Identification of Acetoin Producing Rhizobacteria as Rice Plant Growth Control \textit{(Oryza sativa)} from The Rhizosphere of Elephant Grass Plant \textit{(Pennisetum purpureum)} using Gas Chromatography Mass Spectrometry (GC-MS) and Scanning Electron Microscope (SEM)

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\textbf{Abstract.} The compound of acetoin (3-hydroxybutan-2-butanone) is a volatile compound produced by bacteria that functions as a booster for plant growth or as a biostimulant. Acetoin plays an important role in stimulating the process of organogenesis (morphogenesis) plants so that the formation of plant organs faster and faster plant growth. Besides being able to increase the growth of acetoin, it can also induce plant resistance, increase the formation of branches, roots and flowers so that acetoin can increase plant productivity. The purpose of this study was to identify acetoin-producing rhizobacteria which can stimulate rice growth. Identification of compounds was carried out using Gas Chromatography Mass Spectrometry (GC-MS) and Scanning Electron Microscope (SEM). This study obtained one rhizobacterial isolate capable of producing acetoin which acts as a plant growth booster, namely Rg21. This rhizobacterial isolate was able to increase the number of lateral roots of rice seedlings at the age of 14 days after plants with a percentage increase of 50.00% when compared to controls.

1. Introduction
Rhizobacteria is bacteria that live around plant roots, these live bacteria can symbiosis with plant roots. Rhizobacteria or Plant Growth Promoting Rhizobacteria (PGPR) has the ability to give and increase absorption of nutrients in the soil to improve plant growth. These bacteria live in colonies covering the roots of plants. For plants the presence of these microorganisms will be very good because these bacteria provide benefits in the process of plant physiology and growth [1]. The use of acetoin-producing bacteria is one of the technological innovations to increase the ability of plants to absorb nutrients so that the quality and quantity of plants can be improved [2]. The use of acetoin and urease-producing bacteria has been reported to increase uptake of chemical fertilizers and yields of soybean and rice crops, Khalimi \textit{et al.} [3] reported that BS2a pantoeaagglomerans bacteria were able to increase soybean root dry weight by 190.59% when compared to controls.

In general, the function of PGPR in increasing plant growth is divided into three categories [4, 5, 6] namely, (1) as a growth booster (biostimulant) by synthesizing and regulating the concentration of various substances growth regulators (phytohormones) such as IAA, gibberellins, cytokinins and ethylene in the root environment; (2) as a provider of nutrients (biofertilizer) by tethering N2 from the...
air as symbiosis and dissolving the nutrient P which is bound in the soil; (3) as a pathogenic controller derived from soil (bioprotectans) by producing various anti-pathogenic compounds or metabolites such as siderophore, β-1,3-glucanase, chitinase, antibiotics and cyanide [7, 8].

2. **Methodology**

2.1. *Sampling and isolation of rhizobacteria*

Sampling was carried out from the rhizosphere of elephant grass species (Pennisetum purpureum). Isolation of rhizobacteria from plant roots was carried out by following a procedure developed by Geetha et al [9] modified. A total of 10 g of rhizosphere from each sample macerated on mortal were then diluted with 100 ml of phosphate saline buffer (PBS). Then a series of dilutions with PBS was made until 10^-7 dilutions. The media used to isolate rhizobacteria is Nutrient Agar (NA) medium containing 0.3% beef extract, 0.5% peptone, 1.5% agar and distilled water. This media added benomyl (120 mg / ml) or Nystatin (50 mg / liter) to inhibit fungal growth. A total of 0.2 ml of suspension from each dilution was put into a Petri dish and then mixed with 10 ml NA media with a temperature of about 45 - 50°C. This culture was incubated at room temperature for 24 hours. The growing colonies are then transferred to the new NA media, for the isolation process. The obtained rhizobacterial isolates are coded and moved on the sloping NA media and ready to be used for further testing.

2.2. *Testing of rhizobacterial isolates that produce acetoin*

The biochemical test of rhizobacterial isolates producing acetoin was carried out by the Methyl Red – Voges Proskauer (MR-VP) method [10]. This test is useful in identifying groups of bacteria capable of producing acetoin. If the bacteria ferments carbohydrates into 2.3 butanediol as the main product, there will be a buildup of these ingredients in the growth medium. Then added with 40% KOH and 5% alpha naphthol. If the suspension of rhizobacteria changes color in the medium to red, then the rhizobacterial isolate shows the presence of 2,3 butanediol products as a result of fermentation and the rhizobacteria is positive for producing acetoin (acetyl methyl carbonyl).

