RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2024.020

ScienceAsia 50 (1): 2024: ID 2024020: **1–8**

Improvement of rice growth and yield by seedling pretreatment to induce the artificial coexistence of nitrogen-fixing cyanobacteria and root seedling

Supenya Chittapun*, **Kunthida Lomphengthian, Nuankamol Amnuaysin, Chanitchote Piyapittayanun**

Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Centre, Pathum Thani 12120 Thailand

*Corresponding author, e-mail: supenyac@tu.ac.th

Received 3 Jan 2023, Accepted 18 Jan 2024
Available online 25 Feb 2024

ABSTRACT: The induction of an association between a nitrogen-fixing organism and a plant could potentially offer an alternative approach to ensure a sustainable supply of nitrogen for plants. This research aimed to explore the practicality of inducing the coexistence of *Nostoc commune* TUBT05, a nitrogen-fixing cyanobacterium, and rice seedlings. Seedling roots were facilitated with and without ultrasonic waves and further cocultured in BG-11_0 solution mixed with *N. commune* TUBT05. The effects of this artificial coexistence on the growth and yield of rice were investigated through pot experiments. Untreated and pretreated seedling roots with ultrasonic waves and further immersion in cyanobacterial solution showed an association between the seedling roots with different characteristics. When untreated roots were further cultivated in cyanobacterial solution, a cyanobacterial filamentous film covering the root surface was observed while the cluster of scattered cyanobacteria grown on small fragments of the pretreated root surface was found after immersion in cyanobacterial solution. Both pretreated and untreated seedling roots with ultrasonication before immersion showed significant growth promotion in seedlings and rice ($p < 0.05$). The association of *N. commune* TUBT05 with rice seedling roots significantly enhanced yield ($p < 0.05$). Ultrasonication applied during induction showed no stress effect on seedlings. This study suggested that the creation of artificial coexistence between plants and nitrogen-fixing bacteria via simply soaking seedling roots in nitrogen-fixing cyanobacteria could be used in agronomic processes to enhance the growth and yield of economically important crops. Further assessment of the feasibility and effectiveness of this approach under field conditions is needed.

KEYWORDS: ultrasonication, biofertilizer, nitrogen-fixing cyanobacteria, artificial symbiosis, rice

INTRODUCTION

Overuse of nitrogen fertilizer causes problems in production and utilization. The production of chemical fertilizer releases harmful products and causes environmental issues. An excessive supply of nitrogen in agricultural land enters streams and rivers through runoff and causes eutrophication. To ensure a sustainable supply of nitrogen to plants and environmental safety, alternative practices such as the induction of nitrogen-fixing ability in plants, application of biofertilizer during plantation, and creation of nitrogen-fixing organism symbiosis with the plant are possible [1]. There are many techniques to induce nitrogen-fixing ability in plants, for example, editing and inserting *nif* genes into the plant genome [2], controlling, promoting, and stimulating the nitrogen fixation activity of bacteria in living plants, [3] and inducing a new coexistence of nitrogen-fixing bacteria and plants via cocultivation with plant cells [4–7]. The symbiosis of plants and nitrogen-fixing bacteria such as legumes and rhizobia or Azolla and cyanobacteria is well known to play a role in atmospheric nitrogen fixation and conversion into available ammonium or nitrite for plant growth [2]. The induction of plant and nitrogen-fixing organism coexistence is an interesting issue to ensure nitrogen fixation in plants. However, symbiosis is a natural adaptation and takes a long time to evolve. The creation of artificial coexistence between plants and nitrogen-fixing bacteria is an alternative. Among nitrogen-fixing organisms, cyanobacteria have been intensively suggested to have nitrogen-fixing potential as biofertilizers to improve soil fertility and enhance crop productivity, especially in rice plantations [8–10]. Moreover, cyanobacteria are the source of the organic matter content in soil and synthesize and liberate amino acids, vitamins, and auxins. They are excellent soil conditioners and nitrogen supplementer, and they provide oxygen to the submerged rhizosphere [11].

According to the nitrogen-fixing induction technique, ultrasonic treatment is a promising technology for the induction of the association between plants and nitrogen-fixing cyanobacteria because it is a simple, inexpensive, nonthermal, and eco-friendly technique [12, 13]. In addition, the application of an ultrasonic wave to induce symbiosis is faster than the emergence through evolution. Pretreatment of wheat roots with an ultrasonic wave and cultivation in BG-11_0 mixed with *Nostoc muscorum* supplemented with 2,4 dichlorophenoxyacetic acid induced artificial nodules in wheat containing nitrogen-fixing cyanobacterium, and this cyanobacterium can perform nitrogen fixation activities [14]. In addition, ultrasonication has been proposed as a seed priming technique used to im-

prove seed germination and/or growth in rice [13–15], barley [16], chickpea, wheat, watermelon [17], and sesame [18]. Ultrasonic treatment of rice seeds improved the physiological performance, yield, and grain quality of rice planted under Cd contamination [15]. However, ultrasonication can be stressful and exert an adverse effect on plants depending on the frequency, intensity, and duration of exposure. Whether ultrasonication application has a better effect on plant growth has not been compared. Moreover, the induction of artificial nodules using a chemical supplemented with 2,4 dichlorophenoxyacetic acid involves chemical intervention and is impractical for agricultural purposes. Furthermore, a study on the induction of rice seedling coexistence with nitrogen-fixing cyanobacteria and the long-term effect of this coexistence on growth and yield has not been reported. Therefore, the aim of the present study is to assess the possible induction of the association of rice seedling roots and nitrogen-fixing cyanobacteria with and without facilitated ultrasonic waves and coculture in cyanobacterial solution without chemical supplement. Evidence of coexistence was observed via electron microscopy. In addition, the effect of the coexistence of nitrogen-fixing cyanobacteria and roots on the growth and yield of the rice variety KDML 105' 10GU-TU-70-10 was investigated in a pot experiment.

