Potential of Agriculture By-Product for Improving N-Fixing Bacteria Performance in Acid Soil

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Summary

Nitrogen-fixing bacterial (Nfb) isolates are sensitive to acidic pH, potentially hindering their activity in acidic soils. To address this challenge, a study was conducted to explore the potential of coconut water, rice bran, and molasses—agricultural by-products—in enhancing the performance of nitrogen-fixing bacteria for boosting soybean (*Glycine max* L) productivity in the acidic soil of Inceptisol-Jatinangor, West Java, Indonesia. The study employed a completely randomized design (CRD) with six treatments and three replications. The treatments comprised: (A) control (Nfb), (B) Nfb in compost, and (C to F) Nfb in compost supplemented with molasses, coconut water, rice bran, and a combination of all three organic stimulant. Biodynamic patterns of Nfb, chlorophyll content index, nodule count, plant height and pod number were evaluated. Results indicated that coconut water treatment yielded the highest *Azotobacter* spp. density. Moreover, coconut water and mixed stimulant treatments significantly boosted the chlorophyll content index by 46.77% and 50.99%, respectively, along with notable increases in nodule count, plant height, and pod number. *Azospirillum* spp. density exhibited a positive correlation with the chlorophyll content index, while the latter strongly influenced plant height and pod production. Coconut water emerged as the most effective stimulant, enhancing Nfb effectivity and elevating soybean pod numbers in Inceptisol-acid soil by 62.3% compared to Nfb without stimulant agent.

Key words

Azospirillum spp., *Azotobacter* spp., *Glycine max* L, Inceptisol, organic-stimulant

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Introduction

In Indonesia, the Inceptisol order spans a vast area of 69.62 million hectares, constituting approximately 37.0% of the country's land area (Pusat Penelitian Tanah dan Agroklimat Indonesia, 2000). Despite its extensive coverage, this land faces significant agricultural challenges. Characterized by slightly acidic to acidic soils with low organic matter and nutrient deficiencies, it presents hurdles to farming endeavours. Laboratory analysis of Inceptisol Jatingor soil revealed a pH of 5.48 and low organic matter levels, contributing to suboptimal crop yields, particularly for soybeans—a crop of critical importance in Indonesia due to its role as a protein source. Therefore, enhancing the productivity of Inceptisol is imperative for bolstering soybean output and ensuring food security in the region.

One potential solution to enhance soil productivity involves utilizing Plant Growth Promoting Rhizobacteria (PGPR) as a biofertilizer. However, PGPR may not always yield the desired effects and can even diminish in population density when introduced into acidic soils. Similarly, the efficacy of nitrogen-fixing inoculants in acidic soil can also decrease. Notably, among the most effective nitrogen-fixing bacteria within the PGPR group are *Azotobacter* spp. and *Azospirillum* spp.

Azotobacter spp., a nitrogen-fixing bacterium, converts atmospheric nitrogen gas into a plant-usable form (NH_4). *Azotobacter* genus belongs to the gram-negative, free-living bacteria group within the heterotrophic category (Martyniuk Martyniuk, 2003). During nitrogen fixation, it forms distinct structures, produces abundant Exopolysaccharides (Gomare et al., 2011; Hindersah et al., 2019; Suryatmana et al., 2024); stimulates plant growth and resides in the rhizosphere, although its abundance varies depending on the crop species (Bashan, de-Bashan, 2010). These bacteria also produce indole acetic acid (IAA) and gibberellins (GA), promoting seed germination. However, they are sensitive to acidic soil, high salt levels and extreme temperatures (Jnawali et al., 2015). The viability and abundance of *Azotobacter* spp. in soil are influenced by factors such as soil pH, temperature, moisture and other microbial communities (Kizilkaya, 2009). Thus, careful consideration must be given to the application of these bacteria to address specific challenges like nutrient deficiencies and soil acidity.

Azospirillum, a genus of PGPR, is known to colonize numerous tropical and subtropical plants (Silva et al., 2017). According to Rasool et al., (2015), *Azospirillum* spp. is an N-fixing, aerobic, gram-negative bacterium that not only stimulates plant growth but also produces phytohormones that further enhance plant growth. However, its effectiveness is compromised by sensitivity to pH variations (Bashan de-Bashan, 2010), leading to reduced efficacy when applied to plant seeds.

The fluctuating soil environmental conditions present challenges for maintaining the presence of inoculated *Azospirillum* spp. and *Azotobacter* spp. Consequently, it is imperative to develop an effective strategy to understand the biodynamic performance of these bacteria, thereby enhancing their efficacy when applied to plant seeding in acidic soil conditions. Additionally, successful colonization of plant roots is a crucial prerequisite for achieving optimal PGPR effects. The extent of association establishment and compatibility between the PGPR microbiome and plant roots significantly influence growth stimulation (Mangmang et al., 2015). Therefore, essential strategies are necessary, including fostering a robust association process and improving the viability of both *Azotobacter* spp. and *Azospirillum* spp. with plant roots.

The prospective strategy for enhancing the performance of *Azotobacter* spp. and *Azospirillum* spp. inoculants involves optimizing the formulation of carrier bases and stimulant materials to support their growth. Coconut water, rice bran and molasses stand out as potential agricultural by-products suitable for this purpose. These abundant resources in Indonesia hold significant potential as PGPR inoculant carrier additives and warrant thorough evaluation.

In acidic soil conditions, supplementation with steel is necessary to improve the effectiveness of *Azotobacter* spp. and *Azospirillum* spp. isolates. Studies by Mangmang et al., (2015), Kizilkaya, (2009), and Wang et al., (2015) have reported a decline in the efficacy and activity of N-fixer bacteria (Nfb) when inoculated into acidic soil environments.

