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Short Communication | International Journal of Plant studies | Open Access

The Effect of Flavonoid Naringenin Coupled With the Developed Biofilm *Azorhizobium Caulinodans-Aspergillus* Spp. on Increase in Rice Yields in Conventionally and Organically Grown Rice

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Received: June 22, 2018; Accepted: July 16, 2018; Published: July 18, 2018

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Abstract

Today, the need for economically and environmentally friendly alternatives to substitute inorganic nitrogen fertilizers for rice cultivation is intense than ever before. Our previous studies describe the formation of a successful nitrogen fixing association in the form of a biofilm *in-vitro*. This biofilm was a combination of *Azorhizobium caulinodans and Aspergillus* spp. (AAB). Naringenin (Nar) is a flavonoid capable of increasing Azorhizobial colonization in rice roots. This paper focuses on investigating the ability of naringenin to enhance rice yields when it is applied together with the developed AAB/Nar. It was found that, application of AAB together with naringenin can significantly increase the most important yield parameters such as filled grain weight/plant, total grain weight/plant, filled grain number/plant and the total grain number/plant. The developed AAB/Nar combination on improving the yield of organically grown rice was also investigated. It was observed that the AAB/Nar combination can also enhance the yields of organically grown rice to significantly higher values.

Introduction

The need to adopt environmentally friendly alternatives to agriculture were discussed intensely in the previous few years in the global arena than it was ever before. Rio+20, is a gathering of participants of United Nations and the theme of the summit was the sustainable development. The published document of Rio+20, “The Future We Want” is an agreement of the members to “strive to achieve a land degradation-neutral world in the context of sustainable development” [1]. UN General Assembly in September 2015 defined sustainable Development Goals (SDGs) which are approved by the UN General Assembly depending on the previous “The future we want” document [2]. To achieve these SGD’s, contribution of soil science, sustainable agriculture and healthy soils is immense [2].

The use of inorganic fertilizers to feed the exponentially increasing world population shows a steep increase while the availability of arable lands is a steep decrease. Rice (*Oryza sativa*), is a staple cereal for more than half of the world population, providing 80% of their food requirement. The total global rice production in 2016 July was 495 Million metric tons (mmt) (Food and Agriculture Organization (FAO) (2016)). This on average accounts for 29% of the total output of grain crops [3]. This is a 2.18% increase of the total global rice production than the previous year, 2015 [4].

To cultivate this important crop, major inorganic fertilizers, N, P and K are added in large quantities. Out of these fertilizers nitrogen is considered as the *sine quo none* or the absolute necessity for rice cultivation [5]. Response of rice plant to the nitrogen supply is rapid and shown by rapid change of the growth and yield pattern of the rice plant [6]. However, the detrimental impacts on the environment by nitrogen fertilizers such as damage to the soil microbial biomass, contamination of ground water, eutrophication of rivers, lakes, costal and marine ecosystems, depletion of oxygen in water bodies due to algal blooms, accumulation of toxins and their impact on the aquatic food webs are a few obvious [7, 8]. Other than environmental impacts, nitrogen fertilizer can create a major economic impact in developing countries due to the cost involved in fertilizer imports and fertilizer subsidies. Finding an alternative for nitrogen fertilizers through bio-fertilizers therefore is becoming more and more important.

Azorhiozobium caulinodans is a diazotrophic bacterium and is extremely important in its ability of fixing freely available atmospheric nitrogen non-symbiotically [9], even in the presence of 3% v/v oxygen [10]. These characters are extremely important in the induction of nitrogen fixation in non-legumes such as rice.

