddy, with average productivity of 10 tons per hectare (El Mowfi *et al.,* 2016). Hybrid rice production is an innovative technology to increase further rice productivity, leading to food security and the reduction of poverty in Egypt. This technology can be used to increase the current yield in rice, where the yield levels of the conventional cultivars have stabilized and reported yield advantage of 15–20% over conventional varieties (El Mowfi *et al.,* 2016, Negrao *et al.,* 2008). The heterosis advantage of hybrids may be expressed by superiority over inbred varieties in vigor, number of productive tillers, panicle size, number of spikelets panicle^{-1}, and seed yield.

Hybrid varieties are generally developed by the three lines and the two lines breeding methods. Meanwhile, in the three line method, the cytoplasmic genetic male sterility system is a three lines system that is involved and needed a cytoplasmic male sterile (CMS) source, a maintainer (M), and restorer (R) line is extensively being used in rice hybrids production. Several factors influence the multiplication cytoplasmic male sterile (CMS) and hybrid seed production, such

as seeding time, field condition, planting pattern, weather conditions at flowering, synchronization of flowering between the parental lines, supplementary pollination techniques. Application of GA_3 which is an effective plant growth hormone which stimulates the cells elongation. GA_3 is an key to win high seed yield in hybrid rice seed production. It can increase panicle exertion from the flag leaf, increase the rate of stigma exertion, adjust plant hight, increase the duration of floret opening and make the later branches taller and productive (Yuan 2007, 2010 and Xie *et al.,*2011). It is necessary to identify the appropriate concentration of GA_3 for some hybrid rice varieties. Therefore it is necessary to conduct a research to study the effect of cyanobacteria concentration on hybrid rice seed production by using it as a source of GA_3 hormone.

Used cyanobacteia harvested near the shore in their cultures, both directly or after composting, observing positive effects in soil fertilization. Since this period, algal biomass has been extensively used in agriculture, but in the 20th century, products obtained from cyanobacteia extracts have attracted the attention of farmers worldwide. In fact, a wide variety of biologicallyactive compounds extracted from cyanobacteria (e.g., phenols, terpenoids, free fatty acids FFAs, polysaccharides, and carotenoids) has demonstrated to have promising effects in crops' production. According to the literature, algal metabolites can play an important role in soil decontamination and fertilization; plant protection against biotic and a biotic stress factors; and plant development. In addition, microalgae and cyanobacteria also present phytohormones, which are known for their activity as plant-growth promoters. Taking into account their potential benefits for the development of a sustainable agriculture, both biomass and extracts from microalgae and cyanobacteria are commercially available (Gonçalves, 2021).

Hence it was found of great interest and of significant importance to point out to the dominant cyanobacteria genera, rates of growth of the different isolates as well as their capacities for nitrogen fixation and production of phytohormones (Indole 3 acetic acid and gibberellic acid) were examined. In addition, the effect of cyanobacteria prepared from the most efficient isolates, (*Anabaena oryzae*, *Nostoc centophytum* and mixture) on hybrid rice seed production in a field experiment- as well as on some growth parameters were also studied. This research was expected provide some information concerning appropriate cyanobacteria to support the process of pollination between male sterile lines with restorer line.

MATERIALS AND METHODS

Isolation, purification and identification of cyanobacteria.

The following methods were applied on agricultural waste water samples collected from the experimental farm of Sakha Research Station, Sakha, Kafr EL Sheikh, Egypt using Modified Watanabe medium (El-Nawawy *et al.,* 1958) for isolation and culturing cyanobacteria. Semi-solid medium as described by El-Ayouty and Ayyad (1972) were applied. The unialgal cultures were purified according to Pringsheim (1949). Any colored growth was selected, sub cultured and streaked several times in new agarized Watanabe medium plate. To get unialgal cultures, the previous technique was repeated many times. The identification of cyanobacteria was carried out using the following criteria:

Thallus color, thallus morphology, and dimension and size of heterocysts, vegetative and reproductive cells were put into consideration.

Selection of the most efficient N_2-fixing and geberillin cyanobacteria strain.