Compost is commonly utilized as a carrier base for PGR inoculants due to its organic nature, providing a suitable habitat for microbial isolates. Rich in carbon (C), nitrogen (N), phosphorus (P) and other essential minerals, compost sustains microbial viability, thereby enhancing its effectiveness. Most of these organic substances serve as carriers for biofertilizers. The C/N ratios in most finished compost products are between 12 and 20. Available C to N is a critical parameter for biodegradation, and optimal composting occurs with a C/N ratio between 20 and 30 (Rynk et al., 1992). The chemical characteristics of the varying compost types have pH (6.30 -7.8: neutral), N-total (from 0.95 to 1.68%. : very high), total P_2O_5 (0.27 to 1.13 : very high), K_2O_2 (0.25 to 2.11 % : high), C-organic (16.6 to 25.06% : very high), CEC (125.05 me 100 g^{-1} : very high), compost moisture content (20.15%), C/N ratio (114.22 to 8.52, Ca (0.10%), Mg (0.30%), Fe (0.97%), Mn (0.09%), Cu (7.43 ppm) and Zn (46.64 ppm) (Sebayang et al., 2023, Khater, 2015). The nutrient content of the compost is suitable for carrier inoculant Nfb.

Coconut water, comprising approximately 91.33 % water, and containing sugars (sucrose, glucose, fructose and sorbitol) up to 3 %, along with 0.27 % protein, 0.14 % fat, and 38 % carbohydrates, also contains a diverse array of nutrients including vitamin C, vitamin B complex (riboflavin, thiamine, biotin), and essential minerals such as potassium, sodium, calcium, magnesium, iron, copper, phosphorus, and sulfur (Vijayaram et al., 2014; Prades et al., 2012). Despite coconut water being extensively studied for its chemical composition, its potential contribution to enhancing the effectiveness of N-fixing bacteria such as *Azotobacter* spp. and *Azospirillum* spp. in acidic soils may remain underappreciated.

Molasses, a by-product of the sugar industry, is rich in nutrients. According to Preichardt et al., (2019), it contains significant levels of reducing sugars (12.49 %), non-reducing sugars (35.63 %), as well as sodium (46.12 mg 100g^{-1}), potassium (292.46 mg 100 g^{-1}), manganese (8.36 mg 100 g^{-1}), magnesium (118.38 mg100 g^{-1}), iron (0.83 mg 100 g^{-1}) and phosphorus (65.5 mg 100 g^{-1}). Chikhoune et al. (2014) further identified sucrose (68.36 %), glucose (18.50 %), and maltose (13.14%) in molasses. The molasses used in this study exhibited a protein content of 12.87%, fat content of 0.112 %, crude fiber content of 11.37%, calcium content of 0.08%, available phosphorus content of 0.22%, and magnesium content of 0.21%.

Rice bran, the outer husk of grains removed during processing to obtain rice grains or white flour, is composed of crude fiber (lignin and cellulose) (12.48%), fat (18.80%), protein (13.66%), carbohydrate (40.63%), phytic acid (50.68 mg g^{-1}), oryzanol (3.5%), and tocopherol (40.94 mg g^{-1}) (Moonggarm et al., 2012).

Based on the nutrient content of these three types of by-products, they are highly potential to be used as stimulant materials as carrier agents for inoculants to stimulate their activity and viability. However, further examination is still needed to assess their effectiveness as a stimulating agent.

The aim of this study was to assess the potential of coconut water, rice bran, and molasses as stimulatory additives within a compost carrier base to enhance the performance of a mixed culture of *Azotobacter* spp. and *Azospirillum* spp. when applied to the rhizosphere of *Glycine max* L. in Inceptisol-acid soil. The researchers investigated the stimulator additives' capacity to improve the viability and biodynamic patterns of *Azotobacter* spp. and *Azospirillum* spp., as well as nodulation formation, chlorophyll content index, plant growth, and yield of *Glycine max* L. cultivated in acidic Inceptisol soil in Jatinangor, West Java, Indonesia.

Material and Methods

This experiment was conducted at the Ciparanje Experimental Field, Faculty of Agriculture, Padjadjaran University, West Java, Indonesia. It is located at an average altitude of 774 meters above sea level. The experiment field and layouts are shown in Fig. 1.

Experimental Design

The experimental design employed a completely randomized design (CRD) methodology, testing three types of stimulators at a concentration of 2% (weight/weight of compost base carrier). These were combined with N-fixation using a consortium of *Azotobacter* spp. and *Azospirillum* spp. as Nfb isolates, with a cell density of 10^7 CFU g^{-1} carriers based on compost. Each treatment was replicated three times.

Six treatments were tested in this study:

A. Control (Nfb liquid culture)
B. Nfb in compost
C. Nfb in compost added molasses
D. Nfb in compost added coconut water
E. Nfb in compost added rice bran
F. Nfb in compost added mixture (molasses + coconut water + rice bran).

Preleminary Analysis of Soil

For this experiment, we utilized Inceptisol-acid soil sourced from the Ciparanje area of Jatinanagor, Faculty of Agriculture at the University of Padjadjaran in West Java, Indonesia. Initially, soil analysis was conducted to determine its chemical properties (pH, total N, P-available), physical characteristics (soil texture), and biological density of *Azotobacter* spp. and *Azospirillum* spp. Additionally, waste coconut water, rice bran derived from rice husks ground to 100 mesh and molasses obtained from sugarcane juice waste in the sugar industry were employed.

Culture Enrichment of *Rhizobium japonicum, Azotobacter.* spp. and *Azospirillum* spp.

Rhizobium japonicum, *Azotobacter* spp. and *Azospirillum* isolates stock culture were purchased from the collection of Soil Biology Labortorium of Agriculture, University of Padjadjaran. To cultivate the pure stock cultures of *Rhizobium japonicum*, *Azotobacter* spp. and *Azospirillum* spp., we employed liquid media of Potato dextrose, Ashby and Okon, respectively. The cultures were propagated in 500 ml Erlenmeyer flasks, with each flask filled to 250 ml with the specific medium. Each medium was inoculated with 10 % (v/v) of the respective stock culture, followed by incubation for 72 hours on a rotary shaker at 120 rpm and a temperature of 30 °C.

Figure 1. The experiment field and layouts. The layout preparation of the Inceptisol acid-soil in a test pot (left); The soy bean plant in vegetative phase condition after treatment (right)

Mixing of Organic Stimulant and The Culture of *Azotobacter* spp., and *Azospirillum* spp.