Biofilms are important microbial associations. A proper establishment of a biofilm would provide the benefits to the plant, from nitrogen fixation, suppression of pathogens, PGPRs production, etc. Remedying of the heavily fertilized, damaged soils is also possible with bio filmed biofertilizers [11]. When an *in-vitro* developed bio filmed bio-fertilizer is added to the soil with moderate levels (50% of the fertilizer recommendation) of fertilizer, a drastic improvement of important plant characteristics such as nitrogenase activity, root exudates and soil organic carbon levels, has taken place [12]. Application of three microbial bio-fertilizers coupled with 50% of recommended fertilizers for rice plant, a biomass increment of 55% compared to 100% application of the inorganic fertilizers has been observed [11]

Our previous studies report on development of a successful biofertilizer which is a biofilm formed between the bacterium *Azorhizobium caulinodans* and *Aspergillus* spp. (AAB) [13]. One of the major finding in the previous study was the ability of these biofilm to increase their performance in terms of rice root colonization [13, 14] of rice roots and nitrogen fixation in the presence of the flavonoid naringenin (Perera et al, Unpublished)

Flavonoid naringenin acts as a signaling molecule [15] to induce *A. caulinodans* colonization to significantly high levels, in and around the rice roots. Concentrations of 10^{-4} M (100 $mMolm^{-3}$) and 10^{-5} M (10 $mMolm^{-3}$) naringenin has shown to increase colonization of A. caulinodans in lateral roots, cortical region [16 -18] and xylem of rice roots.

Hence in this study, our first goal was to find out whether the developed AAB [13] is capable of improving rice yields in the presence of flavonoid naringenin.

The second part of this paper deals with organically grown rice. According to the International Federation of Organic Agriculture Movement (IFOAM) definition "Organic Agriculture is a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Even though organic agriculture has ample amount of benefits such as reviving the soil fertility, reduction of nitrate and phosphorous leaching, reduction of greenhouse gases [19] etc., one of the main disadvantages is the lowering of yields compared to the rice cultivated with inorganic fertilizers [20, 21]. If organic agriculture is to be a feasible option in the years to come, increasing the yields is essential to meet the food requirements of the exponentially increasing world population. Hence, our second target was to find out whether the developed biofertilizer is capable of surviving and enhancing the rice yields in organically grown rice cultivation.

Methodology

Experiment 1: Effect of the flavonoid naringenin on conventionally grown rice yields when coupled with the developed biofertilizer AAB/Nar [13] Preparation of pots

This pot experiment was conducted in the Plant house of the Department of Plant Sciences, University of Colombo (Colombo, Latitude DMS 6°55'37.48"N, Longitude DMS 79°51'40.47"E) Sri Lanka from 20 December 2014 up to 10 April 2015 (Maha season). Large rectangular pots with the following dimensions, length x width x height, 1 m x 0.5 m x 0.3 m which were made up of sheets with a material containing aluminum and iron were used for the experiment. The pots were layered with black, thick polythene. Each pot was added with 100 kg of paddy soil sampled from Kurunegala region (Latitude, DMS7°28'22.73"N, Longitude DMS 80°21'17.02"E) which is located in the intermediate zone of Sri Lanka. The paddy soil was manually checked for plant roots and debris.

After that, the soil was air dried and filled in to the pots. The soil pH 6.2, Total Carbon 1.52%, total N, 0.13%, total P, 0.28%. Each pot was covered with a wooden cage lined/covered with an iron mesh and a glass top. The pots were arranged in randomized design.

Planting of Rice

Seeds of the rice (Oryza sativa L.) variety BG 366 were used for the experiment. Pre-germinated rice seedlings were transplanted in to the pots containing 100 kg paddy soil. Twenty four plantlets (24) were planted equidistantly in each pot, one plantlet per hill (distance between two plantlets =20 cm).

Treatments

The experiment contained two treatments and three replicates each.

AAB (Biofilm only)

AAB/Nar only

Preparation of the biofilm and Naringenin

Biofilm and Naringenin were prepared according to the procedures given [13]. For this experiment (Experiment 1), biofilm was prepared using the GFP-labelled *Azorhizobium caulinodans* [14, 22]

Rice root colonization by AAB/Nar

Rice root colonization data were taken on the 30th, 60th and 105th day. A single randomly selected root of each plant was divided in to three and was pressed on to a microscopic slide and was analyzed by micro-imaging as described in the day as described [13]. Green fluorescing regions were photographed by Axiocam ERc 5s. Intensity of fluorescence was measured with ZEN 2012, ZEN light blue edition software as described [13].