MATERIALS AND METHODS

Cyanobacteria cultivation, production, and preparation

Nostoc commune TUBT05 was isolated from organic paddy fields in Chachoengsao Province, Thailand, by Chittapun and Charoenrat [9] and was collected at the Algae and Plankton Research Unit, Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Thailand. The cyanobacterial inoculum was prepared by culturing *N. commune* in BG-11 medium in a 500 ml Erlenmeyer flask and incubated on a rotary shaker at 120 rpm under 25±1 °C with a light/dark cycle of 12 h/12 h per day for 14 days. Then, the large-scale production was conducted by cultivation in an 18.9 l algal culture system [19] containing 15 l BG-11 medium [20] without sodium nitrate (BG-11_0). The agitation was assisted via an air pump, and the system was placed outdoors under natural light for 21 days. Cyanobacterial biomass at the stationary stage was collected by centrifugation at 5,000 rpm for 10 min, and cells were washed twice with distilled water. This wet biomass was used for seedling pretreatment experiments and as supplemented biofertilizer during rice planting. According to the seedling pretreatment, 1 g *N. commune* TUBT05 was suspended in BG-11_0 and well mixed to prepare a suspension of cyanobacterial filaments for the soaking solution. As a biofertilizer, 10 g wet biomass was weighed and suspended in 1 l distilled water before application in a rice pot.

Table 1 Treatments including pretreatment and culture conditions of rice seedling roots before plantation.

Treatment	Seedling treatment	Seedling soaking solution
Control	Without ultrasonication	Distilled water
T1	Without ultrasonication	BG-11_0
T2	Ultrasonication for 30 s	BG-11_0
T3	Without ultrasonication	BG-11_0 mixed with 1 g/l *N. commune* TUBT05
T4	Ultrasonication for 30 s	BG-11_0 mixed with 1 g/l *N. commune* TUBT05

Soil preparations and soil analysis

Soil preparation was performed by mixing 10 kg soil with 10 g *N. commune* TUBT05 biomass. Ten kilograms of supplemented soil was added to a 12-inch plastic diameter pot. Soil before plantation and after rice harvesting was sampled for analysis of organic matter and nitrogen contents. Soil analysis was carried out by Walkey Black modified acid-dichromate digestion and the $FeSO_4$ titration method at the Land Development Department, Ministry of Agriculture and Cooperatives, Thailand.

Rice cultivar and seedling germination

Oryza sativa L. cv. KDML105' 10GR-TU-70-10, a photo-insensitive and drought-tolerant cultivar with an 87–96-day harvesting age [21], was applied in this study. The germination rate was determined by soaking rice seeds in distilled water for 24 h, placing them on wet paper in a plastic petri dish, and sealing the edge with parafilm. The petri dish was incubated at 25±1 °C for 5 days with 5 replications [20]. The germination rate was 95.0%. To obtain seedlings, each rice seed was placed in the middle of a 1 × 1 × 1 inch sponge cube and soaked in distilled water at room temperature and natural light for 8 days.

Seedling pretreatment by ultrasonication and immersion treatments

The 8-day-old seedlings were pretreated with an ultrasonic wave at 50/60 Hz by using an ultrasonication cleaner (Model-575HT) for 30 s (this condition has been preliminarily shown to not destroy the fibrous roots of rice seedling) and was immediately immersed in different solutions including BG-11_0 and BG-11_0 mixed with 1 g/l *N. commune* TUBT05 (Table 1). There were 4 treatments and a control with seedlings soaked in distilled water. Each treatment had 4 replications, and each replication consisted of 16 seedlings. The seedlings were soaked in each treatment at room temperature under natural daylight for 5 days. Four seedlings from each treatment were randomly sampled from each replicate for growth measurements, including root and shoot length and fresh and dry weight of roots and shoots. The root and shoot lengths

were measured using a meter scale and expressed in centimeters (cm). The fresh weight was measured by 4-digit balances. Rice dry weight was determined by leaving the root and shoot at 70 °C in a hot air oven until a constant weight was obtained, and weight measurement was performed by using 4-digit analytical balances. Radicles of 8-day-old seedlings treated with or without ultrasonic waves and 15-day-old roots with or without ultrasonication and coculture in BG-11_0 medium or BG-11_0 mixed with 1 g/l *N. commune* TUBT05 were collected for further associated character analysis.