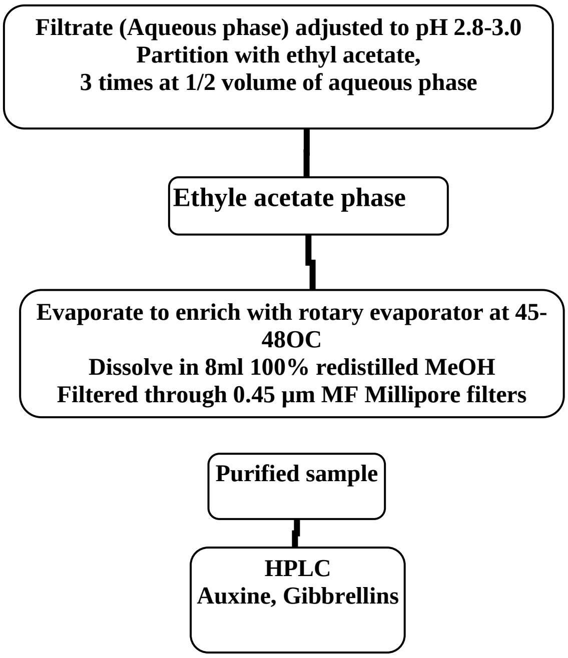
A growth curve experiment was conducted for cyanobacteria isolates to compare their growth activities and their capacities for geberillin and N_2-fixation, so as the most efficient two strains, were further used throughout the present investigation for different ecological and physiological studies.

Determination of fixed nitrogen by cyanobacteria:

Total nitrogen in the cyanobacteria were determined using the micro-kjeldahl method according to Jackson (1973). Results were expressed as mg nitrogen/100 ml culture.

Determination of phytohormones.

Flasks containing cyanobacterial isolates were incubated in the growth chamber under continuous light (2500 lux) for 21 days. Separation and determination of phytohormones (auxin and geberillin) were carried out by gas liquid chromatography in Al-Azhar university (the Regional Center for Mycology and Biotechnology) HPLC analysis was performed on GBC- germey by winChrome Chromatography Ver. 1.3, which equipped with a GBC U.V/vis Detector and Hypercarb (C18, Sum 100x4.6 cm) the detective wavelength was 254nm flow rate of mobilephse was 7 ml/min which 85% Acent: 15% water. Method was according to Van Staden *et al.,* (1973), the procedure can be summarized as follows:



Cyanobacteria preparation extract for spray of rice according to Unyayar *et al.,* (1996)

Experimental Site and Soil Characteristics.

A field experiment was carried out at the Experimental Farm of Rice Research and Training Center, Sakha Agriculture Research Station, Kafr EL-Sheikh, Egypt, during 2019 and 2020, to study the effect of application of cyanobacteria (*Anabaena oryzae*, *Nostoc centophytum* and mixture) on hybrid rice seed production.

Design and Treatments:

The materials included the parental lines of IR69625A and G46A as female (cytoplasmic male sterile or CMS lines) and restorer line (Giza178 R) to produce hybrid seeds. IR69625A was sown on May 1st which is six days earlier than the male parent Giza 178 R (to produce the first hybrid seeds) and the male parent Giza 178 R was sown on May 1st which is sixteen days earlier than the CMS line G46A (as female parent) (to produce the

second hybrid seeds) to get synchronization of flowering. The experiments was performed in A randomized complete block design with three replications with mechanical and chemical properties experimental soil as follows in Table (1).

Field experiment:

Rice seeds at the rate of 20 kg ha^{-1} (15 kg from the CMS lines and 5 Kg from R Line) were soaked in fresh water for 24 h and then drayed and incubated for 48 h to hasten germination. The pre-germinated seeds were uniformly broadcasted in the plastic trays according to the three target sowing dates. The field was well ploughed and dry leveled and then irrigated to make the soil puddled condition. Phosphorous fertilizer as a form of mono-super phosphate (15.5% P_2O_5) at the rate of 100 kg/ha^{-1} and Zinc fertilizer as a form of zinc sulphate (24% Zn So_4) at the rate of 20 kg/ha was added before transplanting. Nitrogen as a form of urea (46% N) was applied in two splits, the first split at the rate of 165 kg/ha^{-1} as a basal and the second split at the rate of 40 kg/ha as a top dress at the panicle initiation stage.

Foliar application of cyanobacteria.

Application of cyanobacterial extract (*Anabaena oryzae* and *Nostoc centophytum* and mixture) 2.5 L/$ha^{-1}$was done in two splits. The first split consisted of 40% of the total amount applied when A and R lines were at 15-20% heading and the second spray 60% was applied when A and R lines were at 35-40% heading. Supplementary pollination serves to enhance the outcrossing rate in order to increase seed set. It was done by shaking the pollen parents (R line) with the rope. This operation was done 2-3 times in between 9 am to 11.30 am and be continued for 10-12 days during flowering.

