The effectiveness of rhizosphere bacteria in inducing the resistance of maize to downy mildew, *Peronosclerospora philipinensis*

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**Abstract.** The aim of study was to determine the efficacy of bacterial isolates from the rhizosphere in influencing the resistance of maize to downy mildew by measuring the accumulated concentration of salicylic acid. Treatments were based on a fully randomized 2-factor design with 3 levels of; factor I (bacteria) 3 levels (b0 = no bacteria, b1 = *Bacillus paramycoides* Ga-3, b2 = *B. cereus* Si-4) and factor II (salicylic acid) 2 levels (as0 = 0 g/L and as1 = 2 g/L). The research was carried out at the greenhouse and plant pathology laboratory, Indonesian Cereal Research Institute (ICERI), from March to July 2020. Each treatment combination was tested on three varieties of maize, namely Anoman, Bima20, and Bima3. Hence there were 18 treatment combinations. Furthermore, the results showed that rhizosphere bacteria and salicylic acid could control downy mildew in maize. In the case of the Bima20 variety, the combined treatment of *B. paramycoides* Ga3 + salicylic acid had the lowest incidence of disease at 13%. Meanwhile, treatment of *B. cereus* Si4 + salicylic acid in the Bima3 variety had the lowest incidence of disease by 26%. Considering the results, the enrichment of salicylic acid content was highest at 9.6 ppm on maize which was applied with the rhizosphere bacterium *B. paramycoides* Ga3 + salicylic acid.

1. Introduction
Corn is grown in temperate and tropical regions of the world, and most (about 80%) are produced in rainy conditions in sub-Saharan Africa, South and Southeast Asia, and Latin America. The eight major corn-growing countries (China, India, Indonesia, Nepal, Pakistan, Philippines, Thailand, and Vietnam) produce 98% of Asia and 28% of the world's maize. Corn plants are very susceptible to abiotic and biotic stress [1, 2]. Downy mildew caused by *Peronosclerospora philipinensis* is a major disease of maize.

*Peronosclerospora philipinensis* is a pathogen that causes downy mildew in corn classified as severe, especially in the South Sulawesi region [3] have reported the causes of downy mildew in other regions in Indonesia, including *P. maydis* and *P. sorghi*. Downy mildew can occur at any stage of maize development from germination to harvest, although it generally infects the host plant as soon as seedlings appear up to one month after planting [4]. The factor variability of the corn genotype resistant to downy mildew was quite high, as indicated by the relatively high level of polymorphism [5].
Meanwhile, corn varieties susceptible to this disease can cause yield losses of 50-100%. In endemic areas, disease incidence can reach tens of hectares [6].

The preventive efforts carried out by farmers by treating seeds with fungicides with metalaxyl reportedly did not succeed because of the resistance effect or resistance to these active ingredients. An alternative control in agriculture currently trending is microorganisms to encourage plant growth and protect host plants from pests and diseases [7, 8]. In Shallot, bacteria community is potentially used as biological control [9,10] but for the case of downy mildew disease in the field, the need for robust microorganisms as biological agents is expectedly to elicit plant resistance.

Salicylic Acid (SA) is an important signal involved in the activation of plant defense responses against abiotic and biotic stresses [11]. SA activity is one of the indicators that systemic resistance is induced in plants. SA is involved in controlling many physiological processes in plants, but SA plays an essential role in the interactions between microbes and plants [10]. The high activity of genes related to plant defense is associated with a slower process of infection. It is associated with lignification and the formation of hydrogen peroxide, which directly inhibits pathogens or free radicals, which have antimicrobial effects [12].

Information regarding rhizosphere bacteria that acts as PGPR to increase plant growth and is expected to reduce the severity of downy mildew is still limited. The disease is currently managed by seed treatment with metalaxyl fungicides, but the development of pathogenic fungi resistance towards synthetic fungicides is a great problem affecting the future of chemical control by fungicides significantly. The use of microorganisms for the induction of systemic resistance against pathogens on maize is well known. This study aimed to determine the effectiveness of rhizosphere bacteria in inducing resistance of corn to downy mildew.