Yield parameters

Yield parameters were taken after the full maturity of the plant after 105 days. The following yield parameters were measured

Filled grain weight/Plant, Unfilled grain weight/Plant, Total grain weight/Plant, Panicle Number/Plant, Number of filled grains/Plant, Number of Unfilled grains/Plant, Total Number of grains/Plant, 100 grain weight

Statistical Analysis

The data of the rice root colonization, and yield were subjected to t-test using SAS V 9. The differences among means were separated by Tuckey multiple range test at 0.05 probability level.

Experiment 2: Contribution of the developed biofertilizer AAB/Nar on organically grown traditional rice yield

Experimental set up

Experiment was conducted in the rice fields of the low country wet zone and the upcountry wet zone in 2017 Yala season (May to end of August). The field site was located at Dombagoda, Horana (Latitude, DMS6°42'59.99" N, Longitude DMS 80°02'60.00" E) – Low country wet zone in the western province (the average annual rainfall 3000 mm and the average annual temperature 30°C). The study site was located in the middle of an abandoned rice field where no cultivation had taken place during last five years.

Rice plant cultivation

Traditional rice variety "*Herath banda*" (3 1/2 month) seeds were collected from the Rice Research and Development Center at Bombuwala. Rice seeds were first soaked in water for 24 hours. The seeds were put in a sack and covered completely with banana leaves for 24 hours for slow warming of the seeds. Germinating seeds were placed in parachute trays (transplanting trays) with the rate of one seed per hill. Soil from the field was the supported medium for seedling growth. Planting trays were placed on water bath to irrigate the system.

Two weeks old rice seedlings were transplanted with a spacing of 20 cm x 20 cm. There were 50 plants per plot in this experiment.

Treatments

Experiment contained two treatments and three replicates

Treatment 1 – Compost only

Treatment 2 – Compost + AAB/Nar

Addition of compost

Amount of compost to be added was calculated according to the recommendations by the Department of Agriculture, Sri Lanka (Compost 4 tons per hectare).

Field maintenance

The water management was done according to the farmer practice and all the plots received water only from rain throughout the research. Proper draining system was established during final land preparation. Hand weeding was performed throughout the experiment.

Yield parameters

Two important yield parameters taken

1.Number of filled grains

2. Total number of grains

Statistical Analysis

Data were analyzed by one-way ANOVA of SAS, using Duncan's Multiple range test (P = 0.05).

Results

Comparison between AAB and AAB/Nar on the biofilm colonization and conventionally grown rice yields

Rice root colonization by the biofilm

In the treatment AAB/Nar, the biofilm colonization shows a drastic decrease from 30 days to 105 days but the treatment AAB shows a steep increase. But the mean fluorescence intensities of AAB/Nar are significantly higher than AAB up until 105 days (figure 1, figure 2) shows a rice root colonized by the AAB/Nar biofilm on the 60th day.

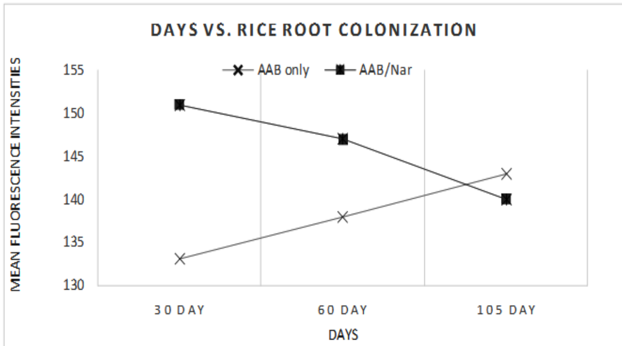

Figure 1: Rice root colonization with treatments, AAB only and AAB/Nar only of the pot experiment using GFP-labelled *Azorhizobium caulinodans.*

