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Consortium Diazotrophic Bacteria for Increasing Rice (*Oryza sativa*) Growth, Physiology and Yield

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Abstract

The research was aimed to examine the effectiveness of the *Bacillus tropicus* (BT)*, Acinetobacter junii* (AJ) and *B. subtilis* (BS) bacterial consortium in increasing the growth and yield of rice (*Oryza sativa* L.). This research was conducted at Experimental Farm and Agronomy & Horticulture Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto from June to October 2023. This study was designed using a non-factorial Randomized Block Design with 9 treatments and repeated three times and there were 27 experimental units. The treatments included A (positive control with NPK fertilization), B (negative control without NPK fertilization), C (BT), D (AJ), E (BS), F (BT + AJ), G (BT + BS), H (AJ + BS), I (BT + AJ + BS). The test plant used Inpari Unsoed 79 Agritan rice variety. Results showed that the diazotrophic bacteria consortium was able to increase plant growth seen from leaf area, plant dry weight, root dry weight and was able to induce nitrate reductase enzyme activity. The *BT + AJ + BS* consortium was the best treatment that was able to provide the best grain yield, reaching 22.89 g per clump.

Keywords: Rice; Consortium; Diazotrophic: Nitrogen; Yield

Introduction

Rice (*Oryza sativa* L.) production in Indonesia is still very dependent on chemical fertilizer intake, especially urea as a source of nitrogen. Nitrogen (N) application occupies the largest portion compared to phosphate (P) and potassium (K) fertilizers so that in the field it has an impact on nutritional imbalance for rice production. N application at the farmer level can currently reach 300-600 kg ha^{-1} and farmers consider that urea tends to quickly affect plant growth (Widowati *et al.* 2011). Fikriawan *et al.* (2024) reported that in soil with moderate nutrient status, the urea dose only ranged from 145.65-236.95 kg ha^{-1}. On the other hand, very high doses will certainly have an impact on the environment, and several studies have shown that they do not significantly increase yields. The negative impact of excessive fertilizer application is pollution of the environment, both soil and surface water, with ecosystem eutrophication, water quality degradation, causing the greenhouse effect, and disrupting the food chain in the ecosystem (Jayanudin *et al.* 2022).

Excessive application of nitrogen fertilizer has an impact on excessive N absorption and has an impact on excess N residue in the soil and conversely, N deficiency affects photosynthesis, resulting in decreased yields (Zhu *et al.* 2023). Zhao *et al.* (2022) reported that excessive N application causes very high N concentrations in plant tissues, increasing N metabolism and increasing carbohydrate consumption, which causes carbohydrate translocation to the grain to decrease, thereby reducing yields. On the other hand, high dose N reduces the level of fertilization efficiency. Gewaily *et al.* (2018) reported that increasing N doses up to 220 kg ha^{-1} gave the highest grain yield, but agronomic efficiency decreased with increasing N doses, where the highest agronomic efficiency was achieved at a dose of 165 kg ha^{-1} N, reaching 4.12 kg grain yield produced per kg of N applied. Furthermore, Liang *et al.* (2021) stated that excessive nitrogen application simultaneously reduces N utilization, yield and rice quality.

High dose N application in addition to reducing efficiency, but also long-term application will reduce soil fertility both physically, chemically and biologically. Ge *et al.* (2018) reported that NPK fertilization significantly reduced the C/N ratio of soil and pH, in soil acidification

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caused by continuous high dose N fertilization. Therefore, it is necessary to find environment friendly technology that can reduce the impact of high dose N fertilization in the long term. Biological approach is one of the rights and environmentally friendly alternatives. The use of beneficial microbes that have N-fixing capabilities is expected to reduce the use of N from chemical fertilizers.

