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## Reactions of banana plantlets *Musa acuminata* L. to extracellular polysaccharides from *Ralstonia zyzygii* subsp. *celebensis* causal agent of blood diseases

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To cite this article: Baharuddin *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **486** 012154

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## Reactions of banana plantlets *Musa acuminata* L. to extracellular polysaccharides from *Ralstonia zyzygii* subsp. *celebensis* causal agent of blood diseases

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**Abstract.** Extracellular polysaccharides generated by bacteria play an important role in inhibiting the translocation of nutrients and water, protecting bacteria in extreme conditions and neutralizing compounds released by plants. Giving filtrate pathogens with different concentrations causes different plant reactions in each variety. This study was aimed to determine the effect of various concentrations of extracellular polysaccharides (EPS) obtained from *Ralstonia zyzygii* subsp. *celebensis* (*rzc*) towards the growth of banana plantlets in-vivo. A randomized complete design was used with parameters of two varieties (Barangan and Ambon) and EPS in different concentration of 0; 10; 11.5; 13, and 14.5%. EPS filtrate culture was obtained from initially growing bacteria on NGA (Nutrient Glucose Agar) medium and subculture (mineral media) and centrifuged at 6000 rpm for 15 minutes then sterilized with a membrane filter. Extracellular polysaccharides were put into MS media, then planting 3-month-old banana plantlets with uniform growth. Culture were placed for inoculation that controlled in a culture room at a temperature  $26 \pm 2^{\circ}\text{C}$  with relative humidity of  $55 \pm 5\%$  and were exposed to 16 h photo period. Observation parameters were number of shoots and leaves, time of appearance of shoots and leaves (days). The results showed that response of Barangan gave better results on number of shoots than ambon in tolerating the effect of extracellular polysaccharides filtrate. Concentration of 13% is tolerance limit for the growth number of banana plantlets. Barangan variety can produce the highest number of shoots at a concentration of 13% extracellular polysaccharides filtrate.

### 1. Introduction

Banana *Musa acuminata* L. is a tropical fruit which is origin from Southeast Asia including Indonesia. Banana has delicious taste, high nutritional content and inexpensive price, leading to one of the most popular fruits elsewhere [1, 2]. The demand of fresh banana in local market alone is high while the supply limits. Attempts to develop banana industry face many difficulties due to, one of main obstacles, is bacterial blood disease [3, 4]. Once pathogen infects, it spreads systemically into banana tissue and succulent plants that source of inoculum is always available causing a limited disease control. The use of resistant cultivars might be rational way, but it seems to face difficulty since most species undergo susceptible variety. Sihotang S et al. [6] evaluates 25 species to blood diseases but no single species is found to resist to the disease. Disease control through seed method is a promising way, but provision of commercial scale seeds is problem and superior seeds with uniform growth is difficult to be achieved in



a short time. Tissue culture with using Barangan Merah and Ambon Lumut is a possible way to solve seed issues in large quantities, quickly and in a short time. Barangan Merah and Ambon Lumut have economical values and long storage. They are also superior varieties with the AAA genome, similar chromosome pairs with superior parents [8].

Seedlings from tissue culture will be able to support the development of agribusiness on a large scale [1, 2, 3, 6]. Hutami S et al. [7] states that various selection components (gamma radiation, pure toxin or filtrate, low Al and pH, PEG, growth regulator etc.) can be used to increase somaclonal variations in various types of plants in producing the desired new genotypes. There was previous study on mechanism of resistance in bananas in several concentrations of filtrate culture bacterial blood diseases through liquid culture on liquid media in vitro. The finding suggested that 2.5% concentration of bacterial filtrate culture was artificially infected to Barangan Merah plantlet and it shown to grow normally. However, once the concentration was increased to 10%, the plantlet dead.

In addition, preliminarily testing 2.5%, 5%, 7.5% and 10% concentrations of extracellular polysaccharides (EPS) to banana plantlets of Barangan Merah and Ambon Lumut varieties demonstrated that normal growth of banana plantlets still occurred, except trial of 10% concentration with a slow growth of plantlet's shoots. Therefore, the study focuses on above 10% concentration.

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## 2. Materials and methods

### 2.1. Preparation of *Pseudomonas celebensis*

Stock of *Pseudomonas celebensis* from Agricultural Biotechnology Laboratory, Research Center was initiated to grow in Tetrazolium Chloride TTC media 10 g peptons, 5 g glucose, 15 g agar, 1000 mL aquades, 8 g Nutrient Broth (NB) and 1% TTC (2.3 TTC) .5-triphenyltetraacolumchlorid). All ingredients were mixed then put into erlenmeyer and added to distilled water. Sterilized media. TTC 1% was sterilized separately with a 0.2  $\mu\text{m}$  membrane filter. After sterilization and before solidification, 5 ml of TTC was added and then poured in petri dish. Propagation of bacteria was carried out following same procedure in growth of bacteria on TTC media with NGA (Nutrient Glucose Agar) media.