Plantation experiment and management

The effect of different seedling pretreatments on rice growth and yield was examined in a pot experiment. The study was conducted outdoors under a nursery house at the Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Thailand, from November 2016 to January 2017. After 5 days of seedling root coculture under different treatments, 5 seedlings from each treatment and replicate were sampled and planted in a 12-inch plastic diameter pot containing 10 kg of supplemented soil. For biofertilizer supplementation, 10 g *N. commune* TUBT05 was added per pot at 10, 30, and 60 days after planting (DAP). Each treatment had 4 replications, and each replication contained 5 seedlings. To determine the growth of rice, 3 rice plants after 30 DAP were randomly sampled from each pot to measure root and shoot length and fresh and dry weight. Rice harvesting was performed after 90 DAP. The number of panicles, grains per panicle, filled grains per panicle, and undeveloped grains per panicle were counted to determine the rice yield.

Analysis of the coexistence between nitrogen-fixing cyanobacteria and seedling roots

The radicles from each treatment were sampled and immediately preliminarily observed under stereo (Olympus, SZ40 Japan) and compound microscopes (Olympus, CX31). These roots were cut and immediately fixed in 2% glutaraldehyde in 50 mM $NaHPO_4$ for 24 h. They were transferred to 50 mM $NaHPO_4$ and restored at 4 °C for further analysis by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For TEM analysis, the sample was further fixed in 1% osmium tetroxide in 0.2 M phosphate buffer for 2 h before dehydration. The dehydration process consisted of serial alcohol dilution at 70%, 80%, 90%, and absolute ethanol. Each dilution was used for 15 min, and absolute alcohol was used for 30 min. For SEM analysis, dehydrated roots were completely dried by a critical point dryer (TOUSIMIS AUTOSAMDRI-931 USA), and samples were placed on carbon tape attached to the stub and coated with gold. The samples were observed using scanning electron microscopy at 5 kV (JEOL JEM-2010, Japan) with an SEM camera (FEI Apreo, Japan). For TEM analysis, the dehydrated roots were embedded in epoxy resin at 70 °C for 12 h. Sections of 90 nm were cut by an ultramicrotome and poststained in uranyl acetate and lead citrate. The root structure was observed using TEM at 160 kV (ZJeM-2010, JEOL) with a TEM CCD camera (model Quemesa, Olympus) [20]. All root sample SEM and TEM analyses were conducted at the Scientific Equipment Center, Prince of Songkla University, Thailand.

Data analysis

The results are shown in terms of the average ± standard error. Data were analyzed by one-way analysis of variance (ANOVA) to determine the significant difference across treatments at $p = 0.05$, and the difference in the mean of data in pairs was compared by Tukey's HSD test ($p \leqslant 0.05$). The data analysis was computed using SPSS version 24.0.

RESULTS

Coexistence between nitrogen-fixing cyanobacteria and seedling roots

In comparison to unpretreated roots (Fig. 1a–1c), pretreatment of rice roots with ultrasonic waves for 30 s produced several cracks on the epidermis of the roots (Fig. 1d–1f). When untreated roots were cocultivated in BG-11_0 mixed with *N. commune* TUBT05, a cyanobacterial filamentous cluster covering the root surface was observed (Fig. 1g–1i). While scattered small fragments of cyanobacteria with many heterocytes growing on the root fractures were found after the immersion of treated roots in BG-11_0 mixed with *N. commune* TUBT05 (Fig. 1j–1l).

When compared to the untreated root (Fig. 2a), the TEM observations showed that the pretreatment of rice seedling roots with an ultrasonic wave caused a loose connection between epidermal cells (Fig. 2c). The walls of some epidermal cells collapsed after pretreatment, producing fractures on the root surface (Fig. 2e). Polysaccharides may act as biofilms and play a major role in cyanobacterial colonization (Fig. 2b and 2d) for both treated and untreated roots. However, the polysaccharide layer of the untreated root was thicker than that of the treated root. Cyanobacteria can colonize the cracks in epidermal cells (Fig. 2f).

Effect of seedling pretreatment and immersion conditions on seedling growth, rice growth, and yield in pots

Seedling pretreatment and soaking conditions significantly affected seedling growth, including root length ($p < 0.01$), leaf length ($p < 0.01$), and leaf dry weight ($p = 0.008$) (Table 2). The maximum dry weight of the leaf and leaf length were recorded from the T4 treatment, which consisted of ultrasonic wave application

