Bacterial support as a biostimulant agent (BPNIII, Azzofor) for marginal soil fertility and stimulating seedlings growth in nursery

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Abstract. A nursery is the first preparation step for the reclamation of marginal land, supported by fertile, strong, and adaptable seedling to the environment. Therefore, the role of bacteria as a bio stimulating agent is needed to stimulate the growth of seedling during nursery and to enable their subsequent adaptation to the environment. The purpose of this study is to determine the effectiveness and survival rate of bacteria as a growing medium in marginal soil fertilization as well as the accelerated effect of the vegetative growth of seedling during the nursery. This research used the Completely Randomized Design with 3 treatments and 10 replications. The two biostimulant agents used were BPN III (Burkholderia Metallica, B.anthina, Rhizobium radiobacter, Azotobacter sp., Azospirillum sp.) and AZZOFOR (Enterobacter sp., Lysinibacillus xylanilyticus, Achrimobacter ruhlandii, Enterobacter hormaechei, Comamonas testosterone, Enterobacter cancerogenus, Pseudomonas morselli, Leclercia adecarboxylata, Citrobacter youngae, Enterobacter hormaechei, Chyuseobacterium indologenes, Achrimobacter xylosoxidans, and Citrobacter farmer), mixed with BPN III and AZZOFOR. The results showed that the biostimulant agents and rhizobacteria (BPN III + AZZOFOR) were suitable and effective in supporting the fertility of the marginal soil with bacterial population 10⁷ CFU gram soil⁻¹. Furthermore, all bacteria stimulated the vegetative growth of seedling, such as plant height, number of leaves, and branch, monthly during the nursery.

Keywords: Bacteria, biostimulant agent, marginal soil, nursery.

1. Introduction
Several areas in Ngemplak village, Windusari, Magelang, Central Java, are prone to erosions and landslides, due to their marginal and hilly land conditions, therefore it is not optimal for sustainable agricultural activities. This condition reduces the nutrient quality of the upper and lower soil, inhibits the activity chain of soil organisms, decreases the soil water content, and adversely affects the fertile soil layer at the top. Furthermore, this soil condition is an unfavorable phenomenon for the environment, crops, and the economy of the local population.

An ecological approach using microbial entrapment technology (MET) and soil conditioner enhancing for plant growth (SCEP) is an alternative process for the reclamation of degraded land. This technology requires the support of bacteria as biostimulant agents and seedling that can adapt to the...
environment. The nursery is usually made the first step in land reclamation, however due to unfavorable conditions, the plants are made to adapt through inoculation and use of polybags to plant seedlings. In addition, marginal soil fertility is successfully restored using functional bacteria as biostimulants, mineral rocks, or zeolites, and hydrogels. The use of these substances is expected to improve soil health conditions, increase fertility, and at the same time accelerate the growth rate of plants in degraded land, especially preliminary experiments in the nursery.

Some functional microbes with the potential to support the acceleration of plant growth and soil fertility are nitrogen-fixing bacteria (NFB) [1], phosphate solubilizing bacteria (PSB) [2], and mycorrhizal fungi [3]. According to Kyuma [4], the types of NFB in the soil have various variations such as Rhodospirillum, Rhodopseudomonas, Rhodomicrobium, Chromatium, Ectothiorhodospira, Triospirillum, Chlorobium, Chloropseudomonas (Photosynthetic bacteria), Azotobacter, Azomonas, Beijerinckia, Derxia, Pseudomonas azotogensis (Gram-negative aerobic bacteria), Klebsiella pneumoniae, Enterobacter cloeae, Escherichia Eintermedia, Flavobacterium sp, Mycobacterium flavum, B. anthina, Burkholderia, Azospirillium, Azotobacter, and rhizobia (Actinomycetes analogue bacteria), and blue green algae.

There are various variations associated with NFB, however, the common and easily available ones are Azotobacter, Azospirillum, and Rhizobium. These bacteria live freely in plant roots and nodules [5], [6], [7], [8], marginal areas such as dry soil [9], acid soils [10], peat [11], saline/mangrove soils [12], [13], as well as forest and mountain soils [14].