Azotobacter spp. and *Azospirillum* spp. cultures with a density of 10^8 CFU ml^{-1} were incorporated into compost powder (100-mesh size) at a 5 (v/wt) ratio. Subsequently, organic additives including coconut water, rice bran, and molasses were added to the microbial carrier at 2% (wt/wt) of the compost to further enhance its efficacy.

Preparation of Planting Medium and N-Fixing Inoculant Application

To cultivate soybeans, we utilized pots measuring 25 cm in height and 20 cm in diameter. Each pot was filled with 8 kg of Jatinangor Inceptisol, into which we incorporated the N-Fixing inoculant based on carrier compost and additives for each treatment. Following an incubation period of one week, soybean seeds were mixed with *Rhizobium japonicum* inoculum and planted at a depth of 2 cm in the soil. The pots were spaced 20 × 20 cm apart, with one seed planted in each hole. Soil moisture was maintained at field capacity, and recommended soybean fertilizer doses of 50 kg urea ha^{-1} (50% recomanded dose), 100 kg SP-36 ha^{-1}, and 150 kg KCl ha^{-1} were applied. The chlorophyll content, total population density of *Azotobacter* spp. and *Azospirillum* spp., number of root nodules, plant height, and pod number per plant until the end of the observation period were observed.

Statistical Analysis

To analyze the experimental data, we utilized Statistical Product and Service Solutions (SPSS) version 15.0. An Analysis of Variance (ANOVA) was conducted to identify significant differences at a 5 % significance level ($P < 0.05$). Furthermore, correlation and regression analyses were performed using the SPSS program version 15.0

Results and Discussion

Characteristic of Inceptisol

Table 1 presents the results of the analysis detailing the chemical, physical and biological characteristics of the Inceptisol soil employed in the experiment. The soil utilized exhibited very low levels of available P_2O_5 (2.71 ppm P) and potential P_2O_5 (14.27 mg 100 g^{-1}), alongside a clay texture (67 %), with 30 % dust and 3 % sand. Additionally, the levels of C-organic (1.58 %), N-total (0.2 %), and Al-dd (0.03 cmol kg^{-1}) were characterized as low. With an acidic pH (5.48) and a cation exchange capacity (CEC) of 21.73 cmol kg^{-1} categorized moderate, the Jatinangor Inceptisols utilized in the study were deficient in essential nutrients such as N and P, featuring a high Mg content and clay texture. The chemical properties of the experimental soil illustrate that the soil is poor in essential nutrients such as N and P, and is acidic, so it is necessary to add an inoculant that is effective and adaptive to the soil conditions.

The Composition of Nutrient in Coconut Water, Rice Bran and Molasses

Table 2. show cases the nutritional constituents present in coconut water, rice bran and molasses. These stimulant materials play a vital role in furnishing the organic compounds and nutrients essential for creating optimal conditions conducive to microbial thriving. The analysis indicates that coconut water and molasses contain sucrose, fructose, and glucose, whereas rice bran lacks these sugars and boasts a high C/N ratio. Moreover, coconut water features biotin and glutamic acid among its components.

Biodynamic Viability of *Azospirillum* spp. Population Density in Rhizosphere during Soybean Plant Growth

Fig 2. illustrates the impact of organic additives on the population dynamics of *Azospirillum* spp. The control treatment (Nfb culture without stimulant) exhibited an initial increase in the population of *Azospirillum* spp. during the third week after soil inoculation. However, this population subsequently declined below the initial level by the following week's vegetative phase. This suggests that the *Azospirillum* spp. culture inoculated into Inceptisol-acid soil without a carrier or additives struggled to adapt to the acidic soil condition. According to Zhou et al. (2013), soil pH significantly influences the activity and viability of *Azospirillum* spp. The optimal pH range for *Azospirillum* spp. is 5.7–7.8, with populations decreasing in pH levels below 5.8. In this study, the pH of the Inceptisols was 5.48, resulting in a lower density of *Azospirillum* spp. compared to the initial population density observed in the control treatment.

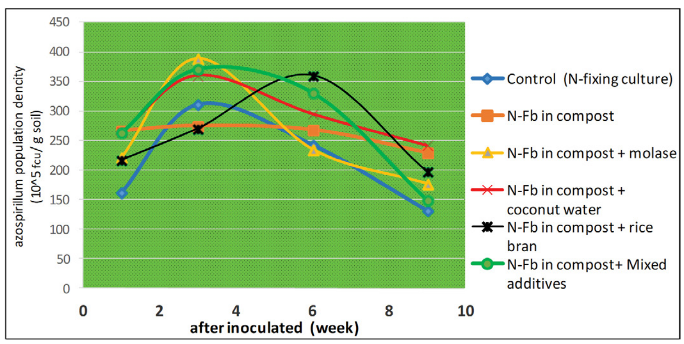

Figure 2. Biodynamic pattern of *Azospirillum* spp. density in the soybean rhizosphere after inoculation to the soil

The population dynamics of *Azospirillum* spp. on the compost base carrier were notably higher after the addition of molasses in the third week compared to other treatments. Additionally, the compost supplemented with coconut water treatment yielded a higher population than the control, compost alone, and compost with bran. This observation suggests that molasses and coconut water possess the capacity to stimulate the growth of *Azospirillum* spp. The potential of coconut water as an stimulant carrier stems from its composition, which includes concentrations of glucose, fructose and sucrose at 1.51%, 1.55%, and 1.80%, respectively (refer to Table 2). These concentrations of nutrient in coconut water likely serve as suitable carbon sources for the growth of *Azospirillum* spp.