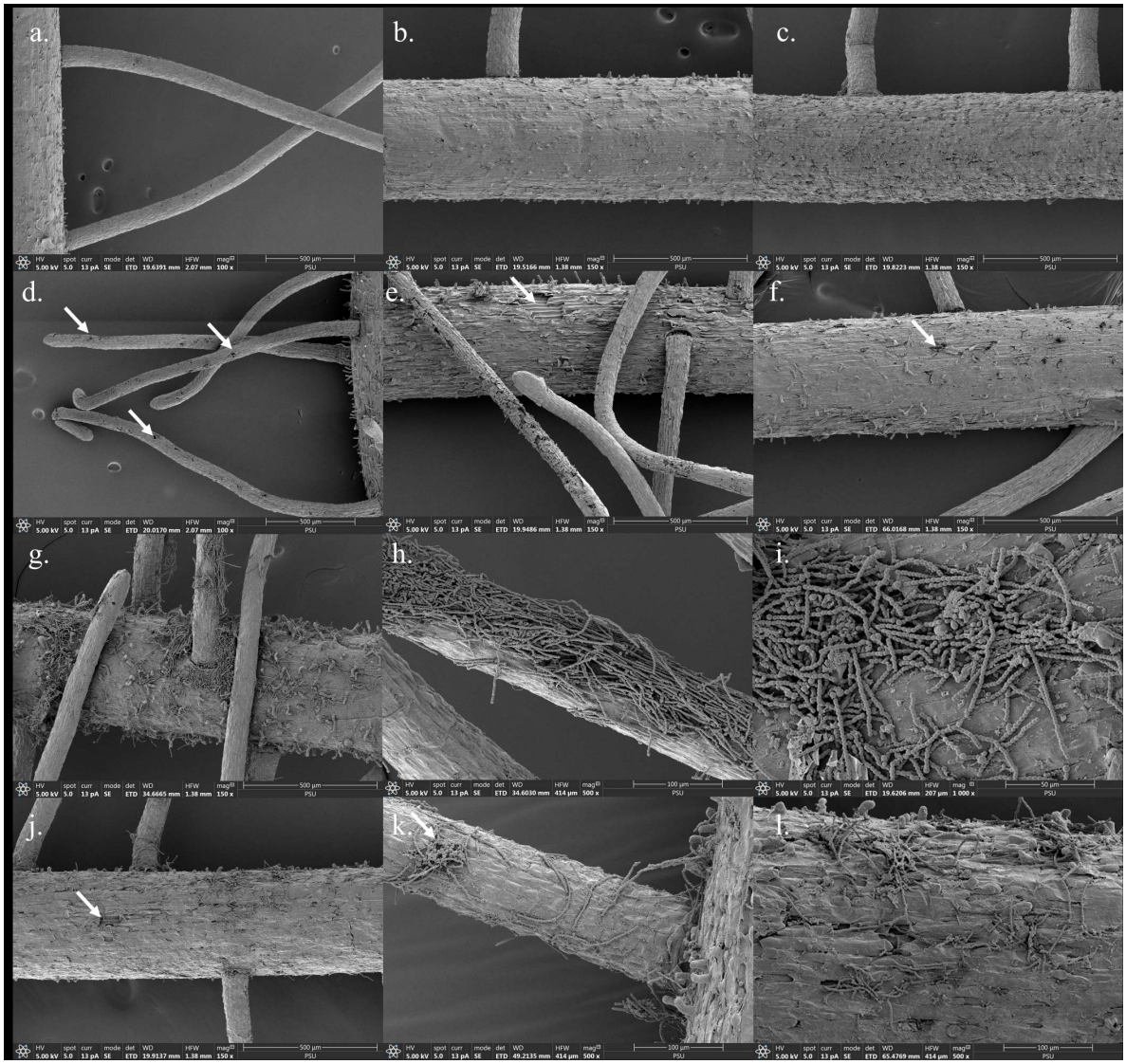

Fig. 1 Scanning electron photographs of roots from different treatments; a-c: untreated root of 8-day-old seedling (a and b) and 13-day-old seedling (c); d-f: root from an 8-day-old seedling treated with ultrasonication (d and e) and that of a 13-day-old seedling (f); g-i: untreated root and coculture in BG-11_0 mixed with *Nostoc commune* at different magnifications [150× (g), 500× (h), and 1,000× (i)]; and j-l: pretreated root with ultrasonication and coculture in BG-11_0 mixed with *N. commune* at different magnifications [150× (j), 500× (k), and 1,000× (l)].

Table 2 The effect of sonication and cyanobacteria inoculation on rice seedlings.

Treatment	Dry weight (g)		Length (cm)	
	root	leaf	root	leaf
Control	0.003±0.000	0.004±0.001^{b}	16.91±0.75^{a}	8.05±0.64^{c}
T1	0.003±0.000	0.005±0.001^{ab}	13.84±0.93^{a}	10.81±0.55^{b}
T2	0.003±0.000	0.005±0.001^{ab}	8.97±0.46^{b}	10.09±0.56^{bc}
T3	0.003±0.000	0.005±0.001^{ab}	9.00±0.44^{b}	11.22±0.55^{ab}
T4	0.003±0.000	0.006±0.001^{a}	10.75±0.56^{b}	13.22±0.57^{a}

Values are presented as the means ± standard errors (SE) ($n = 4$); a different superscript indicates a significant difference (one-way ANOVA and Tukey's HSD, $p < 0.05$); only root length data were transformed by taking the LN before one-way ANOVA.