The advantage of NFB and PSB as biostimulant agent or plant growth in promoting rhizobacteria (PGPR) includes the ability to produce phytohormones, such as auxins using active IAA compounds, gibberellins, and cytokinins, therefore it spurs plant root growth [15]. Widawati and Sudiana [13] stated that it also can separate P from Al, Fe, and Ca bonds. Subsequently, it is easily absorbed by the roots, controls the activity of the pathogen of plant pests (biocontrol), and decomposes agrochemical compounds [16]. According to Glick [17], NFB and PSB also can control pathogens originating from the soil (bioproducts) by several anti-pathogenic compounds or metabolites such as siderophore, chitinase, antibiotics, and cyanide.

Rhizosphere indigenous NFB and PSB are suitable for revegetation of critical land, because after the inoculation process, the bacteria aggressively occupy and colonize plant roots (rhizosphere), therefore the nutrient input required for plant growth becomes quickly available [18]. Several potential NFB in marginal land revegetation are Beijerinckia, Burkholderia, Azospirillum, Azotobacter, and Rhizobium [19].

This study aims to determine the effectiveness of bacteria in fertilizing plant soil from marginal land through reclamation. It also determines the acceleration effect of vegetative growth on seedling during the nursery.

2. Materials and Methods
2.1. Inoculant Making
Inoculants as biostimulating agents are prepared in two groups. The first group is NFB containing nitrogen-fixing bacteria (Burkholderia Metallica InaCCB895, B. anthina InaCCB840, Rhizobium radiobacter InaCCB835, Azotobacter sp., Azospirillum sp.) with the ability to produce IAA, acetylaminase, and nitrogenase activities. The second group is NFB containing the bacteria Enterobacter sp. InaCCB837, Lysinibacillus xylanilyticus InaCCB882, Achromobacter ruhlandii InaCCB883, Enterobacter hormaecheii InaCCB884, Comamonas testosterone InaCCB885, Enterobacter cancerogenus InaCCB886, Pseudomonas mosselii InaCCB887, Leclercia adecarboxylata InaCCB889, Citrobacter youngae InaCCB890, Enterobacter hormaechei InaCCB891, Chyuseobacterium indologenes InaCCB893, Achromobacter xylosoxidans InaCCB894, and Citrobacter faeroer InaCCB896. All bacterial isolates have the ability to produce IAA, acetylaminase, nitrogenase, and phosphomonoesterase activities. Bacteria for the first and second inoculants were respectively
rejuvenated into the LB medium slant in the test tube. After incubating for 5 days at room temperature of 27-30°C, they were transferred into Erlenmeyer glass with 250 liquid LB media, and shaken at 120 rpm for 5 days or until the population reaches $10^5 - 10^9$ CFU mL$^{-1}$ extract. The bacterial extract or liquid inoculant was further transferred to the Erlenmeyer tube containing 2L of LB medium and incubated for 5 days in a fermenter. Furthermore, 40% of the liquid inoculant from the mixture of each bacterium is injected into 100% carrier material containing a mixture of 50% sterile fine compost, 40% sterile zeolite, and 10% hydrocele. Finally, solid inoculants are inoculated on plants after 7 days of incubation or when the bacterial population density reaches $10^8 - 10^9$ CFU per gram solid inoculant.

2.2. Test plants in the nursery
The test plants in the nursery which were later planted on marginal land are Sengon [(Paraserianthes falcataria (L.) Nielsen], coffee (Coffea Arabica L.), and citrus (Citrus sp.). Furthermore, initial preparation was conducted to select 3 months old plants starting from May using sterile soil media with different plant heights. The seedlings are further transferred to a polybag containing 3 kg of marginal soil inoculated with 10 grams of inoculant BPN III, Azzofor, and MIX BPNIII + Azzoor. Furthermore, to maintain soil moisture in the nursery, the plants are watered daily until the seedlings are above ±100 cm and ready to be moved to the marginal land.