Table (1): Physical and chemical properties of the to psoil (0–15 cm) at Sakha Research Station.

Properties	2019	2020
Clay (%)	55	55
Silt (%)	32.4	32.4
Sand (%)	12.6	12.6
Texture	Clayey	Clayey
Organic Matter	1.39	1.39
pH	8.1	8.2
Electrical Conductivity(Ec) (dS/m)	3.30	3.33
Total N (ppm)	512	518
Available P(ppm)	15.09	16.03
CO32−	--	
HCO3−	5.55	5.56
Mg2+	4.3	5
Na+	1.88	1.69
K+	16	16
Fe3+	4.55	4.55
Mn2+	3.13.5	

Data were recorded from 10 randomly selected hills excluding border rows per sub plot. Data were collected for the following growth characteristics; days to heading 50%, plant height (cm), panicle exsertion (%), panicle length (cm), duration of spikelet opening, spikelet opening angle, number of fertile panicles/m2, panicle weight (g), seed set (%), seed yield (t ha^{-1}), and harvest index (%). The crop was harvested when 80% of the grains became golden yellow in color. Grains were sun-dried and adjusted at 14% moisture content to estimate grain yield.

Panicle exertion percentage was estimated as the following formula:

Panicle exsertion % =

$$\frac{\text{Exserted panicle length (cm)}}{\text{Panicle length (cm)}} \times 100$$

Seed set percentage was calculated as the following formula:

Seed set % =

$$\frac{\text{Number of filled grains/panicle}}{\text{Total Spikelet number/panicle}} \times 100$$

The data were collected according to Standard Evaluation System of IRRI (2014) for all the studied characters. All cultural practices were followed as recommended. The data were analyzed following the ANOVA technique and the mean differences were compared by the Duncan's Multiple Range Test (Gomez and Gomez, 1984) using a statistical computer package costat.

RESULTS AND DISCUSSION

Cyanobacterial isolates:

Isolation and purification of cyanobacteria dominated in the wastewater samples collected from different sites in Kafr El- Sheikh, two isolates of cyanobacteria were isolated and tested on the basis of gibberellin production, auxin and nitrogen fixation, and the best two isolates were identified. Isolates were successfully obtained as bacterial free cyanobacteria were also culture (*Anabaena oryzae*, *Nostoc centophytum*) as the most of cyanobacteria they were associated with other microorganisms, hence, these must be purified from any contaminants, they exposed to different trials of purification. However, washing, and mercuric chloride treatments were the most effective method for obtaining cyanobacteria cultures free from bacteria, while the other methods gave some success for killing bacteria in one side and some failure in the other side, which could be lethal for cyanobacteria themselves. These isolates were examined for their morphological and cultural characteristics according to Venkataraman (1981) and Roger and Ardales (1991), in liquid and solid Modified Watanabe medium. After determination of geberillin and N_2-fixation, the most efficient two strains, were further used throughout the present investigation for different ecological and physiological studies.

Nitrogen fixation by cyanobacterial isolates.

Results in Tables (2 & 3) indicated that two isolates were varied in their capacity in the extracellular-nitrogen, Intracellular secreted and dry weight of the cyanobacteria isolates (mg/100 ml-culture) where the lowest content was for isolate *Anabaena oryzae* and the highest was for *Nostoc centophytum* and Mixture. Similar results were also found by Renuka, *et al.,* (2018) which reported that, cyanobacteria are able to fix atmospheric nitrogen (N_2), converting it into organic nitrogen. Air nitrogen fixation, and the increase in nitrogen fixation increases with increasing incubation period with an increase in growth up to forty days (El-Zawawy, 2019).

Table (2): Mean amounts of fixed-nitrogen by cyanobacteria isolates (mg N/100 ml-culture)

Cyanobacterial isolates	Culture age days								
	10			20			30		
	Intracellular	Extracellular	Total	Intracellular	Extracellular	Total	Intracellular	Extracellular	Total
Anabaena oryzae	2.31	0.32	2.63	5.37	1.12	6.49	10.17	1.66	11.83
Nostoc centophytum	3.31	0.56	3.87	6.92	2.11	9.03	12.75	2.72	15.47
Mixture	3.38	0.65	4.03	7.20	3.37	10.57	13.33	2.96	16.29

Table (3): Mean dry weight of the cyanobacteria isolates (mg/100ml-culture)

Cyanobacterial isolates	Culture age days		
	10	20	30
Anabaena oryzae	10	50	112
Nostoc centophytum	11	50	115
Mixture	14	85	141

Phytohormoes production by cyanobacteria.