2. Materials and methods

This research was conducted from March to July 2020 at the Plant Pathology Laboratory and screen house of the Indonesian Cereal Research Institute at Maros. The treatments were arranged in completely randomized design with 2 factors, factor I (3 levels of bacteria): b0 = without bacteria, b1 = Bacillus paramycooides Ga-3, b2 = B. cereus Si-4; and factor II (2 levels of salicylic acid): as0 = 0 g/L and as1 = 2 g/L. Each treatment combination was tested on three corn varieties, namely Anoman, Bima20, and Bima3, so that there were 18 treatment combinations (table 1).

2.1. Propagation of bacterial isolates

Propagation of B. paramycooides Ga3 and B. cereus Si4 was carried out on Nutrient Agar (NA) solid media, then incubated at room temperature (27 °C - 28 °C) for 48 hours to obtain a single colony. The single colony that has been rejuvenated were then completely scratched on a new sterile Petri dish that already contains the media NA, then incubated for 48 hours at room temperature. Bacteria were harvested and dissolved with sterile distilled water and then calculated at OD = 1 at 600 nm with a spectrophotometer to obtain a bacterial concentration of 10⁹ CFU/ml.

2.2. Inoculation of downy mildew spore’s suspension in plants

Corn leaves infected with downy mildew were collected at 5 p.m. The collected corn leaves were then cleaned under running water. The base of the leaves was placed in a container with 2% sugar water and then placed in a dark place with a temperature of 18-23 °C. Downy mildew spores will appear on the underside of the leaves after incubation for 5-7 hours. The spores were harvested by washing the incubated leaves using distilled water. The concentration of spores to be inoculated on corn plants was adjusted to 10⁴ spores/ml using a Haemocytometer. Spore inoculation technique by spraying at the point of plant growth. Each plant was inoculated as much as 5 ml at 10 days after planting (DAP) at 02.00-03.00 a.m.
Table 1. Research treatments.

| Treatment code | Rhyzosphere bacteria | Salicylic acid | Varieties       |
|----------------|----------------------|----------------|----------------|
| b0as0v1        | Without              | Without        | Anoman         |
| b0as0v2        | bacteria             | salicylic acid | Bima 20        |
| b0as0v3        | Without              | Without        | Bima 3         |
| b0as1v1        | bacteria             | salicylic acid | Anoman         |
| b0as1v2        | Without              | Without        | Bima 20        |
| b0as1v3        | bacteria             | salicylic acid | Bima 3         |
| b1as0v1        | Without              | Salicylic acid | Anoman         |
| b1as0v2        | bacteria             | Salicylic acid | Bima 20        |
| b1as0v3        | Without              | Salicylic acid | Bima 3         |
| b1as1v1        | bacteria             | Without        | Anoman         |
| b1as1v2        | Without              | salicylic acid | Bima 20        |
| b1as1v3        | bacteria             | Without        | Bima 3         |
| b2as0v1        | B.                   | salicylic acid | Anoman         |
| b2as0v2        | paramycoides         | Without        | Bima 20        |
| b2as0v3        | Ga3                  | salicylic acid | Bima 3         |
| b2as1v1        | B.                   | Salicylic acid | Anoman         |
| b2as1v2        | paramycoides         | Salicylic acid | Bima 20        |
| b2as1v3        | Ga3                  | Salicylic acid | Bima 3         |
|                | B.                   | Without        |                |
|                | paramycoides         | Salicylic acid |                |
|                | Ga3                  | Without        |                |
|                | B.                   | salicylic acid |                |
|                | paramycoides         | Salicylic acid |                |
|                | Ga3                  | Salicylic acid |                |
|                | B.                   | Salicylic acid |                |
|                | paramycoides         | Salicylic acid |                |
|                | Ga3                  | Salicylic acid |                |
|                | B.                   | Salicylic acid |                |

2.3. Application of rhizosphere bacteria
Rhizosphere bacteria were applied twice, namely seed treatment and application to the planting medium. Seeds were treated by soaking with bacteria before planting for 2 hours. Soaking was done using 50 ml of each bacterium with a concentration of $10^9$ CFU/ml. For corn plants, rhizosphere bacteria each was poured into the growing medium as much as 50 ml/plant a day before inoculation (9 DAP).

2.4. Planting
The soil used as a growing medium for corn was sterilized under the sun for 2 days. The corn seeds were washed and planted in sterilized media as many as 10 seeds per polybag.
2.5. Salicylic acid application
Salicylic acid application was carried out at 7 DAP with a concentration of 10 mL per L water. Spraying was carried out evenly throughout the plant leaves.