2.3. Number of bacterial populations before and after planting from planting media (polybags).
The global bacterial count has used the total plate count method or TPC [20]. This method is used to determine the number of microbes in the sample using selective agar. This method is culture-dependent, aims to count cells that have succeeded in growing and dividing in a medium, under the specified nutrient conditions, time, and temperature. The reclaimed oil samples were also taken at the polybag of each treatment, and aerated. Furthermore, 1 gram of soil sample is mixed with 9 mL of sterile distilled water in a vortex test tube until homogeneous. After that, the soil extract from serial dilution $10^{-3}$ and $10^{-5}$ was planted in triplicate on a Petri dish containing selective medium (5 grams polypeptone + 5 grams yeast extract + 5 grams of glucose + 1 gram of 126 MgSO$_4$.7H$_2$O + 20 grams agar + 1 L Aquadest) [21].

2.4. Research methods
The research method used was a completely randomized design (CRD) with control treatment, NFBIII, AZZOFOR, and MIX inoculant (NFB III, Azzofor). Each treatment was repeated 10 times with the vegetative growth such as plant height, number of leaves, and branches and bacterial populations measured from May to August. The data obtained were analyzed with ANOVA statistical variants followed by DMRT (Duncan Multiple Range Test) at 5% using SPSS version 23 [22].

3. Result and Discussion
The statistical calculation of the measurement result such as vegetative growth of albizia, coffee, and orange plants was determined after growing them in polybags containing marginal soil inoculated with BPN III, AZZOFOR, and biostimulating agents for 4 months as shown in Tables 1, 2, and 3. Furthermore, Table 4 shows the soil fertility as a planting medium indicated by the bacterial population as $10^5$ CFU per gram of soil.

3.1. The Growth of Paraserianthes falcataria (L.) Nielsen seedling
Table 1 shows that the final height varied from the beginning of the measurement (May) due the addition of Azzofor, BPN III, and Azzofor + BPNIII inoculants in the planting medium, which stimulates the growth of Sengon seedling. This can also be seen in the results of the statistical calculations, in the inoculated media, which showed a high growth rate from May (84.92 cm, 80.16 cm, 80.12 cm, and 78.78 cm) to August (97.40 cm, 92.60 cm, 103.44 cm, and 83.44 cm). This yield was better than the seedlings grown on non-inoculated growing media (control). Meanwhile, in the seedling there was no significant difference, therefore, the bacterial inoculant contained in the Azzofor
+ BPNIII mixture stimulated the growth rate such as plant height, number of leaves, and branches as well as a seedling on marginal soils. Nusantara [23] stated that Senigon seedlings inoculated with a mixture of microbes produce plants that are 17.21 cm higher than the control. The same result was reported by Suharti [24], that the microbes in EM4 inoculant in the nursery media tend to increase the Sengon seedling growth rate by 22% at 3 months after planting. Furthermore, Ekamawanti and Ekyastuti [25] reported that rhizosphere microbe isolates were very effective in increasing the height of Sengon seedlings. This is due to the support of nitrogen-fixing and phosphate solubilizing bacteria which provide readily absorbed N and P elements for seedling Sengon, especially root nodule bacteria (Rhizobium radiobacter InaCCB835), Azotobacter and Azospirillum contained in BPNIII. As a biostimulating agent, these bacteria directly and rapidly colonize plant roots (rhizosphere) when inoculated into the soil or plant roots which immediately provides nutritional input for plant growth [26].

| Treatment | Plant height (cm) per month | | | |
|-----------|-------------------------------|---|---|---|
|           | May                           | June | July | August |
| Azzofor   | a84.92±1.73<sup>a</sup>       | a85.56±1.80<sup>a</sup> | a86.16±1.77<sup>a</sup> | b97.40±2.58<sup>ab</sup> |
| BPN III   | a80.16±1.83<sup>ab</sup>      | a84.12±1.94<sup>a</sup> | a85.36±1.95<sup>a</sup> | b92.60±2.16<sup>ab</sup> |
| Mix       | a80.12±1.37<sup>ab</sup>      | a82.64±1.66<sup>a</sup> | a83.72±1.67<sup>a</sup> | b103.44±12.57<sup>ab</sup> |
| Control   | a78.78±1.22<sup>a</sup>       | a80.96±1.20<sup>a</sup> | a82.76±1.26<sup>a</sup> | a83.44±2.70<sup>a</sup> |

