Effect of combination of biofertilizer and nanobiopesticide citronella against mosaic disease on patchouli plant

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Abstract. Nanobiopesticide citronella (Nano-BC) at 1% concentration was effectively reduced the intensity of mosaic disease and yield loss ranging from 23 to 43%. The mixed formulation of Serratia marcescens ARI and Pseudomonas fluorescens LPK1-9 with cow manure (15 kg/tree) and 0.1% nanobiopesticide citronella were able to reduce 20 to 25% the intensity of vascular streak dieback disease (VSD). The combination between biofertilizer and Nano-BC is assumed to be able to reduce the intensity of patchouli mosaic virus and its insect vector and increase the resistance of patchouli plants to the disease. The objective of this study was to obtain a dose of biofertilizer (0, 100, 200, 300 g) and 1% Nano-BC that effectively controlling patchouli mosaic disease and their vectors in the field. Research was conducted on patchouli var Sidikalang at Laing Experimental Garden, Solok from January to December 2018. Application of 1% Nano-BC and 300 g biofertilizer per plant showed as the best combination to reduce the incidence and intensity of patchouli mosaic disease in the field. In addition, the highest content of P₂O₅ was 39.00 ppm available in the soil, in which the efficacy rate ranged from 9.37 to 12.47%.

1. Introduction
Mosaic disease infecting patchouli caused by Potyvirus is economically important and widely spread. In nature, potyviruses are mostly transmitted through insect vectors (aphids) and vegetative propagation [1]. Infection of Potyvirus on patchouli decreased the production of wet and dry weight plant reaching 35% and 41%, respectively [2]. Therefore, disease control is needed to prevent yield loss.

Citronella and clove oil have potential to suppress the development of mosaic viruses on patchouli. Clove oil (1%) and the mixture of clove oil and citronella could reduce 45% and 32% lesion, respectively [3]. Eugenol as one of chemical compounds of clove oil can also increase the resistance of tomato plants to Tomato yellow leaf curl virus [4]. Disease inhibition by 1.2% citronella oil reached 89.78% [5]. Moreover, 1 to 1.5% dosage formula of nanoemulsion citronella (10% oil concentration) was able to suppress the development of Potyvirus by 82.5%; while the suppression of citronella oil formula (20 to 50% oil concentration) was only 65 to 70% [6]. The effectiveness of nano biopesticide formula might be enhanced if combined with biofertilizer.
The use of root bacteria to improve plant resistance has been widely reported. Utilization of *Pseudomonas fluorescens* LPK1.9, *Bacillus subtilis* TD1.3 and *B. subtilis* TD1.8 against yellow leaf curl disease in chili plant has been reported [7]. Another combination, *Serratia marcescens* AR1 and *Bacillus* sp, was applied to control wilt veins (Stewart) on corn plants [8]. Moreover, *S. marcescens* and *P. fluorescens* that formulated with coconut water and cow manure can increase cocoa growth and reduce the cost of using chemical fertilizers [9]. Utilization of *S. marcescens* subsp. *marcescens* could induce plant resistance to the Watermelon mosaic virus (WMV) member of *Potyvirus* group [10]. *S. marcescens* strain 90-166 was effectively controlled Cucumber mosaic viruses (CMV) [11]. These 2 virus species, CMV and WMV were also reported infecting patchouli plant.

The combination between biofertilizer and nanobiosticide citronella (Nano-BC) is assumed to be able to reduce the intensity of patchouli mosaic virus and its vector and increase the resistance of patchouli plants to the disease. The objective of this study was to obtain a biopesticide formula to control patchouli mosaic disease and its vectors.

2. Methods

2.1. Planting preparation and nanobiosticide application

Research was conducted at the Laing Experimental Garden, Solok – West Sumatra, from January to December 2018.

2.1.1. Preparation of biofertilizer (cow manure + root bacteria plant growth trigger/RBPGT)

Bacterial inoculum was prepared by researchers from Andalas University, i.e. *P. fluorescens* LPK1.9 and *S. marcescens* AR1. Biofertilizer mixture contained of $10^8$ cfu/mL media of bacteril inoculum and cow manure with a ratio of 1:10. This biofertilizer mixture was then tightly closed and incubated for 1 mo in a tight closed bag.