The data in Table (4) show that the production of auxin and gibberellic acid increases with the incubation of cyanobacteria and increases gradually with cyanobacteria until twenty days *Anabaena oryzae* 7.90- 16.60 (ug ml-culture), *Nostoc centophytum* 9.00- 51.37 (ug ml-culture) and mixture 11.80- 60.87 (ug ml-culture) respectively then days decreases at the incubation period of thirty days *Anabaena oryzae* 7.15- 15.24 (ug ml-culture), *Nosto centophytum* 8.30-31.55 (ug ml-culture) and mixture 10.50-33.37 (ug ml-culture). The results of indol acetic acid were similar to the results of Gibberellic acid (GA_3), where the production of indol acetic acid increased during the first twenty days and then decreased after that. Similar results were also found by (El-Zawawy, 2016) which mentioned that the produced auxin increased with cynobacteria incubation and gradually increased with cyanobacteria followed various *Nostoc commune*, *Anabaena* sp*.*, *Nostoc calcicola* and *Anabaena variabilis* for gibberellins production. The inoculation with *Anabaena variabilis* gave the highest value while the lowest value was with *Nostoc commune.* The *Anabaena variabilis* , *Nostoc calcicola Anabaena* sp*.* and *Nostoc commune* gave the cytokinin. The cyanabacterial filtrates in suspensions significantly increased the IAA, GA_3 and cytokinin, Tantawy and Atef (2010). Phytohormones are considered signal molecules that are responsible for the regulation of several cellular processes in plants (Lu and Xu (2015).

Effect of spraying cyanobacteria extracts on growth characters.

Poor panicle exsertion of male sterile lines is a major problem in hybrid rice seed production. Hence, the foliar application of GA_3 at the start of panicle emergence has been widely adopted as an essential technology for improving plant height and panicle exsertion. Godha et al., (2020) reported that applied auxins (i.e., NAA, GA_3 and ascorbic acid) increased the highest value of panicle exsertion and the increases ranged between 87.80% and 88.67% when rice plants of A-line were treated with 200 g GA_3 ha-1. Zhen *et al.,* (2018) found that

GA_3 application at the time flowering produced hybrid seeds. Spraying cyanobacteria as a source of GA_3 can help in hybrid rice seed production. In this concern El-Habet and Elsadany (2020) and Abul Majeed *et al.,* (2017) displayed similar findings.

Effect of cyanobacteria on days to 50% heading, plant height, panicle length, panicle exsertion, angle of spikelet opening and duration of spikelet opening characters are given in (Table 5).

The application of Cyanobacteria influences panicle exsertion, spikelet opening angle and other floral traits which increases outcrossing rate of CMS lines leading higher yield because of its ability to secrete GA_3. This is reflected in plant growth and thus on its productivity (Chittoraa *et al.,* 2020 and Didovich, et al., 2020), enhance the growth, plant height, weight of 1000 grain, and grain yield of rice (Chittapun *et al*, 2018 and Jan *et al.,* 2018).

The effect of cyanobacteria has highly significant effect for plant height, panicle length, panicle exsertion, angle of spikelet opening and duration of spikelet opening, but, not significant for days to 50 % heading because of its high capability for nitrogen fixation and plant hormone production. mixture between *Nostoc entophytum* and *Anabaena oryzae* gave the highest values (117.31 and 118.27 cm) for plant height, (23.20 and 23.42 cm) for panicle length, (66.63 and 67.85 %) for panicle exsertion, (27.64 and 28.84^0) for angle of spikelet opening and (171.50 and 175.00 min) for duration of spikelet opening during 2019 and 2020 seasons, respectively. Similar results were also found by Singh et al., (2016) and Yanni *et al.,* (2020). Rahman *et al.,* (2010) reported that, application of GA_3 increased panicle exsertion and out crossing rate and seed yield.

The effect of cyanobacteria as a source of GA_3 as shown that (Table 6) has highly significant effect for number of fertile panicles $plant^{-1}$, panicle weight (g), seed set (%), seed yield (t ha^{-1}) and harvest index. Mixture between *Anabaena oryzae* and *Nostoc entophytum*gave gave the highest values (18.09 and 18.71 panicle $plant^{-1}$) for number of fertile panicles $plant^{-1}$, (2.44 and 2.59 g) for panicle weight, (30.40 and 31.60%) for seed set, (0.881 and 0.914 t/ha^{-1}) for yield and (18.37 and 18.85 %) for harvest index during 2019 and 2020 seasons, respectively. These results are in harmony with those obtained by (Phathka *et al,* 2018, Godlewska *et al.*, 2019, Yassen *et al.*, 2018 and Noroozlo *et al.,* 2019).