| Number of leaves | | | | |
|------------------|---|---|---|
| May              | a2.48±0.21<sup>a</sup>       | b4.00±0.01<sup>a</sup> | b4.00±0.00<sup>a</sup> | b3.76±0.42<sup>ab</sup> |
| BPN III          | a3.20±0.18<sup>bc</sup>      | b4.08±0.08<sup>a</sup> | b4.08±0.10<sup>a</sup> | a3.28±0.26<sup>a</sup> |
| Mix              | a3.28±0.15<sup>bc</sup>      | b4.12±0.17<sup>a</sup> | b4.12±0.17<sup>a</sup> | b4.16±0.19<sup>b</sup> |
| Control          | a3.60±0.20<sup>c</sup>       | a3.80±0.16<sup>a</sup> | a3.88±0.07<sup>a</sup> | a3.60±0.31<sup>ab</sup> |

Note: Letters in front of numbers (rows) indicate differences in growth between months, while letters after numbers (columns) indicate differences between treatments. (Duncan's test of 5% confidence level).

3.2. Coffea Arabica L. seedling growth

Table 2 shows that the growth rate of a Coffee plant from the beginning of May to the end of the measurement in August is insignificantly different. Meanwhile, the number of leaves in the mixed bacterial treatment was different from May (6.59), June (10.12), July (7.94), and August (7.88). Overall, the measurement figures for the initial plant height growth rate (45.52 cm, 43.23 cm, 45.82 cm, 38.28 cm) and the number of early leaves (6.12, 5.62, 5.35, 5.52) show increase in August (plant height: 48.53 cm, 44.29 cm, 46.24 cm, 39.96 cm and the number of leaves: 7.48, 5.08, 7.88, 4.88). The best growth was obtained from coffee seedlings that were inoculated by a mixture of bacteria contained in the biostimulating agents, with plant heights of 47.41 cm, 48.41 cm, 48.53 cm, leaves number of 10.12, 7.94, and 7.88 as well as branch numbers of 2.53, 1.74, 0.941, 0.88 for June, July and August, respectively. The yield of height and number of leaves in this study was better than the Coffee plants inoculated with P diminuta and B subtilis [27]. Furthermore, Junaedil et al. [28], stated that EM4 + 50% fertilizer dose increased plant height growth. The slow growth rate of seedlings per month is due to several factors such as temperature, humidity, aeration, and pH on the polybag planting medium. Furthermore, it is also because there is no match between bacteria and their host (Coffee), which is slow to colonize roots and have an impact on essential plant growth elements such as N and P with less availability. Nitrogen and phosphate are important elements in the rhizosphere of
plants that grow in marginal soils [29] and become key in plant growth, metabolism, and development [30]. Nitrogen and phosphate elements are only absorbed in the form of ammonium and inorganic P by roots. These elements are available due to the role of nitrogen-fixing and phosphate solubilizing bacteria in the soil [31].

Table 2. *Coffea robusta* L seedling growth 4 month after planting

| Treatment    | Plant height (cm) per month | May      | June      | July      | August     |
|--------------|----------------------------|----------|----------|----------|-----------|
| Azzofor      | a45.52±1.81<sup>a</sup>    | a46.12±1.61<sup>abc</sup> | a46.24±1.59<sup>abc</sup> | a46.24±1.59<sup>abc</sup> |
| BPN III      | a43.23±2.25<sup>ab</sup>   | a43.53±1.94<sup>abc</sup> | a43.41±1.81<sup>abc</sup> | a44.29±2.51<sup>abc</sup> |
| Mix          | a45.82±2.10<sup>a</sup>    | a47.41±2.01<sup>abc</sup> | a48.41±2.01<sup>abc</sup> | a48.53±3.01<sup>abc</sup> |
| Control      | a38.28±2.00<sup>a</sup>    | a39.16±2.08<sup>a</sup>   | a39.16±2.08<sup>a</sup>   | a39.96±1.81<sup>a</sup>   |

Table 3 shows that the height growth rate of *Citrus* plants from May to August increased statistically. Nevertheless, the support of Azzofor and BPN III and Azzofor + BPNII slowly helped in growth stimulation than control plants. The growth in leaf numbers worsened and fell from July to August,