2.6. Observations

2.6.1. Percentage of downy mildew infection. Observation of downy mildew incidence was done at 2, 3 and 4 weeks after inoculation (WAI). Percentage of disease incidence was counted by using formula:

\[ I (%) = \frac{a}{b} \times 100\% \]  

(1)

where:

- \( I \) = disease incidence
- \( a \) = number of infected plants
- \( b \) = number of plants grown

2.6.2. Analysis of salicylic acid content. Observation of SA concentration using High Liquid Performance Chromatography (HPLC) following the Association of Official Analytical Chemists [13] procedure was carried out 10 days after inoculation of downy mildew. The SA analysis results are quantified using the formula:

\[ \frac{\text{Sample area}}{\text{Standard area}} \times 100 \text{ ppm} \]  

(2)

2.6.3. Chlorophyll content. Chlorophyll observations were carried out at 21 days after inoculation (DAI) using a SPAD-502 chlorophyll meter. Leaf blade samples taken for measurement were at the base, middle and tip of the leaf.

2.6.4. Stomata density. Observation of the number of stomata was done at 21 DAI. Each treatment was represented by two sample plants. The leaves used were the third leaf from the top. The surface of the leaves was cleaned of sand or soil and then smeared with nail polish covering an area of 1 cm² on the underside of the leaves at the tip, middle and base. After the paint dried, put tape to cover the paint layer. The tape was slowly peeling off so the nail polish is peeling off too. The tape to which the nail polish is attached was taped over the glass object. The surface of the leaves and stomata were recorded on the nail polish layer like a mold. The stomata print results were observed using betaview. The calculation of the number of stomata was carried out on the observation area of 876 × 656 μm².

2.6.5. Soil bacterial populations. The number of bacterial colonies in the soil for each treatment was observed at 15, 30, and 45 DAI. Soil samples were taken as much as 1 g then homogenized using sterile distilled water by shaking until evenly mixed, then taken as much as 1 ml and diluted 10⁻⁸ then grown on NA medium and incubated at room temperature for 24 hours. Calculation of the number of colonies using a colony counter, with the CFU calculation formula:

\[ \frac{\text{CFU's}}{ml} = \text{number of colonies} \times \text{dilution factor} \]  

(3)

2.6.6. Fresh and dried root weight. Fresh and dry weight of plant roots were weighed at harvest. For dry weight it was weighed after being oven at 60 ºC for 48 hours.

Data were analysed using ANOVA and LSD test at the 5% level in advance is applied when the data have shown to have a significant difference to control.

3. Results and discussion

3.1. Effectivity of rhizosphere bacteria to downy mildew disease
The number of infected plants with downy mildew (table 2) at 14 DAI on Anoman ranged from 13% to 44%, while on Bima20 only the *B. paramycoides* Ga3 bacterial treatment showed downy mildew symptoms with 6% infection followed by Bima3 in treatment of control and rhizosphere bacteria single by 5% - 10%. At 21 DAI, downy infected plants on Anoman was 80%-100%, on Bima20 was 13%-51%, and on Bima3 was 15%-65%.

At 28 DAI, disease incidence on Anoman was reached 100%, except for the treatment of rhizosphere bacteria *B. paramycoides* Ga3 + salicylic acid. Meanwhile, the lowest disease incidence (13%) was also observed in the treatment of *B. paramycoides* Ga3 + SA of Bima20 variety, followed by treatment of *B. cereus* Si4 + SA in Bima20 and Bima3 varieties with infections ranging from 23% - 26%. In accordance with the results of research by [14] root inoculation activates changes in systemic gene expression in leaves, increases SA accumulation, and systemic resistance to *Colletotrichum graminicola* infection, suggesting that SA is involved in controlling biotrophic pathogens. [15] suggested that SA is the main plant hormone that mediates the host response to pathogenic microbes. Some of these SA-binding proteins may have direct/indirect roles in plant physiological processes in addition to signaling defenses against pathogen.