2.1.2. Preparation of patchouli seedlings. As many as 4,000 patchouli seeds were planted in the polybag. These seeds then treated using 4 different treatments, (1) patchouli seeds on soil media + manure with 20 g biofertilizer (1: 1); (2) patchouli seeds on soil media + manure with 40 g biofertilizer (1:1); (3) patchouli seeds on soil media + manure with 60 g biofertilizer (1:1); and (4) patchouli seeds in soil media according to SOP (manure + soil without biofertilizer). Nanobiosticide citronella (Nano-BC) dosage 1% (volume 10 ml/ plant) was sprayed on seeds at 1 mo after planting, while the remaining part was treated without spraying Nano-BC.

2.1.3. Planting in the field. The experiment was run using split plots in randomized block design (RBD). The main plots were cow manure + bacteria dose with 4 treatments and sub-plots contained of treatment with Nano-BC and without Nano-BC, each with 2 treatments and each treatment consisted of 10 replications , each replication consisted of 50 plants.

The compositions of main plot were as followed: (1) Cow manure without RBPGT bacteria; (2) Cow manure without RBPGT bacteria + 100 g of biofertilizer; (3) Cow manure without RBPGT bacteria + 200 g of biofertilizer; and (4) Cow manure without RBPGT bacteria + 300 g of biofertilizer. The compositions of sub-plot were: NO, without Nano-BC; and N1, with Nano-BC. The standard dose for manure was 1000 g for each plant hole.

2.1.4. Nanobiosticide citronella application technique. The concentration of 1% Nano-BC (volume 100 ml) was sprayed to all parts of patchouli plants. Spraying was done every month until 4 mo after planting (MAP). Land preparation was carried out according standard protocol for patchouli cultivation.

2.2. Data collection
2.2.1. The incidence of mosaic disease. Disease incidence was recorded based on disease symptom in each row of the experimental plot. Data collection was conducted before treatment and 1 day after treatment. Percentage of disease incidence was assessed by calculating the total number of infected plants in a row divided by the total number of plants multiplied by 100 [12, formula was given below] and statistically analyzed:

\[
\text{% Disease incidence} = \frac{\text{Number of plants showing mosaics symptom}}{\text{Total number of plants}} \times 100
\]

Meanwhile, disease intensity was assessed by calculating the appearance of mosaic symptoms with the score based on infection categories (Table 1).

**Table 1.** Scores and descriptions of mosaic symptoms in patchouli plants.

| Score | Symptom description                                      |
|-------|---------------------------------------------------------|
| 0     | Healthy, asymptomatic plants                            |
| 1     | Mild, striped in some parts of the leaf and chlorosis   |
| 2     | Moderate, all parts of the plants showed mosaic         |
| 3     | Heavy, all parts of the plant showed mosaic and malformations were occurred |

*a Source: [13] modified.

Disease intensity was calculated using a formula [14]:

\[
I = \left( \frac{\sum (n_i \times v_i)}{Z \times N} \right) \times 100\% 
\]

With, \( I \) = disease intensity
\( n_i \) = the number of plants in each infection category
\( v_i \) = score from each infection category
\( Z \) = the highest score from infection category
\( N \) = number of plants observed

2.2.2. The incidence and intensity of Aphis gossypii. The incidence of damage by insect vector was assessed by calculating the number of damaged plants per number of observed plants; whereas its intensity was done by scoring the percentage of leaf shoots that rolled up (due to aphids attack) [13]. The development of the population of *A. gossypii* was calculated before and after the application by counting each shoot that was damaged.

\[
\text{Incidence of aphid damage} = \frac{\text{No. of damaged plants}}{\text{Total no. of plants observed}} \times 100\%
\]

Furthermore, intensity of damage (%) was measured with following formula [15]:

\[
\text{IP} = \sum \left( \frac{n_v}{z \times N} \right) \times 100\% 
\]

With, \( \text{IP} \) = intensity of damage (%)
\( n \) = No. of plants damaged according to categories (score 0, 1, 2, 3, 4)
\( v \) = score of each category
\( z \) = the highest score of damage category
\( N \) = the number of observed plants \((n_0 + n_1 + \ldots + n_6)\)
Table 2. Category and criteria of insect damage.

| Category | Level (%) | Criteria          |
|----------|-----------|-------------------|
| 0        | X = 0     | No damage         |
| 1        | 0 \leq X \leq 25 | Mild damage     |
| 2        | 25 \leq X \leq 50 | Moderate damage |
| 3        | 50 \leq X \leq 75 | Heavy damage    |
| 4        | 75 \leq X \leq 100 | Very heavy damage |

2.2.3. *The level of efficacy.* Efficacy level of the nano pesticide formula for mosaic disease and *A. gossypii* was calculated following the formula below:

\[ EI = \left( \frac{Ca-Ta}{Ca} \right) \times 100\% \]

With, 
- \( EI \) = Effectiveness of the nano pesticide formula tested (%) 
- \( Ca \) = Percentage of plant damage in the control plot 
- \( Ta \) = Percentage of plant damage in treatment plots after application of the nano pesticide

The tested formula is considered effective if the value of the efficacy level (EI) is 30%.