Table 1. Inceptisol acid soil properties

No	Parameters	Unit	Value	Criteria
1	pH H_2O (1 : 2,5)	-	5.48	acid
2	pH KCl 1 N (1 : 2,5)	-	4.61	-
3	Organic Carbon	%	1.58	low
4	N Total	%	0.20	low
5	C/N	-	8	low
6	Available -P (P_2O_5)	ppm P	2.71	very low
7	Potential-P	mg 100 g^{-1}	14.27	very low
8	K_2O $HCl_2P\%$	mg 100 g^{-1}	19.02	low
9	Cation Exchange Capacity (CEC)	cmol kg^{-1}	21.73	moderate
10	Soil Texture:			
	Sand	%	3	
	dust	%	30	Clay
	clay	%	67	

Moreover, studies have indicated that aspartic acid and glutamic acids act as strong attractants (Compton and Sharf, 2021), and also potentially modulate the metabolic system within *Azospirillum* spp. cells. Specifically, they may influence the glutamate metabolism system or glutamate decarboxylase (GAD) system, aiding in overcoming acidic stress conditions (Feehily Karatzas, 2013). The glutamate decarboxylase (GAD) system plays a crucial role in combating acid stress across various bacterial genera, maintaining intracellular pH homeostasis by utilizing protons in a decarboxylation reaction to release γ-aminobutyrate (GABA) from glutamate (Feehily Karatzas, 2013)

Meanwhile, the mixed stimulant contains a more diverse array of carbon sources and nutrients which are present in suitable concentrations for N-fixing bacteria. Molasses, for instance, boasts a high sugar concentration, exceeding 68 %, comprising glucose, sucrose, and maltose (Chikhoune et al., 2014) (see Table 2). This abundance of sugars provides an ample carbon source for energy production, thereby enhancing the growth rate of *Azospirillum* spp.

In contrast, when treated with rice bran, the population of *Azospirillum* spp. reached its peak six weeks later but declined by the ninth week. Rice bran is recognized for its capacity to stimulate the growth of *Azospirillum* spp., albeit at a slower rate compared to molasses or coconut water. This delay can be attributed to the high levels of carbohydrates, cellulose and hemicellulose present in rice bran, which require time to break down into monosaccharides, serving as a carbon source for *Azospirillum* spp. growth. Nevertheless, the abundance of carbohydrates in rice bran renders it a suitable carbon source for sustaining *Azospirillum* spp. growth over an extended duration. It is crucial to acknowledge that rice bran does not offer an immediate carbon source for *Azospirillum* spp. growth, unlike glucose, sucrose, or fructose.

Table 2. Nutritional composition of coconut water, rice bran and molasses

The composition	Coconut water (%)	Rice bran (%)	Molasses (%)
Water content	95.5	14.04	20
Sucrose	1.80	nd	34.5
Glucose	1.51	nd	6
Fructose	1.55	nd	9.5
C-organic	3.8	37.81	3.5
N-total	0.76	0.67	4.2
Phosphorus (P)	0.02	1.03	0.08
Potassium (K)	5	0.98	4.15
C/N	5	56.79	11.90
Biotin	0.02 mgL^{-1}	nd	nd
Glutamic acid	0.165	nd	0.252
Cellulose	nd	11	nd
Hemicellulose	nd	9.5	nd

Noted: nd = not detected

Biodynamic Viability of *Azotobacter* spp. Population Density in Rhizosphere during Soybean Plant Growth

Fig 3. illustrates the biodynamic pattern of *Azotobacter* spp. with and without stimulant treatment. In the compost carrier, the population of *Azotobacter* spp. peaked within three weeks but experienced a significant decline thereafter. Conversely, the control treatment exhibited no increase in *Azotobacter* population. This suggests that the *Azotobacter* spp. inoculant utilized in this study was sensitive to acidic pH. This is evident from the growth curve of *Azotobacter* spp. in the liquid culture of Nfb without a carrier or additives, which is lower. In other words, acidic soil conditions inhibit the growth of *Azotobacter* spp. when no carrier is present. Therefore, the carrier compost and additives serve as protectors, shielding *Azotobacter* spp. from acidic conditions."

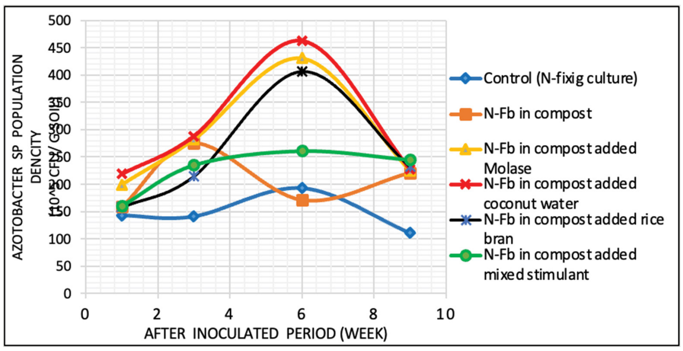

Figure 3. Biodynamic pattern of *Azotobacter* spp. density in the soybean rhizosphere after being inoculated to the soil.

This study found that the growth of *Azotobacter* spp. peaked after six weeks of being added to a mixture of molasses, coconut water and rice bran. The density of *Azotobacter* spp. was higher in this mixture compared to the control, compost and mixed additives. However, the density of *Azotobacter* spp. decreased after nine weeks. Conversely, the density of *Azotobacter* spp. was higher in a mixture of compost, compost-added molasses, coconut water and rice bran compared to the control treatment. The study has also demonstrated that rice bran, molasses and coconut water have the potential to stimulate the growth of *Azotobacter* spp. in acidic Inceptisol. Additionally, the study has found that *Azotobacter* spp. can utilize complex organic materials like rice bran, molasses, and coconut water.

According to previous research, coconut water has been found to be highly effective in increasing the population of *Azotobacter* spp. in acidic Inceptisol soil. *Azotobacter* spp. demonstrates a remarkable ability to utilize the carbon source present in coconut water (Vijayaram et al., 2014). Prades et al. (2012) report that coconut water contains various nutrients, including different types of sugars (sucrose, glucose, fructose, sorbitol), proteins, fats, carbohydrates, vitamins and essential minerals such as potassium, sodium, calcium, magnesium, iron, copper, phosphorus and sulfur. Gutiérrez-Rojas et al., (2011) observed a significant increase in *A. chroococcum* biomass productivity when exposed to 13.06 g l^{-1} of sucrose as a carbon source. In this study, the 1.8% sucrose content in coconut water was found to be suitable for *Azotobacter* spp. as a carbon source, thereby aiding in maintaining the viability of the isolate in acidic soil conditions. Additionally, coconut water contains glutamic acid, which plays a protective role against acidic stress conditions. Glutamic acid helps regulate intracellular pH homeostasis systems, thereby safeguarding against acidic stress conditions, as reported by Feehily Karatzas, (2013).