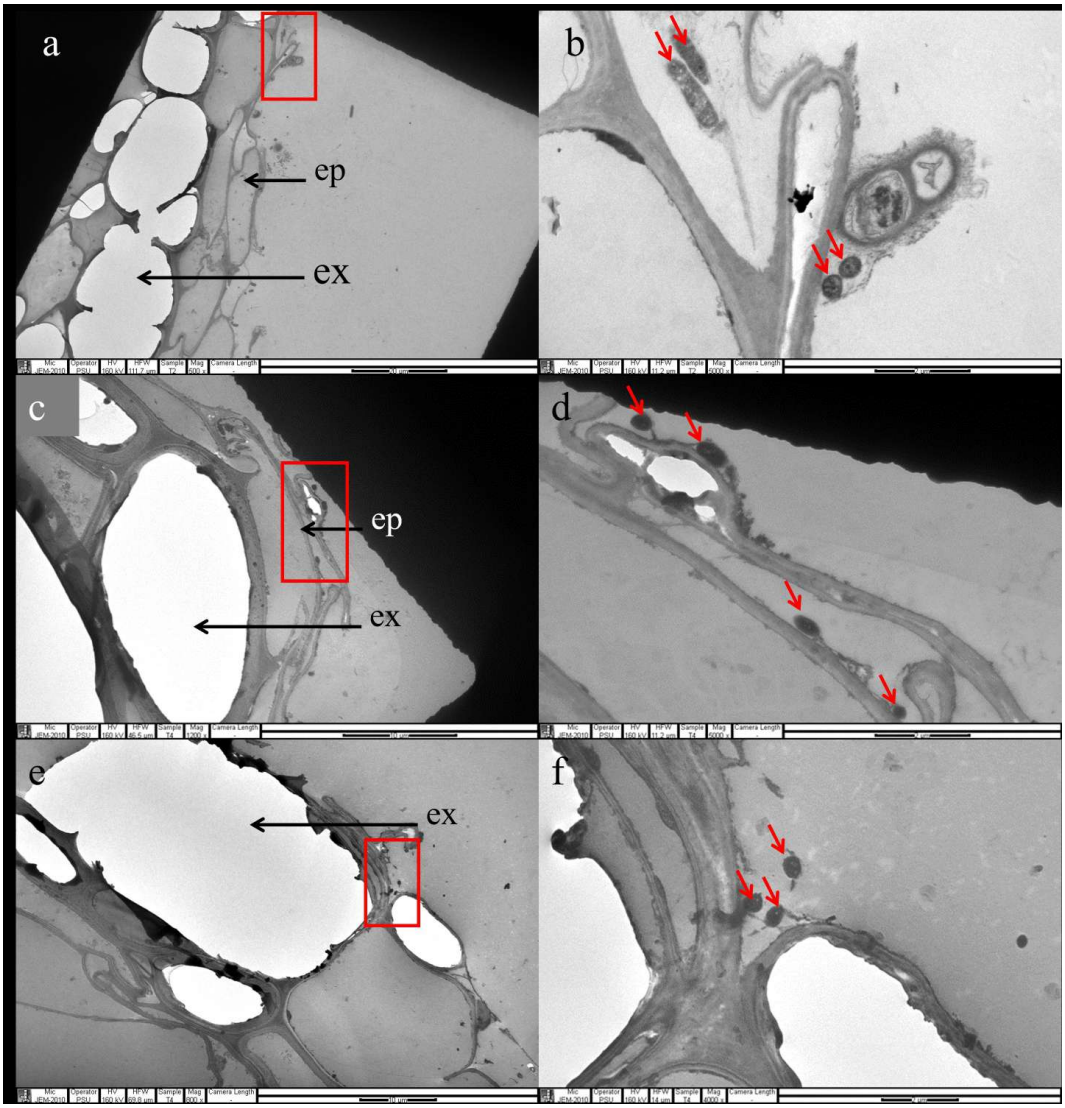

Fig. 2 Transmission electron micrographs of rice seedlings soaked in BG-11_0 mixed with 1 g/l *N. commune* TUBT05; a and b (a magnification of the red rectangle from a) and rice seedlings pretreated with ultrasonic waves and soaked in BG-11_0 mixed with 1 g/l *N. commune* TUBT05; c and d (a magnification of the red rectangle from c); e and f (a magnification of the red rectangle from e). ep: epidermis, ex: exodermis, and red arrow: *N. commune* TUBT05. Magnification: a (500 ×), b (5,000 ×), c (1,200 ×), d (5,000 ×), e (800 ×), and f (4,000 ×). Scale bar: a (20 µm); b, d, and f (2 µm); and c and e (10 µm).

Table 3 The effect of sonication and cyanobacteria inoculation on rice growth.

Treatment	Dry weight (g)		Length (cm)	
	root	leaf	root	leaf
Control	0.021±0.012^{b}	0.013±0.002^{b}	10.84±0.97^{bc}	15.79±0.87^{b}
T1	0.006±0.001^{b}	0.012±0.001^{b}	9.58±1.17^{c}	16.88±0.71^{b}
T2	0.019±0.009^{b}	0.046±0.024^{b}	9.38±0.83^{c}	23.95±3.46^{b}
T3	0.129±0.035^{a}	0.499±0.124^{a}	15.58±1.49^{a}	43.74±4.63^{a}
T4	0.110±0.022^{a}	0.539±0.105^{a}	15.25±1.23^{ab}	48.83±2.93^{a}

Values are presented as the means ± standard errors (SE) ($n = 4$); a different superscript indicates a significant difference (one-way ANOVA and Tukey's HSD, $p < 0.05$). Root dry weight data were transformed by taking the LN before one-way ANOVA, and leaf dry weight.

Table 4 The effect of sonication and cyanobacteria inoculation on rice yield.

Treatment	No. of ears of rice per plant	No. of rice seeds per spike	Whole kernel (number/spike)	Undeveloped kernel (number/spike)
Control	6.75±1.25	69.48±3.00	38.50 ± 2.70^{c}	30.98 ± 2.12^{c}
T1	5.00±0.29	70.70±4.25	45.93 ± 3.01^{bc}	24.73 ± 3.28^{bc}
T2	7.38±0.94	65.07±3.02	43.71 ± 2.73^{bc}	21.37 ± 2.02^{b}
T3	7.12±0.83	59.69±2.19	50.73 ± 1.98^{ab}	7.73 ± 0.81^{a}
T4	6.12±0.43	67.82±2.76	59.08 ± 2.51^{a}	8.73 ± 0.68^{a}

Values are presented as the means ± standard errors (SE) ($n = 4$); a different superscript indicates a significant difference (one-way ANOVA and Tukey's HSD, $p < 0.05$).