2.2.4. *Loss of yield* was assessed by calculating wet (g) and dry biomass (g) of patchouli plants in the 1st harvest (6 MAP). Crop yield losses was calculated based on standard formula and compared with untreated controls. Furthermore, patchouli oil and alcohol content (PA) for each treatment were also analysed.

2.2.5. *Virus detection with serological methods.* Detection of *Potyvirus* in leaf samples from patchouli plants was carried out by dot immuno-binding assay (DIBA) method on nitrocellulose membranes [16] (Thermo Scientific, USA).

2.2.6. *Soil analysis.* Analysis of the complete soil nutrient composition of each treatment was carried out in the Indonesian Spice and Medicinal Crop Research Institute test laboratory.

2.2.7. *Data analysis.* The collected data was analysed using the square root transformation method to ensure the homogeneity of variance and data distribution were normal. The data then underwent an analysis of variance (ANOVA) using the SAS Program.

3. Results and discussion

3.1. *The intensity of patchouli mosaics and rooled shoot*

The lowest percentage of disease incidence and intensity of mosaic disease was analysed by all of treatments compared with control. However, statistic analysis showed that there was no significant difference between with and without Nano-BC treatments (Table 3).

The effect of combination of biofertilizer and Nano-BC on aphids (rooled leaves) was not significantly different, probably due to low aphid population (Table 4).

Observation on average of height of patchouli plants was significantly different between without Nano-BC and with Nano-BC treatment in which the highest height was observed on Cowma treatment plus RBPGT dose of 300 g per plant (Table 5). This results showed that the addition of Cowma plus RBPGT dose of 300 g per plant had ability to induce the height of patchouli plants. However, further test of patchouli plants in different aspects of agro-ecosystems and the application of Nano-BC to the surrounding roots of patchouli plants are still needed.
Table 3. The average percentage of mosaic disease incidence and intensity on patchouli plants var. Sidikalang at 4 mo old after planting a.

| Treatments b | Disease incidence (%) c | Disease intensity (%) c |
|--------------|-------------------------|-------------------------|
|              | Without Nano-BC | With Nano-BC | Without Nano-BC | With Nano-BC |
| Without Cowma Plus RBPGT | 46.37 b | 43.41 b | 17.82 b | 16.70 b |
| Cowma Plus RBPGT 100 g | 46.86 ab | 46.01 ab | 18.18 ab | 18.61 ab |
| Cowma Plus RBPGT 200 g | 45.54 a | 54.47 a | 17.94 a | 22.18 a |
| Cowma Plus RBPGT 300 g | 49.46 ab | 42.15 ab | 18.45 b | 16.15 b |
| CV c | 15.34 | 16.32 |

a Assessment was conducted after 3 times spraying nanobiopesticide citronella.
b Nano-BC, nano Biopesticide Citronella; Cowma, cow manure; RBTPG, root bacteria that triggers plant growth.
c The numbers followed by the same small and capital letters in the same columns or rows, respectively are not significantly different at the level of 5% (LSD).

The efficacy of Nano-BC and Cowma plus RBPGT dose of 300 g/plant compared to untreated control of mosaic disease were 9.37-12.47%. The results of this study could explain a new strategy for viral disease control using rhizobacteria. Similar broad-spectrum resistance given by rhizobacteria was found previously with B. amyloliquefaciens strain EXTN-1 [17], strain P. fluorescens, and S. marcescens [18][19]. Patchouli was harvested at the age 4 MAP, because after this stage many plants are infected by bacterial wilt and nematodes. In the application of Nano-BC and cowma plus RBPGT 300 g/ plant, the weight of wet biomass and dried plants were 190.51 g and 61.90 g, respectively (Table 6).