**Table 2.** Average of downy mildew incidence on three different corn varieties treated with rhizosphere bacteria.

| Treatments                  | Anoman                |                | Bima20               |                | Bima3                |                |
|-----------------------------|-----------------------|----------------|----------------------|----------------|----------------------|----------------|
|                             | 14 DAI                | 21 DAI         | 28 DAI               | 14 DAI         | 21 DAI               | 28 DAI         |
| Control                     | 44.4 d                | 94.4 fg        | 100.0 c              | 0.0 a          | 33.8 abcd            | 33.8 ab        |
| Salicylic Acid (SA)         | 30.0 bcd              | 100.0 g        | 100.0 c              | 0.0 a          | 33.3 abcd            | 41.7 ab        |
| *B. paramycoides* Ga3       | 37.8 cd               | 100.0 g        | 100.0 c              | 6.3 ab         | 33.9 abcd            | 33.9 ab        |
| *B. cereus* Si4             | 37.2 cd               | 100.0 g        | 100.0 c              | 0.0 a          | 51.7 cde             | 56.7 abc       |
| *B. mycoides* Ga3 + SA      | 13.4 abc              | 80.4 efg       | 92.9 c               | 0.0 a          | 13.9 a               | 13.9 a         |
| *B. cereus* Si4 + SA        | 37.8 cd               | 100.0 g        | 100.0 c              | 0.0 a          | 23.8 abc             | 23.8 a         |

Note: Numbers followed by different letters show significantly different results (LSD test α = 0.05).

### 3.2. Effect of rhizosphere bacteria to SA concentration

The observation results of SA levels from each treatment of rhizosphere bacteria were not the same. Figure 1 shows that the treatment of rhizosphere bacteria has a higher SA concentration than the control. The highest SA content was found in the treatment of rhizosphere bacteria *B. paramycoides* Ga3 + SA.
(b1as1), it was 9.6 ppm followed by the treatment of \textit{B. cereus} Si4 + SA (b2as1) bacteria of 7.6 ppm and the lowest concentration of SA in the treatment without bacteria and without SA (b0as0) which was 5.7 ppm.

[16] reported that plants given PGPR were able to increase the concentration of secondary metabolites such as SA and peroxidase as a response to resistance to \textit{Cucumber Mosaic Virus} infection. Total SA levels in uninfected plants were low and strongly induced in infected plants.

![Image: Figure 1. The effect of rhizosphere bacteria \textit{B. paramycoides} Ga3 and \textit{B. cereus} Si4 on SA levels in maize 10 DAI.](image)

Note: b0 = without bacteria, b1= \textit{B. paramycoides} Ga3, b2= \textit{B. cereus} Si4, as0= without salicylic acid, as1= salicylic acid.

**Figure 1.** The effect of rhizosphere bacteria \textit{B. paramycoides} Ga3 and \textit{B. cereus} Si4 on SA levels in maize 10 DAI.

3.3. \textit{Effect of rhizosphere bacteria to stomatal density and chlorophyll content on maize}

After calculating the stomatal density, the results of the variance test were obtained which showed that the average stomatal density of several bacterial treatments in several varieties of corn had no significant differences (table 3). The stomata density of the treatment of \textit{B. paramycoides} Ga3 on Bima20 variety was the highest, which was 75.1/mm². The treatment without bacteria of Anoman variety had the lowest stomatal density value which was 38.5/mm². According to [17] the biotropic pathogen \textit{P. philipinensis} infects plants through the stomata beneath the leaf surface which act as a means for evaporation, a means for exchanging CO₂ in physiological processes related to production. The mechanism of the stomata that opens and closes automatically allows the entry of organisms that play a role in the infection process of plant pathogens. The greater the density of the stomata, the greater the chance of infection. However, the results of the research by [18] stated that there was no relationship between stomata density and the level of resistance of maize to downy mildew.

The results of the variance test (table 3) showed that the mean chlorophyll content of several varieties was significantly different. Observation of chlorophyll content needs to be done to determine the effect of downy mildew on chlorophyll due to the occurrence of chlorosis which causes a decrease in yield [19]. The value of chlorophyll content can be used as a parameter of damage from chlorophyll loss by \textit{P. philipinensis}. The chlorophyll content of Bima20 and Bima3 varieties was not significantly different, but the treatment without bacteria was significantly different from all treatments for Anoman variety. The highest chlorophyll content was showed by the treatment without the Bima3 variety, which was 38.6 units, then followed by the chlorophyll content in the treatment of \textit{B. paramycoides} Ga3 bacteria.
in Bima 3 and Bima20, which was 34.8 units and 34.5 units respectively. The treatment without bacteria of Anoman variety had the lowest chlorophyll content value, that was 20.4 units.