Table (4): Mean amounts of Phytohormones by cyanobacterial isolates (ug ml-culture).

Cyanobacterial isolates	Culture age days					
	Gibberellic acid (GA_3)			Indol acetic acid		
	10	20	30	10	20	30
Anabaena oryzae	2.31	7.90	7.15	8.19	16.60	15.24
Nostoc centophytum	5.17	9.00	8.30	20.69	51.37	31.55
Mixture	6.11	11.80	10.50	30.78	60.87	33.37

Table (5): Effect of spraying cyanobacteria on some growth and floral traits during 2019-2020

Treatments (cyanobacteria)	Days to 50 % heading		Plant height (cm)		Panicle length (cm)		Panicle exsertion (%)		Angle of spikiest opening (°)		Duration of spikelet opening (min)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Control	96.36	95.58	103.19d	104.73d	20.32d	20.64d	42.30d	43.28d	20.38d	20.67d	129.50d	133.00d
Anabaena oryzae	96.41	95.59	108.73c	109.69c	21.55c	21.87c	58.61c	59.61c	24.25c	24.92c	151.50c	155.00c
Nostoc centophytum	96.43	95.61	114.13b	115.66b	22.29b	22.69b	64.73b	65.90b	25.83b	26.93b	166.00b	171.00b
Mixture	96.39	95.60	117.31a	118.27a	23.20a	23.42a	66.63a	67.85a	27.64a	28.84a	171.50a	175.00a
F-test	NS	NS	**	**	**	**	**	**	**	**	**	**

** Highly significant at the 1% level of probability, NS not significant

Table (6): Effect of spraying cyanobacteria on some yield characteristics during 2019-2020

Treatments (cyanobacteria)	No of fertile panicles		Panicle weight (g)		Seed set (%)		Seed yield (t ha^{-1})		Harvest index (%)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Control	11.00d	11.87d	1.85d	2.03d	18.19d	19.45d	0.530d	0.584d	14.96d	15.64d
Anabaena oryzae	13.96c	14.86c	2.15c	2.31c	21.26c	22.22c	0.758c	0.797c	16.29c	16.86c
Nostoc centophytum	15.76b	17.13b	2.31b	2.48b	25.72b	26.39b	0.837b	0.878b	17.26b	17.76b
Mixture	18.09a	18.71a	2.44a	2.59a	30.40a	31.60a	0.881a	0.941a	18.37a	18.85a
F-test	**	**	**	**	**	**	**	**	**	**

** Highly significant at the 1% level of probability, NS not significant

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تأثير رش مستخلصات الطحالب الخضراء المزرقة (السيانوبكتيريا) على انتاج تقاوي الأرز الهجين

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الملخص العربى

الهدف من هذا البحث هو تحديد تأثير مستخلص السيانوبكتيريا (أنابينا أوريزا و نوستوك سينتوفيتوم وخليطها) المعزولة من مياه الصرف المصرية على إنتاج تقاوي الأرز الهجين. كانت السلالات العقيمة الذكرية السيتوبلازمية (IR69625A و G46A) ، بينما كان الأب المعيد للخصوبة جيزة ١٧٨. فى هذه الدراسة تم الحصول على عزلتان من السيانوبكتيريا من مياه الصرف الزراعي بمزرعة محطة البحوث الزراعية بسخا – محافظة كفر الشيخ تم تعريفهما (أنابينا أوريزا ، نوستوك سينتوفيتوم) وبالتالى تم إختبار قدرتهما على تثبيت النيتروجين الجوي وإنتاج الهرمونات و تأثيرهما على إنتاج تقاوي الأرز الهجين برش مستخلص العزلتين وكذلك الخليط منهما على نباتات الأرز. سجلت النتائج تباين العزلات في قدرتها علي تثبيت النيتروجين داخل الخلايا والنيتروجين المفرز خارج الخلية ، وإنتاج الهرمونات والوزن الجاف لعزلتى السيانوبكتيريا حيث كان أقل محتوى فى عزلت أنابينا أوريزا وأعلى محتوى كان لعزلة نوستوك سينتوفيتوم وخليطهما. أعطى استخدام الخليط بين أنابينا أوريزا و نوستوك سينتوفيتوم أعلى القيم في معظم الصفات المدروسة وكذلك في محصول التقاوي في كلا الموسمين.

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