Rice plants in addition to fertilizers get N from mineralization of organic matter and from NH_4 and NO_3, and supplies from biological N fixation activities carried out by prokaryotic diazotrophs, which are widespread among the Bacteria and Archaea domains (Pittol *et al.* 2016). Purwanto *et al*. (2017) reported that N-fixing bacteria originating from the rhizosphere of rice plants not only act as N fixers but also have the ability to stimulate growth with the ability to produce indole acetic acid (IAA). Naher *et al*. (2009) obtained bacteria from the genera *Rhizobium, Burkholderia* and *Corynebacterium* originating from irrigated rice fields with the ability to fix N_2, produce cellulase enzyme and IAA. Purwanto *et al.* (2022) reported that *Bacillus tropicus, Acinetobacter junii* and *B. subtilis* indigenous from saline rice fields on the North Coast of Pemalang had been isolated which had the ability to fix N_2 and were also able to produce IAA. *Stenotrophomonas maltophilia*, *Enterobacter* sp., *Bacillus* sp., *Ochrobactrum haematophilum* and *Pseudomonas aeruginosa* from the rice rhizosphere, in addition to being N fixers, also act as growth stimulants (Mir *et al.* 2022).

The effectiveness of diazotrophic bacteria in supporting the growth and yield of rice plants can be done through single inoculants or consortium inoculants. Consortium inoculants will provide a better effect where with the application of the consortium there is a multi-traits action and reduces the risk of failure of microbial utilization in the field (Pas *et al.* 2015). Purwanto and Suharti (2021) reported that the R11 isolate + *Rhizobium* sp. LM-5 consortium was able to increase yields by up to 177.38% compared to the control. Another study by Kaur *et al*. (2022) reported that the *Erwinia* sp. EU-B2SNL1, *C. arthrosphaerae* EU-LWNA-37 and *P. gessardii* EU-MRK-19 consortium was able to increase barley yields compared to single inoculants, without inoculation and chemical fertilization and was able to reduce the dose of NPK fertilizer. Other study results as reported by Mattos *et al*. (2023) showed that the *A. brasilense* + *P. fluorescens* consortium was able to reduce the use of N fertilizers by up to 30 kg ha^{-1} with higher results compared to chemical fertilization, and increase the efficiency of N use, and increase 37% in economic production with better agronomic performance. This study aimed to examine the effectiveness of the *B. tropicus, A. junii* and *B. subtilis* bacterial consortium in increasing the growth and yield of rice crop.

Materials and Methods

Experimental site and design

This research was conducted at The Experimental Farm, and Agronomy and Horticulture Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto from June to October 2023. This study was designed using a non-factorial randomized block design with 9 treatments and repeated three times so that there were 27 experimental units. The treatments included A (positive control with NPK fertilization), B (negative control without NPK fertilization), C (BT), D (AJ), E (BS), F (BT + AJ), G (BT + BS), H (AJ + BS), I (BT + AJ + BS). The test plant used Inpari Unsoed 79 Agritan rice variety.

Inoculum preparation

The N-fixing bacterial species that have been successfully rejuvenated, each species was inoculated as many as 5 mL into 50 mL of different nutrient broth medium in Erlenmeyer and shaken at a speed of 120 rpm for 24 h. The bacterial stock was then counted colonies using the TPC (total plate count) method. Each N-fixing bacterial isolate had a minimum population abundance of 108 CFU/mL. The bacterial stock culture was applied as much as 2 mL/isolate and applied at the age of 14 and 35 days after planting.

Nursery and planting of rice

The seeds were soaked for 24 h then sterilized by soaking the seeds in 70% alcohol for 1 min, then drained and soaked again in 0.8% bayclin solution for 5 min, then drained again and rinsed with sterile distilled water 4 times for 1 min. The sterilized rice seeds were then sown using soil media and watered until the media was wet. Watering was done every 2 days or when the media was dry. A 25-day old seeds were then transplanted. A 25-day old seeds were transferred to soil media in a bucket. Rice fertilization used various types of fertilizers including urea, KCl, and SP-36. Fertilization was done 2 times. The first fertilization was 7 weeks after planting; the second fertilization was 28 weeks after planting.

Observation variables

The variables observed in this study included plant height (cm), leaf area (cm^2), number of tillers per clump, plant dry weight (g), chlorophyll a, b and total content (mg g^{-1}), nitrate reductase activity (NRA), N uptake (g per plant) and grain weight per clump (g).