2.3. *Identification of compounds with gas chromatography - mass spectroscopy (GC-MS)*

Identification of compounds found in the rhizobacterial filtrate was carried out using Gas-Spectroscopy Gas Chromatography (GC-MS). Extracts or ethyl acetate fractions dissolved in 5 ml of methanol were then analyzed by GC-MS. The results of detection were carried out through matching molecular weights and fragmentation patterns of isolated compounds with compounds in the GC-MS library. By using GC-MS, the compounds produced can be known the molecular weight and molecular structure of these compounds.

2.4. *Observation of rhizobacterial root colonization of rice plants by scanning electron microscope (SEM)*

Ultra structure analysis by observing ultra structure response with SEM was carried out based on the method of Souissi et al. [11]. Observations with SEM focused on the root morphology of rice plants colonized by rhizobacteria. Preparations for SEM originated from seeds that had been soaked with rhizobacterial suspension and then fixed with a solution of 2.5% glutaraldehyde in a 100 mM buffer phosphate buffer (pH 7.0) at 40°C for 24 hours and after that it was allowed to stand at room temperature (250°C) to next observation.

3. **Results and discussion**

The results of biochemical testing using the Methyl Red-Voges Proskauer method [12] showed that 3 rhizobacterial isolates were proven to produce acetoin from 29 isolates. This can be seen in the mixture of rhizobacterial suspension after the addition of 40% KOH and 5% alpha naphthol which changes colour to cherry red. Acetoin (C4H8O2) contained in the media is oxidized in the presence of air and KOH becomes diacetyl. Diacetyl reacts with guanidine from peptone, and the presence of aphaphaholol produces red which functions as a catalyst and colour enhancer (Figure 2).
appearance of cherry red shows a positive (+) result and the brown yellow colour shows a negative (-) result (Table 1) [13].

| No | Isolate | Acetoin | No | Isolate | Acetoin |
|----|---------|---------|----|---------|---------|
| 01 | Rg 1    | -       | 16 | Rg 16   | -       |
| 02 | Rg 2    | -       | 17 | Rg 17   | -       |
| 03 | Rg 3    | -       | 18 | Rg 18   | -       |
| 04 | Rg 4    | -       | 29 | Rg 19   | -       |
| 05 | Rg 5    | +       | 20 | Rg 20   | -       |
| 06 | Rg 6    | -       | 21 | Rg 21   | +       |
| 07 | Rg 7    | -       | 22 | Rg 22   | -       |
| 08 | Rg 8    | -       | 23 | Rg 23   | -       |
| 09 | Rg 9    | -       | 24 | Rg 24   | -       |
| 10 | Rg 10   | -       | 25 | Rg 25   | -       |
| 11 | Rg 11   | -       | 26 | Rg 26   | +       |
| 12 | Rg 12   | -       | 27 | Rg 27   | -       |
| 13 | Rg 13   | -       | 28 | Rg 28   | -       |
| 14 | Rg 14   | -       | 29 | Rg 29   | -       |
| 15 | Rg 15   | -       |    |         |         |

Description: +: produces acetoin, and -: does not produce acetoin

In Table 1 it can be seen that of the 29 isolates tested for their ability to produce acetoin 3 isolates were positive (+) producing acetoin. namely isolates Rg5, Rg21 and Rg26. Furthermore, the three isolates were tested for their ability as growth boosters in increasing lateral roots in rice seeds and the results of isolates Rg 21 increased lateral roots 50.00% when compared to controls (Table 2).

| No | Isolate | Number of roots | Percentage Increase (%) |
|----|---------|-----------------|-------------------------|
| 1  | Kontrol | 14              | -                       |
| 2  | Rg 5    | 10              | -                       |
| 3  | Rg 21   | 21              | 50.00                   |
| 4  | Rg 26   | 9               | -                       |

The effect of rhizobacterial treatment on rice seeds growth can bee seen in Figure 1. The visual comparisons between control treatment (A) and rhizobacterial treatment (B) can be easily observed, were the seeds with rhizobacterial treatment shows a better lateral roots growth.
3.1. Identification of compounds with gas chromatography - mass spectroscopy (GC-MS)

The results of acetoin compounds detection in the rhizobacterial filtrate of Rg21 isolates using GC-MS showed that the compounds detected were not only acetoin compounds but also other compounds were detected. This information showed in Table 2.

Table 2. Results of Detection of Compounds in Rhizobacteria Isolate Rg21, by using the GC-MS