Through this investigation, it was revealed that *Azotobacter* spp. could effectively utilize diverse carbon sources present in rice bran, including mannitol and various monosaccharides, as energy sources. The viability and dynamic behavior of the soil microbiome in the interactions among *Azospirillum* spp., *Azotobacter* spp., and *Rhizobium japonicum* are pivotal for establishing successful attachment and colonization of plant roots, ultimately influencing the plant's nutrition and overall health.

Chlorophyll Content Index of Soybean Leaves

After planting soybean plants, leaf chlorophyll levels were observed during the vegetative phase three weeks later. The results of the statistical analysis are presented in Table 3. The control treatment exhibited the lowest chlorophyll content index, which was significantly different ($P < 0.05$). Conversely, the treatment involving mixed additives, such as coconut water, molasses and bran, demonstrated the highest increase in leaf chlorophyll content. Although not significantly different from the coconut water treatment alone, the addition of mixed additives led to a 46.77% and 50.99% increase in the chlorophyll content index compared to the control treatment. Notably, the inclusion of molasses did not result in a significant increase in the chlorophyll content index.

Coconut water and a mixture of additives (mollases, coconut water and rice bran) offer rich nutrients that are particularly advantageous for the growth and activity of *Azospirillum* spp. and *Azotobacter* spp. These bacteria play a crucial role in nitrogen fixation, contributing to the provision of essential nutrients for plants and enhancing nitrogen absorption by plant roots. By fostering the activity of nitrogen-fixing bacteria, soybean plants can leverage their capabilities to fulfil their nitrogen requirements for chlorophyll synthesis. This elevation in chlorophyll levels, a pivotal pigment for plant growth (Li et al., 2018; Wang et al., 2012), correlates positively with nitrogen absorption by plants, thereby serving as an indicator of photosynthesis rate. Ensuring adequate nitrogen availability is paramount to bolster chlorophyll content and facilitate photosynthesis, thereby fostering improved plant growth.

The study underscores that the combination of coconut water and mixed additives positively influences the population of N-fixing bacteria and leaf chlorophyll content in soybean plants. A significant correlation was observed between the population of *Azospirillum* spp. and the Chlorophyll Content Index (CCI) of the leaves, as illustrated in Fig 4. The linear regression equation,

$$Y = 4.33 \times 10^{-7} X + 21.357,$$

where Y represents the Chlorophyll Content Index (CCI) and X represents the population of *Azospirillum* spp. (CFU g^{-1}), suggests that a one-unit increase in the *Azospirillum* spp. population will correspond to a 4.33×10^{-7} increase in the CCI of the leaves. This indicates a significant relationship between the population of *Azospirillum* spp. (independent variable) and the chlorophyll index of the leaves (dependent variable). Moreover, coconut water and

Table 3. Effect of adding organic additives to the content index of chlorophyll in soybean leaves

Treatment Code	Treatment	Chlorophyll content index of soybean leaves (CCI)	Increasing of chlorophyll content index of soybean leaves (%)
A	Control (Nfb liquid culture)	25.3000[a]	-
B	Nfb in compost	30.6667[b]	21.21
C	Nfb in compost added molasses	25.9000[a]	2.37
D	Nfb in compost added coconut water	37.1333[c]	46.77
E	Nfb compost added rice bran	28.3667[b]	12.12
F	Nfb in compost added mixed additive	38.2000[c]	50.99

Note: According to Duncan's multiple distance test, numbers followed by the same letter indicate no significant difference at a $P < 0.05$ significance level. Nfb = Nitrogen-fixing bacteria

mixed additives exhibited potential in enhancing the performance of *Azospirillum* spp., thereby contributing to an elevation in the CCI of soybean plants under acidic soil conditions.

The Number of Root Nodules of Soybean Plant

Soybean plants establish a symbiotic relationship with Rhizobia symbiotic nitrogen-fixing bacteria, by forming nodules their roots. The impact of various types of organic additives on the formation of root nodules is depicted in Table 4. Positive interactions occur between *rhizobium* and non-symbiotic Nfb (*Azotobacter* spp. and *Azospirillum* spp.), as evidenced by the increased nodule formation in Nfb inoculant with carrier and additives.

Table 4. illustrates the application of various organic additives. The findings reveal that the use of organic additives such as molasses, coconut water and mixed additives led to a significant increase in the formation of root nodules ($P < 0.05$). However, rice bran and compost showed no significant effect compared to the control treatment. Specifically, the coconut water treatment exhibited the highest number of nodules (88.33 per plant), representing a 35.20 % increase compared to the control. Conversely, the number of root nodules in the Nfb culture treatment without additives tended to be lower than in the control treatment.

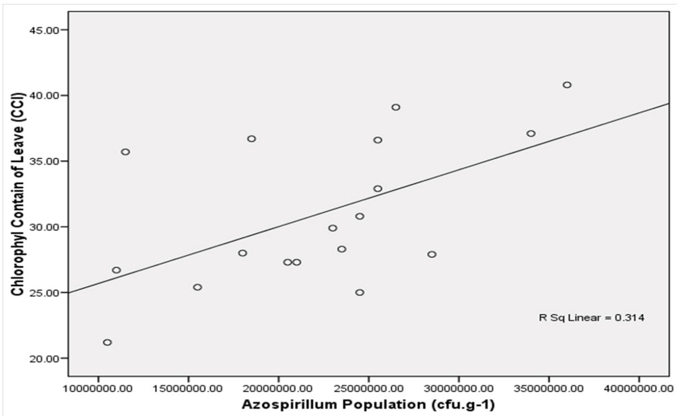

Figure 4. The graph of correlation – regression between chlorophyll contain index and *Azospirillum* spp. density population (CFU g^{-1}). The linear regression equation is $Y = 4,33.10^{-7}X + 21,357$, where Y = Chlorophyll contain index (CCI) of leaves (CCI), and X = *Azospirillum* spp. population density (CFU g^{-1}).