Data analysis

The research data were analyzed using ANOVA, and if the results showed a significant difference, it was continued with DMRT α: 5% in DSAASTAT v. 1.514.

Results

Vegetative growth

Inoculation of consortium of diazotrophic bacteria gave significant effect only on leaf area of rice plants, but did not give any effect on plant height. Plant height did not show any difference between consortium of diazotrophic bacteria treatment compared to without inoculation treatment or plants given inorganic fertilization. Leaf area of rice plants increased with application of diazotrophic bacteria with both single inoculant and consortium. Results showed that inoculation with consortium of diazotrophs gave average leaf area of 881.95 cm^2, higher than leaf area in single inoculant treatment (698.31 cm^2). Inorganic fertilization treatment and without fertilization only gave leaf area of 642.95 and 536.12 cm^2, respectively. The consortium of *B. tropicus + A. junii + B. subtilis* gave the highest leaf area of 910.65 cm^2, although it was not significantly different from the consortium of AJ + BS, BT + BS and BT+AJ (Table 1).

The results showed that inoculation of diazotrophic bacteria, either single inoculant or in the form of a consortium, significantly increased the dry weight of roots and dry weight of shoots. The consortium of AJ + BS and BT + AJ + BS gave the highest dry weight of roots, respectively, at 7.78 and 6.0 g $plant^{-1}$. On the other hand, the dry weight of shoots also increased significantly with inoculation of single inoculum or diazotrophic bacterial consortium. The highest dry weight of shoots was seen in the consortium of AJ + BS was 16.85 g $plant^{-1}$, although it was not different from the application of single inoculant or other diazotrophic bacterial consortia (Table 1).

Table 1: The effect of consortium diazotrophic bacteria on vegetative growth of rice plant

Treatments	Plant height (cm)	Leaf area (cm^2)	Root dry weight (g)	Plant dry weight (g)
With NPK	87.94 a	642.95 ab	2.96 a	10.55 ab
Without NPK	91.81 a	536.12 a	2.79 a	7.27 a
BT	90.57 a	635.52 ab	4.23 ab	11.84 abc
AJ	98.22 a	734.95 bc	4.01 ab	14.89 bc
BS	91.54 a	724.46 bc	3.91 ab	10.63 ab
BT + AJ	92.60 a	879.32 d	3.55 a	11.03 abc
BT + BS	93.20 a	844.64 cd	3.38 a	12.29 abc
AJ + BS	97.16 a	893.18 d	7.78 c	16.85 c
BT + AJ + BS	94.64 a	910.65 d	6.00 bc	14.61 bc

Description: numbers followed by the same letter in the same column are not significantly different according to DMRT α: 5%

Table 2: The effect of different treatments on chlorophyll contents of rice plant

Treatments	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophylls (mg/g)
With NPK	0.7083 a	0.7088 a	0.7082 a
Without NPK	0.7084 a	0.7095 a	0.7087 a
BT	0.7085 a	0.7099 a	0.7090 a
AJ	0.7084 a	0.7095 a	0.7087 a
BS	0.7084 a	0.7089 a	0.7082 a
BT + AJ	0.7082 a	0.7091 a	0.7085 a
BT + BS	0.7086 a	0.7098 a	0.7089 a
AJ + BS	0.7084 a	0.7097 a	0.7089 a
BT + AJ + BS	0.7083 a	0.7094 a	0.7086 a

Description: numbers followed by the same letter in the same column are not significant different according to DMRT α: 5%

Photosynthetic pigments

The application of diazotrophic bacteria consortium or single inoculant has not given significant effect on chlorophyll a, b and total chlorophyll contents. Total chlorophyll a content in diazotrophic bacteria inoculation treatments did not show any increase compared to inorganic fertilization treatments or treatments without diazotrophic bacteria inoculation. Chlorophyll a content in various diazotrophic bacteria inoculation treatments ranged from 0.7082-0.7086 mg g^{-1}; while inorganic fertilization treatments and treatments without diazotrophic bacteria inoculation had chlorophyll a contents of 0.7083 and 0.7084 mg g^{-1}, respectively. Chlorophyll b contents showed no significant difference between treatments. Chlorophyll b contents in various diazotrophic bacteria treatments ranged from 0.7089-0.7089 mg g^{-1}; while the inorganic fertilization treatment and the treatment without diazotrophic bacteria inoculation had contents of 0.7088 and 0.7095 mg g^{-1} respectively. The total chlorophyll contents in various diazotrophic bacteria treatments did not display shown significant differences but still were in the range of 0.7082 - 0.7090 mg g^{-1}; whilst the total chlorophyll levels in the inorganic fertilization treatment and without inorganic fertilization were 0.7082 and 0.7087 mg g^{-1} respectively (Table 2).