### 2.2. Preparation of extracellular polysaccharides (EPS) filtrate culture

Bacteria grown on NGA media were re-grown on KWB media (Keen and Williams liquid culture) 2.3 g  $\text{KH}_2\text{PO}_4$ ; 1.32 g  $(\text{NH}_4)_2\text{SO}_4$ ; 0.08 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 8 mg  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 50 mg Ammonium ferrocitrate; 2.5g Casamino Acis; 1 g Yeast Extract; 12 g Saccharose; 1000 mL aquades; pH 7. Ammonium ferrocitrat solution was added after the media was sterilized by filtering a 0.2  $\mu\text{m}$  membrane filter. Furthermore, the bacteria were reproduced again with mineral media (6 g  $(\text{NH}_4)_2\text{SO}_4$ ; 5.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 50 g Ca  $(\text{NO}_3)_2$ ; 10  $\mu\text{g}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 3  $\mu\text{g}$   $\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$ ; 30  $\mu\text{g}$   $\text{H}_3\text{BO}_3$ ; 20.5  $\mu\text{g}$   $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 1  $\mu\text{g}$   $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ; 2  $\mu\text{g}$   $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ; 3  $\mu\text{g}$   $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ; 12 g saccharosa; 50 mM potassium-phosphate buffer; 1000 mL aquades; pH 7.6) sterilization with a 0.2  $\mu\text{m}$  membrane filter. Incubation for 48 hours at 110 rpm. Bacteria growing in mineral media were centrifuged at a speed of 6000 rpm 15 minutes. The supernatant was centrifuged again for 5 minutes 10.000 rpm and sterilized with a 0.2  $\mu\text{m}$  membrane filter before incubated 48 hours and shaken with a 110 rpm. To obtain EPS of bacteria on mineral media, bacteria were centrifuged 6000 rpm for 15 minutes. The supernatant was centrifuged again for 5 minutes 10.000 rpm and then filtered with a 0.2  $\mu\text{m}$  membrane filter.

### 2.3. Preparation of murashige and skoog (MS) media

All components of MS media were mixed and added with a growth regulator BAP 5 ppm then sufficient volume up to 1000 mL. After pH 6.7, 6 g of agar was added. Media were sterilized for 15 minutes 121°C.

### 2.4. Extracellular polysaccharides treatment on culture media

Administration of EPS-inducing agents was undertaken before medium of MS + BAP solidifies and commenced from the lowest to the highest concentration of 10%; 11.5%; 13%; and 14.5% until mixture of MS media and EPS liquid reached at 10 mL per bottle. Then banana plantlet was planted.

2.5. *Planting of plantlets*

3 months-old Banana plantlets were used with uniform growth. Planting was carried out in laminar air flow, banana plantlets sub-cultured on the treatment media.

2.6. *Culture condition and observation parameters*

In vitro culture was incubated at tissue culture room with a temperature of 21°C, ± 1500 lux 8 hours dark and 16 hours light. Observations were made on number of shoots, number of leaves, time of shoots emergence and time of leaves emergence.

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3. **Results and discussion**

3.1. *Number of shoots*

Concentrations of EPS and interaction of both significantly affected to the number of plantlets. Table 1 shows that Barangan Merah varieties grown at 13% EPS concentration produced the highest number of shoots nameely 6.67 shoots, but not significantly different from other treatments. While Ambon Lumut varieties grown in treatment without EPS showed the highest number of shoots 3.67 and was very significantly different from other treatments.

**Table 1.** Average number of banana plantlets

Varieties	Extracellular Polysaccharides (%)				
	0	10	11.5	13	14.5
Barangan Merah	5 (2.35 <sup>ab</sup> )	1.33 (1.34 <sup>c</sup> )	2 (1.58 <sup>c</sup> )	6.67 (2.66 <sup>a</sup> )	1 (1.22 <sup>c</sup> )
Ambon Lumut	3.67 (2.04 <sup>b</sup> )	1 (1.22 <sup>c</sup> )	1.67 (1.46 <sup>c</sup> )	2 (1.58 <sup>c</sup> )	0 (0.71 <sup>d</sup> )

Note: The numbers followed by the same letter mean that they are not significantly different at the LSD<sub>α=0.01</sub> test level

3.2. *Number of leaves*

Table 2 shows that the planting of two varieties had no significant effect and the various EPS concentrations significantly affected while the interaction of both did not significantly affect number of plantlet leaves.

**Table 2.** Average number of banana plantlets

Varieties	Extracellular Polysaccharides (%)				
	0	10	11.5	13	14.5
Barangan Merah	3 (1.87)	0.33 (0.88)	0.33 (0.88)	1.00 (1.22)	0.00 (0.71)
Ambon Lumut	3.67 (2.04)	0.33 (0.88)	0.33 (0.88)	2.00 (1.58)	0.00 (0.71)
Average	3.33 (1.95 <sup>a</sup> )	0.33 (0.88 <sup>c</sup> )	0.33 (0.88 <sup>c</sup> )	1.50 (1.40 <sup>b</sup> )	0.00 (0.71 <sup>c</sup> )

Note: The numbers followed by the same letter mean that they are not significantly different at the LSD<sub>α=0.01</sub> test level

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Table 2 shows that control treatments produced the highest number of leaves at 3.33 and were significantly different from other EPS concentrations.