Table 5 Percentage of organic matter and nitrogen composition in planted soil and soil after harvesting from different treatments ($n = 3$).

Treatment	Planted soil	Soil after harvesting				
		Control	T1	T2	T3	T4
%OM	13.17±0.07	7.41±0.26	8.05±0.21	7.17±0.11	8.02±0.28	6.93±0.47
%N	0.69±0.00	0.39±0.01	0.42±0.01	0.37±0.01	0.42±0.01	0.36±0.02

OM: Organic matter; N: nitrogen.

and immersion in BG-11_0 mixed with 1 g/l *N. commune* TUBT05. Seedling pretreatment showed a significant effect on rice growth ($p < 0.01$). The seedlings with or without ultrasonic treatment immersed in BG-11_0 mixed with *N. commune* TUBT05 showed the greatest root and leaf weights and lengths (Table 3). The maximum value for leaf dry weight and length was recorded from T4. However, T3 and T4 were not significantly different.

Rice pretreated with ultrasonic waves and immersed in BG-11_0 mixed with cyanobacteria showed improved yield ($p < 0.05$). Seedling pretreatment through ultrasonic waves followed by immersion in BG-11_0 mixed with cyanobacteria (T4) yielded the highest count of intact kernels and the lowest count of underdeveloped kernels. This was followed by untreated seedlings immersed in BG-11_0 and cyanobacteria mixture (T3), pretreated root soaked in BG-11_0 (T2), and finally untreated roots soaked in BG-11_0 (Table 4). Moreover, immersion in BG-11_0 mixed with cyanobacteria can shorten the grain filling period. This implies that rice is healthy and has a rapid growth; therefore, the grain filling period was earlier than that in the control and immersion only in BG-11_0 treatments. Hence, the results showed that soaking seedling roots in cyanobacterial solution enhances the growth of seedlings in terms of leaf length and weight, and immersion of the seedlings in BG-11_0 mixed with *N. commune* TUBT05 enhanced rice growth during plantation. Furthermore, post-plantation measurements indicated a decrease in soil organic matter and nitrogen content with no significant differences observed among the treatments (Table 5).

DISCUSSION

The cyanobacterial genus *Nostoc* has been reviewed as a common cyanobiont of plants, including angiosperms, gymnosperms, pteridophytes, and bryophytes [22, 23]. In cycads, *Nostoc* filaments have been identified as a symbiont in specialized lateral roots called coralloid roots. The invasion was suggested to arise from an injured root part, lenticels, papillose region, or breaking of the dermal layer, where the cyanobacterial fragment could enter the root. The infection process could be achieved because hormogonia, short motile cyanobacterial filaments, enter the root and proceed to revert to vegetative cells and differentiate into heterocytes, where nitrogen fixation occurs [22, 23]. Hormogonia can be induced by low nitrogen conditions [23, 24]. Our study used ultrasonic waves to make several fissures in an epidermal layer of the lateral root and then soaked the pretreated roots in cyanobacterial BG-11_0 solution to induce hormogonia that could deliver cyanobacteria associated with the lateral roots of rice seedlings. After hormogonium made contact with root crack cavities, the vegetative cells were reformed, and the heterocytes were later differentiated. Numerous heterocytes could be noticed at the crack opening, where they were exposed to light and oxygen. Heterocyte characteristics provide the conditions for nitrogenase enzyme protection from oxygen penetration via cell walls and oxygen production from photosynthesis [22]. The numerous heterocytes resulted in less growth, which was observed in this study. Cyanobacteria growing on roots pretreated with ultrasonic waves showed fewer filaments than

those growing on and covering untreated roots. In addition, some plants can secrete hormogonia-inducing factors (HIF) that can promote *Nostoc* spp. filaments to convert to hormogonia [25]. HIF has been proven and reported in *Oryza sativa* to induce *Nostoc* strains associated with rice roots [26]. This can correspondingly promote the success of the association process. The biochemical composition of HIF in the coralloid root of cycads has been investigated, and it has been reported that HIF is a mixture of diacylglycerols (DAGs), which are mainly composed of 1-palmitoyl-2-linoleoyl-*sn*-glycerol, 1-palmitoyl-2-ikeitk-*sn*-glycerol, 1-stearoyl-2-linolenoyl-*sn*-glycerol, and 1-stearoyl-2-linoleoyl-*sn*-glycerol [27]. DAGs are commonly distributed primary metabolites found in cyanobacteria and plants; however, the significant function of DAGs is still unknown [27]. Therefore, the combination of the creation of several fractures on the lateral root epidermis via ultrasonic waves, an induction of hormogonia cells and their motility by coculture in cyanobacterial BG-11_0 solution, and a chemotactic attraction through a secreted HIF are the vital factors that influenced the success of rice root and *N. commune* TUBT05 associations.