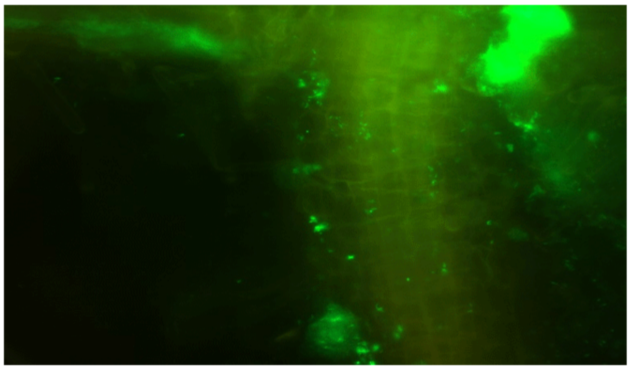

Figure 2: An epifluorescent micrograph shown bright green fluorescing AAB colonization on the rice plant roots taken on the 30 days from a treatment with AAB/Nar. (x10x40)

Yield parameters

Almost all the parameters including Filled grain weight/Plant, Unfilled grain weight/Plant, Total grain weight/Plant, Panicle Number/Plant, Number of filled grains/Plant, Number of Unfilled grains/Plant, Total Number of grains/Plant, 100 grain weight showed a significant increment in the treatment AAB/Nar compared to AAB only (Figure 4).

Organically grown rice Compost + AAB/Nar

Addition of the developed AAB/Nar combination have significantly incremented the number of filled grains per plant and the number of total grains per plant in comparison to the treatment AAB only.

Discussion

Significantly higher rice yields when AAB is added with naringenin to conventionally grown rice

This study was based on the biofilm (AAB) that was developed in a previous study [13]. Biofilms are extremely advantageous associations between microorganisms [11]. Biofilms can have the forms with irreversible differentiations, division of labour among different cell types and intercellular cooperation [23]. Also they can contribute to increment of soil fertility and soil cycles enhancing the activity of soils [24].

Our previous studies conducted with 15N isotopic analysis resulted in extremely higher nitrogen fixation values in the presence of the AAB/Nar in the rice plants compared to only AAB (data not published). In this study one of our main goals was to find out the effect of naringenin and biofilm on increasing the rice plant yields compared to only biofilm application.

When the yield results obtained for the experiment 1 are compared (Figure 3), each and every important yield component, the filled grain weight/plant (Figure 3A), the total grain weight/plant (Figure 3C), the filled grain number/plant (Figure 3D), total grain number/plant (Figure 3F) showed a significant increment for the treatment AAB/Nar compared to AAB. Ladha et al. (2016) [5], has stated that nitrogen as the *sine qua non* (absolute necessity) of today's high yielding agriculture. Higher amount of nitrogen application can increase the amount of photosynthate which influences dry matter production resulting higher root volume and dry matter content [25].