The number of root nodules in the Nfb culture treatment without additives tended to be lower than in the control treatment, owing to various factors such as the compatibility between *Rhizobium japonicum* and its host, external environmental conditions and interactions with surrounding microbes. Coconut

Table 4. The number of root nodules of soybean plants

Treatment Code	Treatment	Number of root nodules	Increasing of number of root nodules (%)
A	Control (Nfb culture)	65.33[a]	-
B	Nfb in compost	58.33[a]	-10.71
C	Nfb in compost with molasses	85.67[b]	31.12
D	Nfb in compst with coconut water	88.33[b]	35.20
E	Nfb compost with rice bran	68.33[a]	4.08
F	Nfb in compost with mixed additive	84.67[b]	29.59

Note: According to Duncan's multiple distance test, numbers followed by the same letter indicate no significant difference at a $P < 0.05$ significance level. Nfb = Nitrogen-fixing bacteria water, molasses and stimulant mixtures were identified as effective stimulants for soybean root nodule formation. Meanwhile, rice brand and compost did not significantly increase the nodules formation. Previous studies have highlighted that coconut water contains crucial amino acid compounds like leucine, lysine, tyrosine, histidine, tryptophan, threonine, phenylalanine, valine, methionine and cysteine, as well as fructose (Li et al., 2018), all of which play vital roles in nodule formation. Additionally, compounds such as glutamic acid, sucrose and glucose found in coconut water and molasses serve as effective attractants for *Rhizobium japonicum* and can facilitate nodule formation. Extracellular polymeric substance (EPS) plays a crucial role in facilitating interactions between *Azotobacter chroococcum* strain AC.C112 and *Rhizobium* species strain RS.C112. (Compton Scharf, 2021; Stambulskaa Lushchak, 2015).

Sugars from coconut water or molasses can also stimulate to produce EPS by *Azotobacter* spp. (Helmy et al., 2008). Moreover, EPS's produced by *Azotobacter* spp. impacts the number of effective nodules in soybean roots, since EPS's acts as an adhesive substance, facilitating the attachment of *Azotobacter* spp. to the root surface and aiding in the mobilization of nitrogen from the surrounding environment into the plant roots. EPS acts as an adhesive substance, facilitating the formation of channels for *Rhizobium japonicum* attachment to the root surface, thereby initiating nodulation and ultimately leading to increased formation of nodules. This mechanism serves as a model for the attachment of *Rhizobium japonicum* and *Azotobacter* sp to plant roots.

Soybean Plant Height

The plant height serves as an indicator of growth progress, and the statistical analysis assessing the effects of various treatments on plant height is detailed in Table 5.

The treatment utilizing N-fixing bacteria supplemented with coconut water emerged as the most effective, yielding a plant height of 36.4 cm seven weeks after planting—an impressive increase of 46.77% compared to the control treatment. Incorporating Nfb into compost, along with coconut water, significantly enhances ($P < 0.05$) the population and activity of both *Azotobacter* spp. and *Azospirillum* spp., consequently supplying plants with essential nitrogen nutrients.

Coconut water provides the ideal nutrients and carbon sources for *Azotobacter* spp. and *Azospirillum* spp., leading to accelerated bacterial growth and enhanced nitrogen fixation. This research has found that the presence of *Azospirillum* spp. bacteria expedites the process of root surface colonization and nodulation in soybean plants. This is advantageous as these nitrogen-fixing bacteria contribute to plant growth by supplying essential nutrients and phytohormones such as cytokinin and indole-3-acetic acid (IAA) (Reddy et al., 2018; Sumbul et al., 2020). Studies indicate that *Azotobacter* spp. and *Azospirillum* spp. produce proteins, amino acids and growth substances that bolster plant growth. Cytokinin facilitates cell division during vegetative growth by interacting with nucleic acids precursor substances.

Moreover, the efficacy of inoculants on plant growth hinges on their ability to colonize effectively. This study has revealed that colonization of *Azotobacter* and *Azospirillum* on soybean roots occurs through an attachment process, which is believed to be influenced by the presence of coconut water stimulant containing glucose, sucrose and fructose. These sugars serve as suitable carbon sources for the growth of these bacteria and facilitate their attachment and colonization onto soybean roots. Velmourougane et al. (2017) note that *Azotobacter chrocooccum* produces EPS during biofilm formation in individual cultures in Jensen's broth media, containing 2% sucrose as a carbon source. This study suggests that glucose and sucrose present in coconut water stimulate *Azotobacter* spp. or *Azospirillum* spp. to produce EPS and extracellular material, thereby enhancing the effectiveness of bacteria attachment to soybean roots. As a result of the roles of *Azotobacter* spp. and *Azospirillum* spp. in appropriate stimulant compounds, there is an increase in maximum nutrient absorption by the roots, thereby promoting increased plant height growth.

The essential yield component investigated in this study was the number of filled pods per plant. The statistical analysis results regarding the pod production attributed to these treatments are presented in Table 6. The inoculation treatment of N-fixing bacteria with added coconut water exhibited a significant effect ($P < 0.05$) on the number of soybean pods, yielding an average of 37.30 pods per plant, marking a remarkable increase of 62.32 % compared to the control . Conversely, the treatment involving Nfb without additives in compost and rice bran did not show any significant difference among each other.

Table 5. Effects of inoculation with N-fixing bacteria and additives on soybean plant height

Treatment Code	Treatment	Plant height (cm)	Increasing of plant height (%)
A	Control (Nfb liquid culture)	25.3000[a]	-
B	N-fb in compost	30.6667[bc]	21.21
C	N-fb in compost with molasses	25.5667[a]	1.05
D	N-fb in compost with coconut water	37.1333[d]	46.77
E	N-fb compost with rice bran	28.7000[ab]	13.44
F	N-fb in compost with mixed additive	34.8667[cd]	37.81

Note: According to Duncan's multiple distance test, numbers followed by the same letter indicate no significant difference at a 5% significance level. Nfb = Nitrogen-fixing bacteria

Table 6. Effect of N-fixing bacteria and additives on the number of soybean pods

Treatment Code	Treatment	Number of soybean pods/ plant	Increasing of number of soybean pods/ plant (%)
A	Control (Nfb liquid culture)	23.00[a]	-
B	Nfb in compost	25.00[a]	8.70
C	Nfb in compost with molasses	29.33[b]	27.54
D	Nfb in compost with coconut water	37.30[c]	62.32
E	Nfb compost with rice bran	26.33[ab]	14.49
F	Nfb in compost with mixed additive	33.67[b]	46.38