Table 3. Average stomatal density and chlorophyll content of leaves treated with rhizosphere bacteria on three varieties of maize at 21 DAI.

| Treatments     | Stomatal density (l/mm²) | Chlorophyl content (units) |
|----------------|--------------------------|---------------------------|
| **Anoman**     |                          |                           |
| Without bacteria | 38.5                     | 20.4 d                    |
| *B. paramycoides* Ga3 | 51.7                     | 28.7 c                    |
| *B. cereus* Si4 | 57.2                     | 29.6 bc                   |
| **Bima20**     |                          |                           |
| Without bacteria | 61.4                     | 33.6 abc                  |
| *B. paramycoides* Ga3 | 75.1                     | 34.5 ab                   |
| *B. cereus* Si4 | 65.9                     | 34.3 abc                  |
| **Bima3**      |                          |                           |
| Without bacteria | 60.9                     | 38.6 a                    |
| *B. paramycoides* Ga3 | 67.7                     | 34.8 ab                   |
| *B. cereus* Si4 | 61.8                     | 34.1 abc                  |

Note: Numbers followed by different letters show significantly different results (LSD test α = 0.05).

Losing chlorophyll content in plants results in plants becoming stunted and even not producing yield. When all environmental factors are in suitable conditions, the presence of chlorophyll will be very high in a plant [20]. When the presence of chlorophyll in a plant is low, while the need for chlorophyll formation has been met, it can be explained that the presence of pathogens or plant pests that interfere with plant physiology. Loss of chlorophyll or chlorosis is the main symptom of maize with downy mildew [21].

3.4. Effect of rhizosphere bacteria to growth of plants
Corn varieties treated with rhizosphere bacteria tended to show better growth compared to plants that were not treated with bacteria. However, observations of plant height from 20 to 30 DAP on treatment *B. paramycoides* Ga3 and *B. cereus* Si4 were not significantly different from the control (figure 2). At 20 DAP, Bima3 variety responded to the treatment of *B. paramycoides* Ga3 with the best plant height compared to other treatments, while the treatment of *B. paramycoides* Ga3 on Bima20 variety showed a better plant height at 30 DAP. Meanwhile, the treatment without bacteria on the Anoman variety showed the lowest plant height among other treatments both at 20 and 30 DAP. It seems that the use of rhizosphere bacterial isolates was able to stimulate plant height growth compared to treatment without bacteria. The treatment of rhizosphere bacteria are able to increase the growth of corn plants [22].

3.5. Effect of rhizosphere bacteria to population of bacterial colonies on soil
Results of observations on bacteria the number of soil bacteria colonies in the treatment of rhizosphere bacteria and SA can be seen in table 4. Application of rhizosphere bacteria and SA on three varieties of maize had no significant effect on the bacterial population in the soil. At 15 DAI, the treatment of *B. cereus* Si4 without SA was observed to be the highest number of bacterial colonies with 38 x 10⁸ cfu/g, at 30 DAI, treatment of *B. cereus* Si4 + SA, the highest bacterial population was observed at 230 x 10⁸ cfu/g, whereas in 45 DAI the highest bacterial colony population was observed in the treatment of *B. paramycoides* Ga3 + SA with 40.7 x 10⁸ cfu/g. It is suspected that the high bacterial population causes the ability of plants to develop optimally in limiting pathogenic activity. The results of this study were supported by [23] application of the rhizosphere bacteria *B. paramycoides* Ga3 and *B. cereus* Si4 each
combined with SA provides the highest suppression of downy mildew infection compared without rhizosphere bacteria.

![Graph showing plant height in cm for 20 and 30 HST with treatments]

Note: b0 = without bacteria, b1 = *B. paramycoides* Ga3, b2 = *B. cereus* Si4, as0 = without salicylic acid, as1 = salicylic acid, v1 = Anoman, v2 = Bima20, v3 = Bima3.