The application of RBPGT formulation (cowma plus 300 RBPGT) without Nano-BC showed the highest patchouli oil production per kg of dried biomass (19.67 ml) and % oil yield (1,887) while this treatment had no effect on the yield of patchouli and salicylic acid levels [Table 7]. The highest % oil yield was 1.758% in the cowma plus 300 RBPGT treatment without Nano-BC. However, patchouli alcohol content was only 27.84% in which it was still categorized below the national standard. This might be due to early harvesting, i.e. at 4 mo-old plants. Harvest time at 4 MAP was the most optimal harvesting age both in terms of wet weight, dry weight and yield in leaves, branches and total, even though the oil quality was still low [20].

3.2. Verification of the mosaic virus
Detection of Potyvirus using ELISA on patchouli leaf samples before and after Nano-BC treatment showed that 100% patchouli leaves were positively infected by Potyvirus (Table 8). However, after the application of Nano-BC and biofertilizer package, there was a reduction in the percentage of Potyvirus mosaic virus-infected patchouli plants.
### Table 4. The average percentage of rooled leaf incidence and intensity on patchouli plants var. Sidikalang at 4 mo old after planting.

| Treatmentsb | Leaf roller incidence (%)c | Intensitay of leaf roller (%)c |
|-------------|-----------------------------|---------------------------------|
|             | Without Nano-BC | With Nano-BC | Without Nano-BC | With Nano-BC |
| Without cowma Plus RBPGT | 9.27 a | 9.98 a | 2.48 a | 2.84 a |
| Cowma Plus RBPGT 100 g | 8.57 a | 12.24 a | 2.44 a | 3.27 a |
| Cowma Plus RBPGT 200 g | 10.89 a | 11.95 a | 2.90 a | 3.42 a |
| Cowma Plus RBPGT 300 g | 10.05 a | 10.59 a | 2.83 a | 2.85 a |

CV 41.76 52.97

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a Assessment was conducted after 3 times spraying nanobiopesticide citronella.
b Nano-BC, nano Biopesticide Citronella; Cowma, cow manure; RBTPG, root bacteria that triggers plant growth.
c The numbers followed by the same small and capital letters in the same columns or rows, respectively are not significantly different at the level of 5% (LSD).

### Table 5. The average of height of patchouli plants var. Sidikalang and the number of primary branches at 4 mo old after planting.

| Treatmentsa | Plant height (cm)b | No. primary branchesb |
|-------------|-------------------|-----------------------|
|             | Without Nano-BC | With Nano-BC | Without Nano-BC | With Nano-BC |
| Without cowma Plus RBPGT | 31.79 b | 34.49 b | 8.57 b | 9.07 b |
| Cowma Plus RBPGT 100 g | 33.01 ab | 37.87 ab | 9.16 a | 10.47 a |
| Cowma Plus RBPGT 200 g | 35.49 ab | 34.21 ab | 10.08 ab | 9.12 ab |
| Cowma Plus RBPGT 300 g | 34.60 a | 37.16 a | 10.05 a | 10.87 a |

CV 11.84 16.15

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a Nano-BC, nano Biopesticide Citronella; Cowma, cow manure; RBTPG, root bacteria that triggers plant growth.
b The numbers followed by the same small and capital letters in the same columns or rows, respectively are not significantly different at the level of 5% (LSD).
Table 6. The average of weight of wet biomass per plant (g) and dry weight per plant (g) of patchouli var. Sidikalang 4 mo old after planting.

| Treatments \(^a\)   | Weight of wet biomass per plant (g) | Weight of dry biomass per plant (g) |
|---------------------|-------------------------------------|-------------------------------------|
|                     | Without Nano-BC | With Nano-BC | Without Nano-BC | With Nano-BC |
| Without cowma Plus RBPGT | 176.95 a        | 115.99 a     | 47.26 b         | 34.22 b      |
| Cowma Plus RBPGT 100 g | 136.14 a        | 166.51 a     | 43.49 ab        | 57.86 ab     |
| Cowma Plus RBPGT 200 g | 214.02 a        | 143.52 a     | 66.99 ab        | 45.05 ab     |
| Cowma Plus RBPGT 300 g | 188.78 a        | 190.51 a     | 59.85 a         | 61.90 a      |
|                      | A                | A             | A                | A             |

CV 30.26 29.80

\(^a\) Nano-BC, nano Biopesticide Citronella; Cowma, cow manure; RBTPG, root bacteria that triggers plant growth.