### 3.3. Time of shoots emergence

Analysis of variance showed that planting of two varieties had no significant effect and various EPS concentrations significantly affected whereas interaction of both did not significantly affect the average of shoot age.

**Table 3.** Average time of shoots emergence on banana plantlets

Varieties	Extracellular Polysaccharides (%)				
	0	10	11.5	13	14.5
Barangan Merah	5.33	19.00	20.00	20.67	20.67
Ambon Lumut	6.67	18.67	20.00	20.33	21.00
Average	6.00 <sup>c</sup>	18.83 <sup>b</sup>	20.00 <sup>ab</sup>	20.50 <sup>a</sup>	20.83 <sup>a</sup>

Note: The numbers followed by the same letter mean that they are not significantly different at the LSD  $\alpha=0.01$  test level

Table 3 shows that the concentration without EPS treatment produced the fastest time to emerge shoots that was 6 days and was significantly different from other EPS concentrations.

### 3.4. Time of leaves emergence

Analysis of variance showed that planting of two varieties had no significant effect and various EPS concentrations significantly affected whereas interaction of both did not significantly affect the average time of leaves emergence.

**Table 4.** Average time of leaves emergence on banana plantlets

Varieties	Extracellular Polysaccharides (%)				
	0	10	11.5	13	14.5
Barangan Merah	11.33 (2.27)	25.00 (2.89)	25.33 (2.91)	14.00 (2.43)	31.00 (3.15)
Ambon Lumut	12.33 (2.34)	25.00 (2.89)	25.00 (2.89)	16.33 (2.55)	31.00 (3.15)
Average	11.83 (2.30 <sup>c</sup> )	25.00 (2.89 <sup>ab</sup> )	25.16 (2.90 <sup>ab</sup> )	15.16 (2.49 <sup>bc</sup> )	31.00 (3.15 <sup>a</sup> )

Note: The numbers followed by the same letter mean that they are not significantly different at the LSD  $\alpha=0.01$  test level

Table 4 shows that concentration without EPS treatment produced the fastest leaves emergence time of 6 days but was not significantly different from the concentration of 13% EPS and was significantly different from other EPS concentrations.

## 4. Discussion

Two banana varieties did not have a significant effect on parameters of number of leaves, time of emerging shoots and time of emerging leaves. In Barangan Merah, number of emerging shoots were more than another variety but the growth of explant in both Barangan Merah and Ambon Lumut was similar. Phenotyping appearance of explants between Barangan Merah and Ambon Lumut deferred although genotyping character in both varieties are the same (AAA). The findings suggest that response of both varieties to EPS concentration given were different and the main factor responsible for the difference in responding EPS might be a genetic-based expression which is fragile to environmental pressure. The phenotyping expression may fluctuate in different environments between in vitro and in vivo [9].

The average number of shoots and leaves, time of emerging shoots and leaves with and without EPS concentration shows that the trial without EPS concentration performed to have a better growth due to absent of pathogen. The explant can grow and develop better since nutrient is available and no limiting factor occurs to delay its growth. This is in line with the statement of [10], [11] that the media is a determining factor in multiplication of tissue culture due to media containing minerals, vitamins and hormones that plants need to grow.

Concentration of EPS filter released by *Pseudomonas celebensis* greatly affected to all parameters. The more concentration was given, the earlier malformation of explant occurred. Time of emerging shoots and leaves on media given shows that once the EPS concentration was increased to 14.5%, leaf and shoot growth were limited. The finding suggests that lethal concentration commences to 14.5% affecting to malformation growth of explant tissue. The inhibition of explant growth is generally caused by the presence of EPS compound *Pseudomonas celebensis* which is responsible for inhibited growth activity of host such as translocation of nutrients, host cell metabolic processes and cell. According to Obgonna et al. [12] several pathogens have an extra infection mechanism to penetrate the tissues such as chemical absorption. Chemical absorption by utilizing the binding of polysaccharides that are neutral or containing homopolysaccharides or heteropolysaccharides that are modified to carry non-sugar residues such as acid groups or pyruvic acid compounds. It is this pyruvic acid compound that plays a role in penetrating plant tissues, thereby affecting the normal growth activity of host. Sarker R H and Biswas A [13] and Dhanavel D et al. [14] state that the increase of EPS concentration causes metabolic disorders in explants.

In contrast, with a concentration of 13% EPS the plantlets developed much greater number of shoots and leaves than plantlets with other concentrations. The increase of emerging shoots and leaves may be due to compatibility of defense mechanism activation and genotype differentiation [7]. Defense mechanism activation of plant tissue rises resistance on the specific selection pressure [15]. Once the plant tissue can undermine the influence of EPS activity, the plant cells perform health and generate new emerging shoots and leaves, but if not, growth of emerging shoots and leaves is interrupted until killed.

## 5. Conclusion

Barangan Merah produced many more shoots and was more escape from negative effect of EPS filtrate from *Pseudomonas celebensis* than Ambon Lumut. 13% EPS filtrate concentration of *Pseudomonas celebensis* rose to have the highest shoots of Barangan Merah and this concentration was a tolerant limit for leaf development.

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