Surface polysaccharides such as exopolysaccharides involved in the colonization of roots can be observed in plant-bacteria or plant-cyanobacteria associations, including *Nostoc* sp. [23, 28]. Cyanobacteria excrete exopolysaccharides as a biofilm to enhance cell adhesion to solid surfaces [29]. In untreated roots with ultrasonication, colonization may begin from cyanobacteria anchoring onto the root system, proliferating, and forming microcolony-biofilm structures at the surface of the root. This formation contributes to an optimal microenvironment and has ecological significance for nutrient uptake during rice planting in pots. *Nostoc* sp. associated with rice roots improves the growth of rice plants by increasing their nitrogen fixation [26]. The use of *N. commune* TUBT05 as biofertilizer in this study followed the report by Chittapun et al [20], which demonstrated that the application of *Nostoc* sp. as biofertilizer in rice planted in pots increases rice growth and yield. This study also demonstrated that induction of the association of cyanobacteria and rice seedling roots either using ultrasonic waves or no treatment enhanced the growth of rice seedlings and the growth and yield of rice. Therefore, the creation of an artificial association between *Nostoc* sp. and rice seedling roots can be created and improve the growth of rice seedlings and rice growth and yield. Furthermore, treated roots with ultrasonication prior to immersion in BG-11_0 mixed with cyanobacteria exhibited slightly enhanced plant growth and yield compared to untreated roots immersed in the same BG-11_0 and cyanobacteria mixture without significant divergence. These results convincingly demonstrate that ultrasonic does not induce stress in seedlings. Moreover, the colonization of cyanobacteria within the cracks in epidermal cells in the treated root appears to have a greater profound impact on plant growth than that of the formation of biofilm on the root surface. These phenomena could be further investigated through transcriptome analysis. This study suggests that untreated roots before immersing them in a mixture of BG-11_0 and cyanobacteria could provide an economically feasible and uncomplicated option for agricultural practices. Further assessment of the feasibility and effectiveness of this approach under field conditions is needed.

Based on our results, cyanobacterial symbiosis was found to play an important role in promoting rice growth; however, to date, the molecular mechanism of this positive effect remains unknown. Elucidation of the gene expression profile of cyanobacteria by transcriptome analysis in the association with both extra- and intracellular patterns of rice root exudates will allow us to gain deep insight into the mechanism of cyanobacterial-plant interactions. It has been previously reported that the expression of genes involved in the metabolism and chemotaxis of *Pseudomonas aeruginosa* was altered in response to sugar beet root exudate [30]. In addition, Fan et al [25] showed that many genes in *Bacillus amyloliquefaciens* FZB42 involved in nutrient utilization, chemotaxis, and cell motility were upregulated in response to maize root exudates. Moreover, since compositions in plant exudates likely play important roles in the differential gene expression of cyanobacteria and result in various effects on plant growth, knowledge gained from this study could shed light on putative pathways responsible for cyanobacterial symbiosis and could then be applied for the establishment of artificial associations between nitrogen-fixing cyanobacteria and other high economic value plants to reach maximum yield and high quality.

CONCLUSION

This study shows the feasibility of the pretreatment of rice seedling roots with or without ultrasonication and coculture in cyanobacterial solution to improve the growth and yield of rice. The coexistence of nitrogen fixing-cyanobacteria on rice seedling roots has a positive impact on the number of completed grains resulting in increasing yield.

The induction of an association by soaking seedling roots in nitrogen-fixing cyanobacterial solution provides an alternative option for farmers to create artificial symbiosis and promote plant growth instead of using chemical fertilizers. Therefore, the coexistence of plants and nitrogen-fixing cyanobacteria could be a fundamental goal for engineering rice crops to reduce the demand for chemical nitrogen fertilizers. These could be an alternative avenue to support a sustainable agricultural approach to food production.

Acknowledgements: This work was partially supported by the Thailand Science Research and Innovation Fundamental Fund, contract no. TUFF03/2565. We are thankful to the Algae and Plankton Research Unit, Department of Biotechnology, Faculty of Science and Technology, Thammasat University for equipment facilitation.