3.3. Seedling growth of *Citrus* sp.

Tabel 3. Laju pertumbuhan seedling *Citrus* sp. 4 bulan setelah tanam

| Treatment    | Plant height (cm) per month | May      | June      | July      | August     |
|--------------|----------------------------|----------|----------|----------|-----------|
| Azzofor      | a48.47±3.58<sup>ab</sup>   | b62.29±6.08<sup>bc</sup> | ab52.83±3.49<sup>ab</sup> | ab53.29±3.84<sup>ab</sup> |
| BPN III      | a45.53±2.03<sup>a</sup>    | a43.65±1.98<sup>b</sup>   | b55.23±3.28<sup>b</sup>   | b55.76±3.27<sup>b</sup>   |
| Mix          | a52.28±2.63<sup>ab</sup>   | a54.40±2.35<sup>bc</sup>  | a56.20±2.51<sup>bc</sup>  | a59.52±2.62<sup>b</sup>   |
| Control      | a54.00±1.71<sup>a</sup>    | a46.28±1.60<sup>b</sup>   | a46.88±1.64<sup>a</sup>   | b51.28±1.55<sup>a</sup>   |

Note: Letters in front of numbers (rows) indicate differences in growth between months, while letters after numbers (columns) indicate differences between treatments. (Duncan’s test of 5% confidence level)
especially in seedlings treated with BPNII (44.29, 47.82, 44.59, 44.41) and Mix BPNII + Azzofor (51.00, 45.20, 42.84, 42.24). Although the biostimulant agent was slowly effective, it had a significant effect on orange seedling for 4 months in the nursery. Several studies on Citrus fertilization made no yet use of inoculants, by using organic fertilizers such as manure, compost, and chemical fertilizers. Sutopo [32] stated that the fertilization of citrus plants depends on the type, age, biomass, and environmental factors.

### 3.4. Number of bacterial populations before and after planting from planting media (polybags)

The population was calculated before and after inoculation through dilution to separate the bacterial colonies that were originally clustered into colonies, thereby facilitating their visibility. Colony-forming units (CFU) are living individual (bacterial cells) capable of dividing and forming colonies as shown in Table 4. There is usually an increase in the number of bacterial populations before and after inoculation globally. This phenomenon supports the growth rate of seedling in the nursery because the bacteria's effectiveness helps to provide the nutrients needed by plants such as N and P. Subsequently, the presence of bacteria and its populations in the planting medium is important. Obaton [33] stated that soil fertility is indicated by a minimum bacterial population of $\leq 10^7$ CFU per gram of soil. Similarly, Wijebandara et al. [34] and Hayat et al. [35] reported that the presence of functional bacteria in the soil plays an important role in the food chain and improves soil quality biologically. Functional microbes are an important part of the biogeochemical cycle such as carbon, nitrogen, sulfur, and phosphorus cycle [36].

| Soil samples from seedlings | Populasi bakteri dalam tanah (CFU/gram tanah) | Before inoculation | After inoculation |
|----------------------------|---------------------------------------------|-------------------|-----------------|
|                            | Control | AZZOFOR | BPN III | MIX |
| Sengon                     | 1.83 x 10^7 | 3.59 x 10^7 | 2.60 x 10^7 | 2.06 x 10^7 | 4.59 x 10^7 |
| Coffee                     | 1.28 x 10^7 | 3.67 x 10^7 | 2.40 x 10^7 | 2.09 x 10^7 | 4.20 x 10^7 |
| Orange                     | 1.97 x 10^7 | 3.02 x 10^7 | 2.38 x 10^7 | 2.04 x 10^7 | 3.18 x 10^7 |

### 4. Conclusion

The biostimulant agents, namely BPN III and Azzofor as well as the collaboration between rhizobacteria (BPNIII + Azzofor) are suitable and effective in supporting the fertility of marginal soil with a bacterial population of $10^7$ CFU per gram of soil. Furthermore, all bacteria stimulate vegetative growth of Sengon, Coffee, and Orange seedlings such as plant height, number of leaves, and branches every month during the nursery.

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