| Peak number | RT (Mins) | Area (%) | The compound detected                                      | Molecular Formula          |
|-------------|-----------|----------|-----------------------------------------------------------|----------------------------|
| 1           | 4.45      | 40.99    | N-Hydrazine                                               | N\(_2\)H\(_4\)            |
| 2           | 6.33      | 50.52    | 2,3 butanediol, diacetat                                  | C\(_8\)H\(_{14}\)O\(_4\)  |
| 3           | 6.67      | 0.79     | L-Valine,N-acetyl-                                        | C\(_7\)H\(_13\)NO\(_3\) |
| 4           | 6.72      | 1.39     | 2-Heptanone,3-methyl                                      | C\(_8\)H\(_9\)O            |
| 5           | 7.01      | 0.44     | 1,5-Dimethyl-6,8-dioxabicyclo[3.2.1]octan                 | C\(_8\)H\(_{16}\)O\(_2\)  |
| 6           | 9.32      | 0.39     | 2,5-Hexanidine,3,4-dihydroxy-3,4dimethyl                  | C\(_8\)H\(_{14}\)O\(_4\)  |
| 7           | 9.72      | 0.55     | Pyrazine, tetramethyl                                     | C\(_8\)H\(_{12}\)N\(_2\)  |
| 8           | 23.00     | 1.52     | Tris(tert-butyldimethylsiloxyl)                           | C\(_{18}\)H\(_{45}\)AsO\(_3\)Si\(_3\) |
| 9           | 23.07     | 1.53     | (E)-2-bromobutyloxychalcone                               | C\(_{10}\)H\(_{16}\)BrO\(_2\) |
| 10          | 25.25     | 1.31     | Arsenous acid                                             | AsH\(_3\)O\(_3\)         |

The results of compound identification in rhizobacteria isolate Rg21 showed that of the 11 compounds detected only one compound identified as acetoin compound was 2.3 butanediol, diacetate with a percentage of 50.52%. The chemical structure of compound 2,3 butanediol, diacetate as presented in figure 2.
GC-MS results showed that compound 2,3 butanediol, acetate on Isolate Rg21 appeared on peak 2 with a retention time of 6.33 minutes (Figure 3).

The chromatographic results from the chromatography in Figure 3 show that Rg21 rhizobacterial isolates contained 11 chromatogram peaks namely peak 1 was hydrazine with retention time of 4.45 minutes with an area of 40.99%, peak 2 was 2,3 butanediol, diacetate with retention 6.72 minutes with a percentage of area 50.52%, peak 3 is L-Valine, N-acetyl with retention time of 6.67 minutes with percentage area of 0.79%, peak 4 is 2-Heptanone-3-metyl with retention time of 6.87 minutes with percentage area 1.39%, peak 5 is 1,5- Dimethyl-6,8-dioxaoctahydrothiabicyclo (3,2,1) octane with retention time 7.01 minutes with percentage area of 0.44%, peak 6 is 2, 5-Hexanedione, 3,4-dihydroxy-3,4 dimethyl is with a retention time of 9.32 minutes with a percentage area of 0.39%, peak 7 is Pyrazine, tetramethyl with a time of retention 9.72 with a percentage area of 0.55%, peak 8 is Tris (tert-butyldimethylsilyloxy) arsane with a retention time of 23.00 minutes with a percentage area of 1.52%, peak 9 is (E)-2-bromobutyloxylchalcone with retention time 23.07 minutes with a percentage of 1.53%, peak 10 is 1,4-Phthalazinone with retention time 23.22 minutes with percentage area 0.57% and peak 11 is Arsenous acid with retention time 25.25 minutes with percentage area 1.31%.

3.2. Rhizobacterial colonization of rice plant roots
The observation of the roots of rice plants using a scanning electron microscope (SEM) showed that the treatment of rhizobacteria in Rg21 isolates successfully colonized the roots of rice plants. This results showed in figure 4.

Figure 3. Chromatogram composition of GC-MS results Isolate Rg21

Figure 4. The observation of rhizobacterial colonization on the roots of rice plants using SEM. A. Control, B. treatment of Rg2 rhizobacteria.
The success of colonizing rice roots is a prerequisite for interactions between rice plants and rhizobacteria so that they can benefit each other. Rhizobacteria benefit from root exudates which contain nutrients needed by rhizobacteria, while plants can be boosted by root growth because rhizobacteria produce acetic. Observations of rhizobacterial colonization on plant roots using SEM were reported by several previous researchers, such as the results of the study that Bradyrhizobium japonicum GD3, Pseudomonas putida GD4, and Bacillus megaterium GP4 colonized and associated with the roots of Ipomoea spp. [15], Bacillus spp. Bacteria appear to colonize and associate with rice plant roots [16]. Other researchers such as Levanony et al. [17] showed colonization of Azospirillum brasilense Cd bacteria with wheat plant roots. Souissi et al. [11] showed colonization of the bacterium Pseudomonas fluorescens LS102 and Flavobacterium balustinum LS105 at the root of the Euphorbia esula plant.

4. Conclusion
Based on the results of tests in the field, it shows that there is one rhizobacterial isolate that produces aceticin, capable of acting as a driver of plant growth, namely Rg21 isolates. This isolate is able to increase the number of lateral roots by 50.00% when compared to the control. The scanning electron microscope (SEM) showed that rhizobacterial treatment of Rg21 isolates successfully colonized the roots of rice plants.

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