**Figure 2.** Average of plant height of 3 maize varieties treated with rhizosphere bacteria at 20 and 30 DAP.

**Table 4.** Total population of bacterial colonies on soil treated with rhizosphere bacteria and SA.

| Treatments               | Bacterial population in soil (x 10⁸ cfu/g) |
|--------------------------|-------------------------------------------|
|                          | 15 DAI | 30 DAI | 45 DAI |
| **Without bacteria**     |        |        |        |
| Without salicylic acid   | 15.0   | 33.3   | 5.3    |
| Salicylic acid           | 15.7   | 34.9   | 9.7    |
| **B. paramycoides Ga3**  |        |        |        |
| Without salicylic acid   | 22.7   | 163.3  | 28.7   |
| Salicylic acid           | 24.0   | 202.6  | 40.7   |
| **B. cereus Si4**        |        |        |        |
| Without salicylic acid   | 38.0   | 157.0  | 35.2   |
| Salicylic acid           | 22.3   | 230.0  | 12.0   |

**3.6. Effect of rhizosphere bacteria to weight of roots**

The effect of rhizosphere bacteria on three corn varieties on the parameters of fresh weight and dry weight of the roots is shown in table 5. Regarding root fresh weight, Anoman variety (*B. paramycoides* Ga3 + SA and 5.35 g root fresh weight) was significantly higher than other treatments. Similarly, a previous study was conducted to show the highest yield in *B. paramycoides* Ga3 treatment reaching 5.4 kg plant fresh weight [24]. The Bima20 variety showed no significant difference between treatments, and the control treatment on Bima3 was significantly lower than the other treatments.

The root dry weight in the Anoman variety was no insignificant difference with other treatments, whereas in the Bima20 variety the control treatment showed the highest root dry weight with 10.45 g, which was not significantly different from the treatment of rhizosphere bacteria *B. paramycoides* Ga3 and *B. cereus* Si4 respectively each singly. Meanwhile, in Bima3, the treatment that responded the best
to dry weight of corn plant roots was \textit{B. paramycoides} Ga3 that was observed to be significantly different from the control treatment and the combination treatment of \textit{B. cereus} Si4 + SA.

Table 5. Average fresh weight and dry weight of roots treated with rhizosphere bacteria on three corn varieties.

| Treatments          | Root fresh weight (g) | Root dry weight (g) |
|---------------------|-----------------------|---------------------|
| **Anoman**          |                       |                     |
| Control             | 1.15 c                | 0.50 d              |
| Salicylic acid (SA) | 1.20 c                | 0.70 d              |
| \textit{B. paramycoides} Ga3 | 2.60 bc              | 1.45 d              |
| \textit{B. cereus} Si4 | 2.60 bc              | 1.30 d              |
| \textit{B. paramycoides} Ga3 + SA | 5.35 abc            | 3.40 bcd            |
| \textit{B. cereus} Si4 + SA | 1.00 c               | 0.70 d              |
| **Bima20**          |                       |                     |
| Control             | 23.85 a               | 10.45 a             |
| Salicylic acid (SA) | 6.70 abc              | 2.30 cd             |
| \textit{B. paramycoides} Ga3 | 23.85 a             | 9.60 ab             |
| \textit{B. cereus} Si4 | 15.20 abc            | 6.00 abcd           |
| \textit{B. paramycoides} Ga3 + SA | 17.35 abc        | 3.40 bcd            |
| \textit{B. cereus} Si4 + SA | 7.60 abc             | 2.80 bcd            |
| **Bima3**           |                       |                     |
| Control             | 2.60 bc               | 1.35 d              |
| Salicylic acid (SA) | 8.15 abc              | 3.55 abcd           |
| \textit{B. paramycoides} Ga3 | 19.95 ab             | 8.90 abc            |
| \textit{B. cereus} Si4 | 12.30 abc            | 4.20 abcd           |
| \textit{B. paramycoides} Ga3 + SA | 13.20 abc        | 5.50 abcd           |
| \textit{B. cereus} Si4 + SA | 6.05 abc             | 2.85 bcd            |

Note: Numbers followed by different letters show significantly different results (LSD test $\alpha = 0.05$).

4. Conclusions

- The application of the rhizosphere bacteria \textit{B. cereus} Si4 combined with salicylic acid application in Bima3 was relatively more effective than control in suppressing downy mildew with disease infection 26%.
- Application of a combination bacteria \textit{B. paramycoides} Ga3 and salicylic acid increases concentration of salicylic acid up to 9.6 ppm.

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