Note: According to Duncan's multiple distance test, numbers followed by the same letter indicate no significant difference at a $P < 0.05$ significance level. Nfb = Nitrogen-fixing bacteria

This study revealed that coconut water was a stimulating agent that enhanced the interaction among *Rhizobium japonicum*, *Azotobacter* spp., and *Azospirillum* spp., thereby bolstering nitrogen supply to plants. According to Jnawali et al. (2015), the availability of nitrogen significantly influences the formation of proteins, amino acids, protoplasm, chlorophyll and ultimately, plant yield. Moreover, as highlighted by Vijayaram et al., (2014) and Prades et al., (2012), coconut water serves as a nutrient source for soil microbes, containing vital compounds such as sugars (sucrose, glucose, fructose and sorbitol) up to 3%, protein 0.29%, fat 0.15%, carbohydrates 7.27%, and vitamins C and B complex (riboflavin, thiamine and biotin), along with essential amino acids. These constituents enhance the efficacy of *Azotobacter* spp. and *Azospirillum* spp., consequently promoting improved plant growth and increased soybean pod numbers.

Conclusion

The addition of coconut water, molasses and their combination led to an increased population density of *Azospirillum* spp. compared to the control treatments (Nfb liquid culture and Nfb in compost). *Azospirillum* spp. reached its peak population density in three weeks, while in rice bran, it took six weeks to achieve the highest population peak (late exponential phase). Coconut water emerged as the most effective stimulant in enhancing the population density of *Azotobacter* spp., with the peak density occurring in the sixth week. Nodule formation increased by 31.12%, 35.20 %, and 29.59 % with the addition of molasses, coconut water and mixed additives, respectively. Furthermore, coconut water elevated plant height by 46.77 % and the number of pods by 62.32% compared to the control treatment. The population density of *Azospirillum* spp. exhibited a positive correlation with the increase in the chlorophyll content index of soybean leaves, while plant height positively correlated with the number of pods produced. Overall, coconut water emerged as the most effective stimulant in enhancing the performance of N-fixing bacteria (*Azotobacter* spp. and *Azospirillum* spp.), resulting in a higher pod yield compared to the control treatment in Inceptisol-acid soil. This research holds significant relevance and potential utility for the agricultural sector in Indonesia. Given Indonesia's surplus of agricultural by-products, leveraging these resources could enhance the economic value of agricultural by-products and boost soybean yields in acidic soil.

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CRediT authorship contribution statement

Pujawati Suryatmana: conceptualization, performed most of the experiments, supervision, writing original draft, funding acquisition, methodology. **Diyan Herdiyantoro:** contributed to the investigation, formal analyses data, validation, software, resources. **Nadia Nuraniya Kamaluddin:** contributed to data curation, data validation, visualization. **Mieke Rochimi Setiawati:** project administration, contributed to writing-review and editing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest, no competing financial interests or personal relationships that could influence the work reported in this paper.

References

Bashan Y., de-Bashan L. E. (2010). Chapter Two—How the Plant Growth-Promoting Bacterium *Azospirillum* Promotes Plant Growth—A Critical Assessment. In D. L. Sparks (Ed.), Advances in Agronomy 108: 77–136. Academic Press. doi: 10.1016/S0065-2113(10)08002-8

Chikhoune A., Bedjou F., Oubouzid S., Boukefoussa R., Bechri B., Tarmoul H., Abdeladim T., Tounsi A., Hamitri M., Chikh S., Kouadri L. (2014). Development of Sugar Cane Molasses in Formulations of Madeleines, Mini Croissants, and Buns Incorporated with Interesterified Oil. J Chem 2014: 936780. doi: 10.1155/2014/936780

Compton K. K., Scharf B. E. (2021). Rhizobial Chemoattractants, the Taste and Preferences of Legume Symbionts. Front. Plant Sci. 12. doi: 10.3389/fpls.2021.686465

Feehily C., Karatzas K. A. G. (2013). Role of Glutamate Metabolism in Bacterial Responses towards Acid and Other Stresses. J Appl Microbiol 114 (1): 11–24. doi: 10.1111/j.1365-2672.2012.05434.x

Gomare K. S., Mese M., Shetkar Y. (2011). Isolation of *Azotobacter* and Cost Effective Production of Biofertilizer. Indian J Appl Res 3 (5): 54–56. doi: 10.15373/2249555X/MAY2013/14

Gutiérrez-Rojas I., Torres-Geraldo A. B., Moreno-Sarmiento N. (2011). Optimising Carbon and Nitrogen Sources for *Azotobacter chroococcum* Growth. Afr J Biotechnol 10 (15): 2951–2958. doi: 10.5897/AJB10.1484

Helmy Q., Suryatmana P., Kardena E., Funamizu N., Wisjnuprapto. (2008). Biosurfactants Production from *Azotobacter* spp. and Its Application in Biodegradation of Petroleum Hydrocarbon. Journal of Applied and Industrial Biotechnology in Tropical Region 1 (Special Edition): 1-5.

Hindersah R., Risanti R. R., Haikal I., Mahfud Y., Nurlaeny N., Rachmadi M. (2019). Effect of *Azotobacter* Application Method on Yield of Soybean (*Glycine max* (L.) Merill) on Dry Land. Agric 31 (2): 136–145. doi: 10.24246/agric.2019.v31.i2.p136-145

Jnawali A. D., Ojha R. B., Marahatta S. (2015). Role of *Azotobacter* in Soil Fertility and Sustainability–A Review. Adv Plants Agric Res 2 (6): 250-253 doi: 10.15406/apar.2015.02.00069

Khater E. S. G. (2015.) Some Physical and Chemical Properties of Compost. Int J Waste Resour 5 (1): 2-5. doi:10.4172/2252-5211.1000172

Kizilkaya R. (2009). Nitrogen Fixation Capacity of *Azotobacter* spp. Strains Isolated from Soils in Different Ecosystems and Relationship between Them and the Microbiological Properties of Soils. J Environ Biol 30 (1): 73—82.