NRA

The NRA of rice plants increased with diazotrophic bacteria inoculation, either single inoculant or consortium. The NRA values in various diazotrophic bacteria treatments did not differ from inorganic fertilization treatments. Inoculation with diazotrophic bacteria had higher NRA compared to treatments without inorganic fertilization (B). The highest NRA was achieved in the inoculation treatment of BT + BS reaching 0.93 μmol NO_2/g/hour, but did not differ from the inoculation treatments of AJ, BS, BT + AJ, AJ + BS and BT + AJ + BS (Fig. 1).

N uptake

The results showed that the application of diazotrophic bacterial consortium was able to increase N uptake of rice plants compared to treatments without fertilization or treatments with NPK fertilization. The highest N uptake was achieved in the AJ + BS consortium, followed by the BT + AJ + BS consortium treatment of 205.61 and 170.98 mg $plant^{-1}$, respectively (Fig. 2).

Rice grain yield

The results of the study showed that inoculation of diazotrophic bacteria consortium was able to significantly increase rice yields. The diazotrophic bacteria inoculation treatment was able to provide an average rice yield of 15.65

g/hill, and this result was higher than the inorganic fertilization and without inorganic fertilization treatments of 10.17 and 11.01, respectively (experiencing an increase of 53.88 and 42.14%, respectively). The diazotrophic bacteria consortium treatment was able to produce an average of 18.36 g/hill (an increase of 52.53%), while the single inoculant treatment only produced 12.04 g/hill of rice. The highest results were achieved in the BT + AJ + BS treatment which was able to produce 22.89 g/clump of grain, although it was not different from the AJ + BS treatment which produced 21.41 g/clump of grain (Fig. 3).

Discussion

Rhizosphere bacteria are bacteria that originate from the rhizosphere of plants, and the interaction between beneficial bacteria and plants will have a positive impact on plant growth. The ability of bacteria to support growth through the ability as N fixer is one of the abilities of bacteria in supporting plant growth and is an environment friendly strategy in crop management. Diazotrophic bacteria are able to support plant growth through multiple traits in addition to the ability to fix N, they are also able to produce IAA (Islam *et al.* 2009). Purwanto *et al*. (2017) reported that diazotrophic bacteria isolated from the rhizosphere of rice in addition to having the ability to fix N are also able to increase rice plant growth through the ability to produce IAA.

The results of this study showed that inoculation of diazotrophic bacteria in a consortium can increase plant growth as seen from the variables of leaf area, plant dry weight, and root dry weight which increased significantly. Application of a consortium of dizotrophic bacteria increases plant vegetative growth. This is in line with the provision of N that can be absorbed by plants for vegetative growth. Application of a consortium of *B. tropicus, A. junii*, and *B. subtilis* bacteria can support plant growth not only through nitrogen-fixing ability but these bacteria have the ability to produce IAA (Purwanto *et al.* 2022), so that it can have an impact on increasing root growth. This is in line with the opinion of Shin *et al.* (2016) where diazotrophic bacteria can directly affect plant growth through phytohormone synthesis, improving nutrient absorption, solubilization of inorganic phosphate and mineralization of organic phosphate.