REFERENCES

1. Kollmen J, Strieth D (2022) Review: The beneficial effects of cyanobacterial co-culture on plant growth. *Life* **12**, 223.
2. Preininger É, Gyurján I (2001) Trials to create artificial nitrogen-fixing symbioses and associations using *in vitro* methods: an outlook. *In Vitro Cell Dev Biol Plant* **37**, 139–148.
3. Ryu M, Zhang J, Toth T, Khokhan D, Geddes BA, Mus F, Garcia-Costas A, Peters JW, et al (2020) Control of nitrogen fixation in bacteria that associate with cereals. *Nat Microbiol* **5**, 314–330.
4. Gusev MV, Korzhenevskaya TG, Pyvovarova LV, Baulina OI, Butenko RG (1986) Introduction of a nitrogen-fixing cyanobacterium into tobacco shoot regenerates. *Planta* **167**, 1–8.
5. Preininger É, Zatykó J, Szũcs P, Koráyni P, Gyurján I (1997) *In vitro* establishment of nitrogen-fixing strawberry (*Fragaria* × *Ananassa*) via artificial symbiosis with *Azomonas insignis*. *In Vitro Cell Dev Biol Plant* **33**, 190–194.
6. Gantar M (2000) Co-cultivation of N_2-fixing cyanobacterium *Nostoc* sp. strain 2S9B and wheat callus. *Symbiosis* **29**, 1–18.
7. Gorelova OA, Kleimenov SY (2003) The accumulation and degradation dynamics of cyanophycin in cyanobacterial cells grown in symbiotic associations with plant tissues and cells. *Microbiology* **72**, 361–369.
8. Saadatnia H, Riahi H (2009) Cyanobacteria from paddy fields in Iran as a biofertilizer in rice plants. *Plant Soil Environ* **55**, 207–212.
9. Chittapun S, Charoenrat T (2015) Isolation and growth of N_2-fixing cyanobacteria from organic agricultural areas in Sanamchaikate, Chachoeng-Sao province, Thailand. *Thammasat Int J Sci Tech* **20**, 27–32.
10. Majeed A, Muhammad Z, Islam S, Ullah Z, Ullah R (2017) Cyanobacterial application a bio-fertilizers in rice fields: role in growth promotion and crop productivity. *PSM Microbiol* **2**, 47–50.
11. Singh NK, Dhar DW, Tabassum R (2016) Role of cyanobacteria in crop protection. *Proc Natl Acad Sci India Sect B Biol Sci* **86**, 1–8.
12. Bonto AP, Tiozon Jr RN, Sreenivasulu N, Camacho DH (2021) Impact of ultrasonic treatment on rice starch and grain functional properties: A review. *Ultrason Sonochem* **71**, 105383.
13. Rao G, Ashraf U, Huang S, Cheng S, Abrar M, Mo Z, Pan S, Tang X (2018) Ultrasonic seed treatment improved physiological and yield traits of rice under lead toxicity. *Environ Sci Pollut Res Int* **25**, 33637–33644.
14. Fadl-Allah EM, El-komy HM, Al-Harbi NA, Sholkamy EN (2011) *In vitro* creation of artificial nitrogen fixing cyanobacterium (*Nostoc muscorum*) association with wheat. *Afr J Microbiol Res* **5**, 302–310.
15. Huang S, Rao G, Ashraf U, Deng Q, Dong H, Zhang H, Mo Z, Pan S, et al (2021) Ultrasonic seed treatment improved morpho-physiological and yield traits and reduced grand Cd concentrations in rice. *Ecotoxicol Environ Saf* **214**, 112119.
16. Yaldagard M, Mortazavi SA, Tabatabaie F (2008) Application of ultrasonic waves as a priming technique for accelerating and enhancing the germination of barley seed: optimization f method by the Taguchi approach. *J Inst Brew* **114**, 14–21.
17. Goussous SJ, Samarah NH, Alqudah AM, Othman MO (2010) Enhancing seed germination of four crop species using an ultrasonic technique. *Expl Agric* **46**, 231–242.
18. Shekari F, Mustafavi S, Abbasi A (2015) Sonicaion of seeds increase germination performance of sesame under low temperature stress. *Acta Agric Slov* **105**, 203–212.
19. Chittapun S, Limbipichai S, Amnuaysin N, Boonkerd R, Charoensook M (2018) Effects of using cyanobacteria and fertilizer on growth and yield of rice, Pathum Thani I: a pot experiment. *J Appl Phycol* **30**, 79–85.
20. Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue green algae (Order: Chroococcales). *Bacteriol Rev* **35**, 171–305.
21. Chongkid B (2013) Gamma-ray induced mutation of KDML105 for pho insentivity, short harvest age and drought tolerance. *Am J Eng Appl Sci* **2**, 265–270.
22. Meeks JC (2009) Physiological adaptations in nitrogen-fixing *Nostoc*-plant symbiotic associations. *Microbiol Monogr* **8**, 181–205.
23. Santi C, Bogusz D, Franche C (2013) Biological nitrogen fixation in non-legume plants. *Ann Bot* **111**, 743–767.
24. Campbell EL, Christman H, Meeks JC (2008) DNA microarray comparisons of plant factor-and nitrogen deprivation-induced hormogonia reveal decision-making transcriptional regulation patterns in *Nostoc punctiforme*. *J Bacteriol* **190**, 7382–7391.
25. Fan B, Carvalhais LC, Becker A, Fedoseyenko D, Wirén N, Borriss R (2012) Transcriptomic profiling of *Bacillus amyloliquefaciens* FZB42 in response to maize root exudates. *BMC Microbiol* **12**, 116.
26. Nilsson M, Rasmussen U, Bergman B (2005) Competition among symbiotic cyanobacterial *Nostoc* strains forming artificial association with rice (*Oryza sativa*). *FEMS Microbiol Lett* **245**, 139–144.
27. Hashidoko Y, Snishizuka H, Tanaka M, Murata K, Murai Y, Hashimoto M (2019) Isolation and characterization of 1-palmitoyl-2-linoleoyl-*sn*-glycerol as a hormogonium-inducing factor (HIF) from the coralloid roots of *Cycas revoluta* (Cycadaceae). *Sci Rep* **9**, 4751.
28. Gantar M, Rowell P, Kerby NW, Sutherland IW (1995) Role of extracellular polysaccharide in the colonization of wheat (*Triticum vulgare* L.) roots by N_2-fixing cyanobacteria. *Biol Fertil Soils* **19**, 41–48.
29. Rossi F, De Philippis R (2015) Role of cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial mats. *Life* **5**, 1218–1238.
30. Mark GL, Dow JM, Kiely PD, Higgins H, Haynes J, Baysse C, Abbas A, Foley T, et al (2005) Transcriptome profiling of bacterial response to root exudates identified genes involved in microbe-plant interactions. *Proc Natl Acad Sci USA* **102**, 17454–17459.