Li Y., He N., Hou J., Xu L., Liu C., Zhang J., Wang Q., Zhang X., Wu X. (2018). Factors Influencing Leaf Chlorophyll Content in Natural Forests at the Biome Scale. Front Ecol Evol 6. doi: 10.3389/fevo.2018.00064

Mangmang J. S., Deaker R., Rogers G. (2015). Early Seedling Growth Response of Lettuce, Tomato and Cucumber to *Azospirillum brasilense* Inoculated by Soaking and Drenching. Hort Sci(Prague) 42 (1): 37–46. doi: 10.17221/159/2014-HORTSCI

Martyniuk S., Martyniuk M. (2003). Occurrence of *Azotobacter* spp. in Some Polish Soils. Pol J Environ Stud 12 (3): 371–374.

Moongngarm A., Daomukda N., Khumpika S. (2012). Chemical Compositions, Phytochemicals, and Antioxidant Capacity of Rice Bran, Rice Bran Layer, and Rice Germ. 3rd International Conference on Biotechnology and Food Science (ICBFS 2012), April 7-8, 2012, 2: 73–79. doi: 10.1016/j.apcbee.2012.06.014

Prades A., Dornier M., Diop N., Pain J.-P. (2012). Coconut Water Uses, Composition and Properties: A Review. Fruits 67 (2): 87–107. doi: 10.1051/fruits/2012002

Preichardt L. D., Haubert L., Sawitzki M. C., Bertol T. M., Vicenzi R., Meinhart A. D., Silva, W. P., Fiorentini Â. M. (2019). Multivariate Optimization of *Staphylococcus xylosus* AD-1 Biomass Production Using Sugarcane Molasses plus Yeast Extract and Soybean Meal. Acta Sci Biol Sci 41 (1): 2-10. doi: 10.4025/actascibiolsci.v41i1.47487

Pusat Penelitian Tanah dan Agroklimat Indonesia. (2000). In *Sumberdaya lahan Indonesia dan pengelolaannya* (1st ed., pp. 169–172). Pusat Penelitian Tanah dan Agroklimat, Badan Penelitian dan Pengembangan Pertanian, Departemen Pertanian.

Rasool L., Asghar M., Jamil A., Rehman S. U. (2015). Identification of *Azospirillum* Species from Wheat Rhizosphere. J Anim. Plant Sci 25 (4): 1081–1086.

Reddy S., Singh A. K., Masih H., Benjamin J. C., Ojha S. K., Ramteke P. W., Singla A. (2018). Effect of *Azotobacter* sp and *Azospirillum* sp on Vegetative Growth of Tomato (*Lycopersicon esculentum*). J Pharmacogn Phytochem 7 (4): 2130–2137.

Rynk R., van de Kamp M., Willson G. B., Singley M. E., Richard T. L., Kolega J. J., Gouin F. R., L. Laliberty L. Jr., Kay D., Murphy D. W., Hoitink H. A. J., Brinton, W. F. (1992). The Composting Process, Characteristics of Raw Materials, in On-Farm Composting Handbook, pp. 106 - 113. NRAES-54, Cornell University Press, Ithaca, NY

Sebayang N. U. W., Akasah W, Manurung V. R., Sinamo K. N., Perangin-angin, G. A., Lesmana A. F., Rahma K. A. (2023). Chemical Characteristics and SEM Analysis of Compost from Cassava Peel Waste and Cow Manure: Production by Pendawa I Farmer Group, Candi Rejo Village. ABDIMAS TALENTA Jurnal Pengabdian Kepada Masyarakat 8 (1): 604 – 610.

Silva L. B. da Silva L. L., da Ferreira E. P., de Santos B., d'Eça N. K. F., Martin-Didonet C. C. G. (2017). Polyphasic Vharacterization of Endophytic Bacteria of Sorghum Grown on Cerrado Soil of the Goiás State – Brazil. Int J Dev. 7: 9287

Sumbul A., Ansari R. A., Rizvi R., Mahmood I. (2020). *Azotobacter*: A Potential Bio-Fertilizer for Soil and Plant Health Management. Saudi J Biol Sci 27 (12): 3634–3640. doi: 10.1016/j.sjbs.2020.08.004

Suryatmana P., Handayani S., Bang S., Hindersah R. (2024). Screening and Profiling of Mercury-Resistant *Azotobacter* Isolated from Gold Mine Tailing in Pongkor, West Java. J Degraded Min Lands Manag 11 (2): 5287–5300. doi: 10.15243/jdmlm.2024.112.5287

Velmourougane K., Prasanna R., Singh S. B., Kumar R., Saha S. (2017). Sequence of Inoculation Influences the Nature of Extracellular Polymeric Substances and Biofilm Formation in *Azotobacter chroococcum* and *Trichoderma viride*. FEMS Microbiol Ecol 93 (7): 1-13. doi: 10.1093/femsec/fix066

Vijayaram S., (2014). Natural Sources of Coconut Component Used for Microbial Culture Medium (NSM). Int. J Pharm Sci Rev Res 26 (2): 28–32.

Wang H., Liu S., Zhai L., Zhang J., Ren T., Fan B., Liu H. (2015). Preparation and Utilization of Phosphate Biofertilizers Using Agricultural Waste. J Integr Agric 14 (1): 158–167. doi: 10.1016/S2095-3119(14)60760-7

Wang M., Shi S., Lin F., Hao Z., Jiang P., Dai G. (2012). Effects of Soil Water and Nitrogen on Growth and Photosynthetic Response of Manchurian Ash (*Fraxinus mandshurica*) Seedlings in Northeastern China. PLOS One 7 (2): e30754. doi: 10.1371/journal.pone.0030754

Ya Stambulskaa U., Lushchak I. V. (2015). Efficacy of Symbiosis Formation by Pea Plants with Local Western Ukrainian Strains of *Rhizobium*. Journal of Microbiology, Biotechnology and Food Sciences 5 (2): 92–98. doi: 10.15414/jmbfs.2015.5.2.92-98

Zhou S., Han L., Wang Y., Yang G., Zhuang L., Hu P. (2013). *Azospirillum humicireducens* spp. Nov., a Nitrogen-Fixing Bacterium Isolated from a Microbial Fuel Cell. Int J Syst Evol Microbiol 63 (7): 2618–2624. doi: 10.1099/ijs.0.046813-0

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