Direct interaction of plants with soil and root microbes occurs in the root area so that the direct effect of the inoculation of the diazotropic bacteria consortium can be seen from root growth. The results showed that the application of the diazotrophic bacteria consortium significantly increased the dry weight of the roots. Inoculation of the AJ + BS and BT + AJ + BS consortium gave the highest dry root weights of 7.78 and 6.00 g plant^{-1}, respectively. These weights were significantly greater compared to the treatment without fertilization, which were 178.85 and 114.93%, respectively, and increased by 162.54 and 102.36% against the NPK fertilization treatment. The

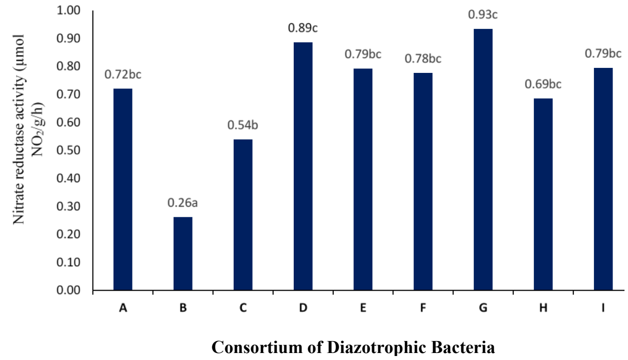

Fig. 1: The effect of consortium diazotrophic bacteria on nitrate reductase activity. A (positive control with NPK fertilization), B (negative control without NPK fertilization), C (BT), D (AJ), E (BS), F (BT + AJ), G (BT + BS), H (AJ + BS), I (BT + AJ + BS)

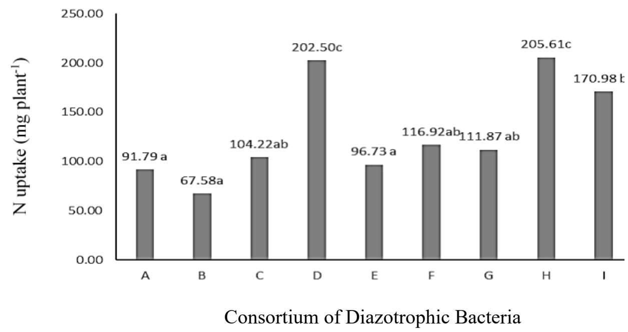

Fig. 2: The effect of consortium diazotrophic bacteria on N uptake. A (positive control with NPK fertilization), B (negative control without NPK fertilization), C (BT), D (AJ), E (BS), F (BT + AJ), G (BT + BS), H (AJ + BS), I (BT + AJ + BS)

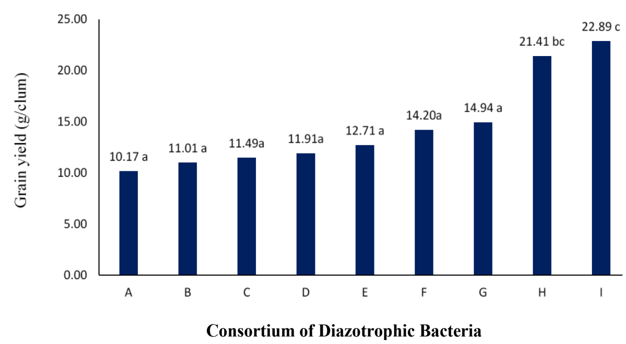

Fig. 3: The effect of consortium diazotrophic bacteria on rice yield. A (positive control with NPK fertilization), B (negative control without NPK fertilization), C (BT), D (AJ), E (BS), F (BT + AJ), G (BT + BS), H (AJ + BS), I (BT + AJ + BS)

ability of diazotrophic bacteria as PGPR with the ability to produce IAA affected root growth, both in terms of root length and size. Moreover, application of the consortium with the genus *Bacillus* sp. gave the best root dry weight. *Bacillus* sp. is a species of bacteria that has the ability to fix N, solubilize P and produce auxin (Cortes *et al.* 2020). Thus, the ability to support growth through auxin production can modify root architecture and root tissue structure through its ability to balance hormonal levels in plants (Vacheron *et al.* 2013).