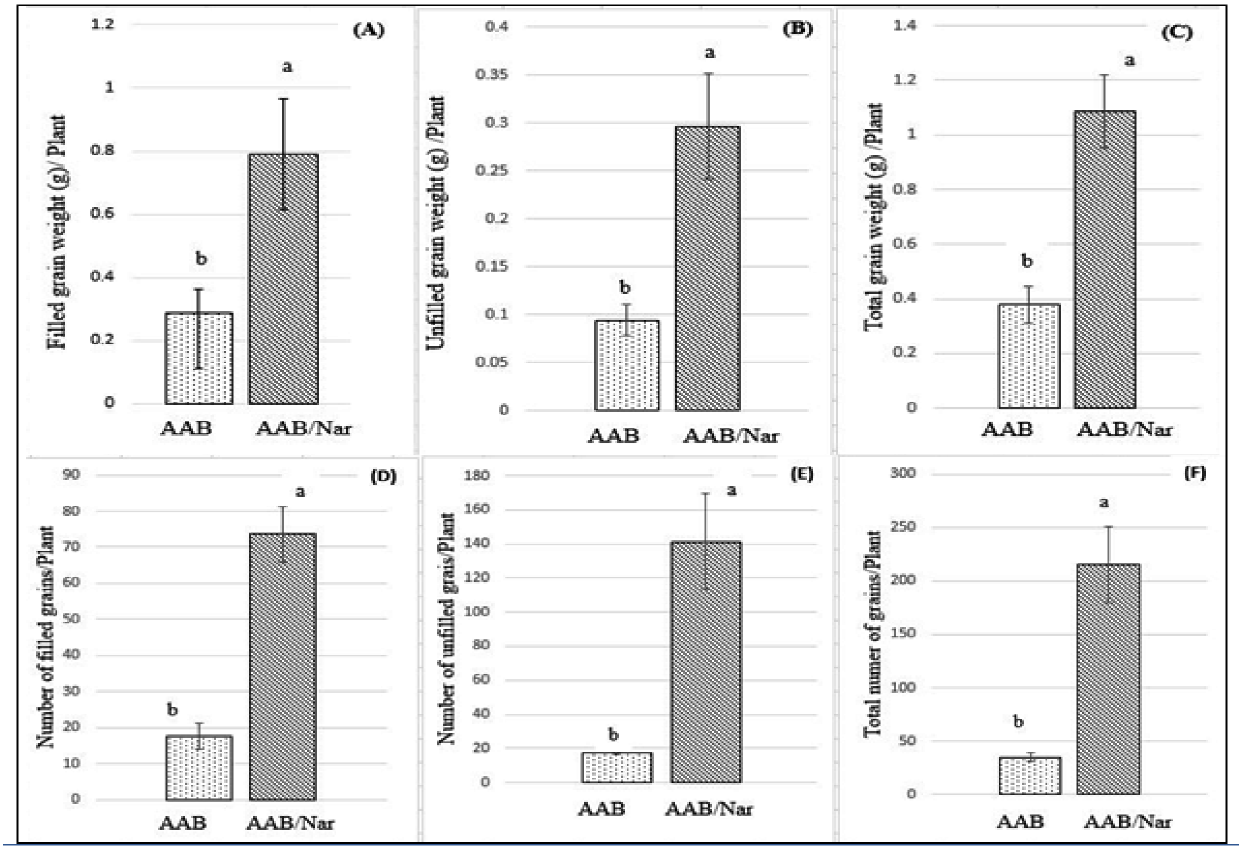

Figure 3: Comparison between the treatments, AAB Vs AAB/Nar Treatment on Filled grain weight /Plant (A), Unfilled grain weight/Plant (B), Total grain weight/Plant (C), Number of filled grains/Plant (D), number of unfilled grains/Plant (E), Total number of grains/Plant (F). Data analyzed by one-way ANOVA of SAS, using Duncan's Multiple range test (P = 0.05). Data followed by different letters differ significantly.

Nitrogen becomes a limiting factor to obtain the maximum yield of rice [26]. For a plant to gain the maximum yield, the optimum source, sink balance should be maintained [27]. In rice plant, the period of panicle primordial initiation and late stage of spikelet formation, nitrogen absorbed, contribute mostly to the formation of spikelets [6]. Addition of nitrogen in the neck node initiation stage is very important for spikelet production [6]. Nitrogen accumulation in panicles is a result of nitrogen absorbed by the plant in all growth stages, from the seedling stage to the grain filling stage [6]. Number of panicles depends on the number of primary, secondary and tertiary tillers [28]. Higher tillering leads to higher number of panicles and thereby more spikelets [29]. Hence the Number of tillers/area is an important determinant of the rice plant yield [30], and more fertile tillers will result more yield [31]. Therefore it is clear that the tillering ability of the plant affect the number of panicles, and the degree of panicle branching and seed setting rate of the spikelets determine the number of spikelets which determines the number of grains [29].

Physiological transformation of apical meristem in to panicle meristem demarcates the transformation of the vegetative phase to reproductive phase [28].