Increased root growth, in this case an increase in root dry weight will be followed by an increase in other characters, especially an increase in the length and surface area of the roots that interact with soil colloids so that it is expected to increase nutrient absorption from the soil. This can be seen that the inoculation of diazotrophic consortium AJ + BS and BT + AJ + BS was able to increase N uptake in rice plants. This result is in line with Hossain *et al.* (2020) that the application of diazotrophic bacteria in this case is *Bacillus cereus* was able to increase N contents in tissue by up to 1.52% and N absorption reaches 48.12 mg g^{-1} with N application of 50% of the recommendation. This is in line with Purwanto and Suharti (2021) where the application of PGPR and diazotrophic bacteria, both single inoculants and consortia, was able to increase N absorption and the PGPR consortium was able to increase N absorption by up to 83.27%. Increased N uptake by rice plants in various inoculations of diazotrophic bacteria consortium, although not showing significant differences, but the amount of plant chlorophyll, a, b and total was increased. N is one of the components of the leaf chlorophyll structure, so that increasing chlorophyll levels will increase plant photosynthesis, thereby improving plant growth (Putri *et al.* 2017). This is supported by the increasing leaf area of the plant so that it can support plant photosynthesis. The direct effect of this process is an increase in plant dry weight as a net result of photosynthesis. Silva *et al.* (2019) showed that inoculation of diazotrophic bacteria in pineapple plants was able to support plant growth through the production of IAA and nitrogen for plant metabolism, resulting in increased plant dry weight.

Diazotrophic bacteria inoculation, both single inoculation and consortium, can increase the activity of the NR enzyme, compared to the control, although it does not show any difference with the NPK fertilization treatment. The NR enzyme is the first enzyme that catalyzes the reaction and rate of nitrate assimilation, the reduction of nitrate to nitrite (Koyama *et al*. 2020). N is absorbed by plants in the form of nitrate or ammonium, and nitrate must be reduced to ammonium in plant tissue before amino acid synthesis is carried out and ammonium is directly synthesized into amino acids after being absorbed by plants. Diazotrophic bacteria inoculation increases the NRA, so that it can increase plant N absorption, thereby encouraging plant growth (Silva *et al*. 2019). Santos *et al.* (2019) reported that inoculation of a consortium of diazotrophic bacteria was able to increase the activity of the NR enzyme and was able to induce leaf area growth, besides biomass and nutrient accumulation in stems of sugarcane.

The rice grain yield was seen to increase with the inoculation of diazotrophic bacteria consortium. The highest grain yield was achieved in the inoculation treatment of BT + AJ + BS followed by the AJ+BS consortium of 22.89 and 21.41 g $clump^{-1}$, respectively. The diazotrophic consortium showed better results than single inoculants. This is related to the multi-trait ability of the diazotrophic bacteria where as PGPR it also acts as N fixer. N is able to increase the vegetative organs of plants, especially in root characteristics such as root dry weight so that the plant's ability to absorb nutrients increases. This is in line with the finding of Mboyerwa *et al.* (2022) where there was a correlation between the characteristics of rice plant roots, especially root dry weight and rice yield. On the other hand, consortium inoculation can increase leaf area, photosynthetic pigments, and N absorption in plant tissue so that metabolism, both photosynthesis and N assimilation, increase through assimilate partitioning from the source to the sink. This is supported by increased N absorption and nitrate reductase enzyme activity with diazotrophic consortium inoculation, compared to control. Increased N absorption will be followed by increased NRA, producing ammonium which is then incorporated into protein (Zhu *et al.* 2023). Thus, inoculation of diazotrophic bacteria consortium with multi-traits characters as biofertilizer will have a positive impact on the growth and yield of rice plants.

Conclusion

Based on the research results, it can be concluded that the diazotrophic bacteria consortium was able to increase plant growth seen from leaf area, plant dry weight, root dry weight and was able to induce nitrate reductase enzyme activity. The *B. tropicus* + *A. junii* + *B. subtilis* consortium was the best diazotrophic bacteria consortium being able to provide the best grain yield, reaching 22.89 g per clump.

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Author Contributions

P: Conceptualization, Methodology, Data Curation, Supervision, writing, Funding acquisition. NWAL: data analysis, supervision, AS: validation, language review. RNKS: data validation, writing, LNB: data analysis and writing, JEBM: data collection, and data analysis.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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