Higher yields obtained for AAB/Nar in experiment 1 could be due to the better vegetative growth that has taken place during the vegetative and reproductive phases (up to 105 days). When the colonization data are compared, as given in Figure 1, AAB/Nar has significantly higher colonization values in all critical vegetative and reproductive growth stages where nitrogen is highly required and utilized by the plant, compared to AAB. Previous studies have explained the direct contribution that flavonoid naringenin has in *Azorhizobium caulinodans* colonization through acting as a signaling molecule in enhancing the bacterium *Azorhizobium caulinodans* colonization in rice plant roots [13, 16-18, 32]. Flavonoids are usually secreted by leguminous plant roots and they induce the transcription of nod genes. Naringenin is referred to as the most efficient nod gene inducer [33].

Presence of 10^{-5} M or 10^{-4} M flavonoid naringenin is reported to increase *A. caulinodans* colonization of rice lateral root cracks, xylem region and the root cortex [13, 16-18, 32]. Flavonoids have also been found to induce the expression of other bacterial genes with unknown functions [33, 34]. These are extremely important findings as the colonization of a non-legume plant by a diazotroph can be led to the possibility of assimilating atmospheric nitrogen by a non-leguminous plant such as rice, which is otherwise carried out only by leguminous plants.

Significantly higher rice yields when AAB/Nar is added to organically grown rice

After it was observed that addition of AAB/Nar can improve rice yields in to significant levels, our next attempt was to find out the impact on rice yield by the developed bio fertilizer in an organically grown rice field. A field experiment was conducted in an organically grown rice field in the low country wet zone of Sri Lanka. The main organic fertilizer used was compost. Here, it was found out that, by the addition of the developed bio fertilizer AAB/Nar coupled with recommended levels of organic fertilizers, rice yields, or the number of filled grains and the total number of grains incremented in to significantly higher levels (Figure 4). The developed AAB must have supplemented the compost through the unique beneficial characteristics a bio fertilizer gains in the biofilm mode of life. Biofilms are considered as bio-fertilizers with substantial amount of benefits on plant growth and yield. The benefits are far greater than the application of conventional microbial monocultures or the application of fertilizers [11]. Vessey (2003) [35] defines a bio-fertilizer as "A substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant".

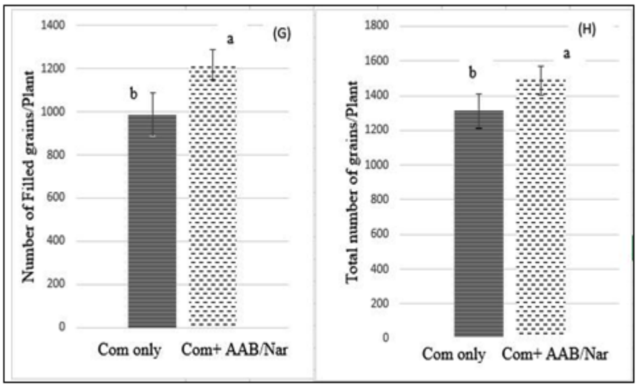

Figure 4: Comparison between the treatments, Compost only Vs Compost + AAB/Nar. Data analyzed by one-way ANOVA of SAS, using Duncan's Multiple range test (P = 0.05). Data followed by different letters differ significantly.

When a fungal-rhizobial biofilm or a fungal-bacterial biofilm is added to a growing medium of a plant, several beneficial effects are provided to the increment of the growth and yields of the plant [11]. To increase the growth and yield of a plant, biofilms contribute in numerous ways. Competition suppression, increase of available oxygen in the fields, action of the biofilms as plant growth promoting rhizo-bacteria, pest and disease control, are some major methods [11, 36]

Conclusions

It can be concluded that the developed *Azorhizobium caulinodans-Aspergillus* spp. biofilm enhances the rice yields when coupled with flavonoid naringenin. When this combination, is applied to conventionally grown rice fields or to organically grown rice fields, the rice yields showed a significant increase. This is an extremely important finding with a possibility of aiding future challenges reduction of inorganic fertilizer usage in agriculture.

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