\(^b\) The numbers followed by the same small and capital letters in the same columns or rows, respectively are not significantly different at the level of 5% (LSD).

Table 7. The amount of patchouli oil production per kg dry biomass (ml), oil yield (%), patchouli alcohol level (%), and salicylic acid level (mg / kg) at 4 mo old after planting.

| Treatment \(^a\)   | Patchouli oil production per kg dry biomass (ml) \(^c\) | Oil yield (%) \(^c\) | Patchouli alcohol content (%) \(^c\) | Salicylic acid content (mg/kg) \(^c\) |
|---------------------|---------------------------------------------------------|---------------------|--------------------------------------|-------------------------------------|
|                     | Without Nano-BC | With Nano-BC | Without Nano-BC | With Nano-BC | Without Nano-BC | With Nano-BC | Without Nano-BC | With Nano-BC |
| Without cowma Plus RBPGT | 18.67           | 19.31        | 1.790           | 1.852       | 22.42           | 35.23       | Nd          | 1.375     |
| Cowma Plus RBPGT 100 g | 19.58           | 16.67        | 1.878           | 1.598       | 30.68           | 29.96       | 1.545       | 1.505     |
| Cowma Plus RBPGT 200 g | 19.33           | 16.27        | 1.854           | 1.561       | 28.78           | 29.50       | 1.335       | 1.485     |
| Cowma Plus RBPGT 300 g | 19.67           | 18.33        | 1.887           | 1.758       | 27.08           | 27.84       | 1.400       | 1.140     |

\(^a\) Nano-BC, nano Biopesticide Citronella; Cowma, cow manure; RBTPG, root bacteria that triggers plant growth.

\(^b\) Nd, not detected.

\(^c\) The numbers followed by the same small and capital letters in the same columns or rows, respectively are not significantly different at the level of 5% (LSD).
Table 8. The average absorbance value of Potyvirus in patchouli leaf samples for each treatment tested before and after Nano-BC application.

| Treatments                                      | Before application of nano pesticida citronella | After application of nano pesticida citronella |
|------------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|                                                 | Absorbance value (%) | ELISA result (%) | Absorbance value (%) | ELISA result (%) |
| Buffer                                         | 0.114                                         | 0.106                                         |
| Negative Control                               | 0.205                                         | 0.109                                         |
| Positive Control                               | 1.441                                         | 0.877                                         |
| Withoutcowma Plus RBPGT                        | 1.264                                         | 100                                           | 0.173             | 30               |
| Cowma Plus RBPGT 100 g                         | 1.298                                         | 100                                           | 0.143             | 20               |
| Cowma Plus RBPGT 200 g                         | 1.192                                         | 100                                           | 0.137             | 10               |
| Cowma Plus RBPGT 300 g                         | 1.247                                         | 100                                           | 0.367             | 50               |
| Withoutcowma Plus RBPGT                        | 1.234                                         | 100                                           | 0.755             | 90               |
| Cowma Plus RBPGT 100 g                         | 1.229                                         | 100                                           | 0.496             | 80               |
| Cowma Plus RBPGT 200 g                         | 1.197                                         | 100                                           | 0.500             | 60               |
| Cowma Plus RBPGT 300 g                         | 1.096                                         | 100                                           | 0.285             | 50               |

*RBTPG, root bacteria that triggers plant growth; NO, without Nano-BC; N1, with Nano-BC.

3.3. Soil analysis

Limited factors of land suitability for patchouli cultivation including slope, altitude, temperature, rainfall, soil texture, soil pH, CEC, K2O, P2O5 are found in land units 1, 2, 3, 4, 5 [21]. In the treatment of Nano-BC and addition of 300 g of biofertilizer per plant, it had the highest amount of P2O5 content compared to other treatments (data not shown). The addition of P2O5 and FMA mycorrhizal fertilizers can increase patchouli production and better total nutrient uptake of P, N and K [22].

Nutrient uptake effectiveness needs to be increased due to its influence in the availability and ability of nutrients absorption, which comes from inorganic and biological fertilizers, including soil organic matter, soil moisture, the presence of soil microbes and other environmental factors [22]. Additional biofertilizer contains LPK1.9 P. fluorescens and S. marcencens AR1 bacteria during patchouli planting needs to be considered in the further study.

The role of organic fertilizers on soil physical properties including (a) improving soil structure to help organic matter “bind” soil particles into solid aggregates, (b) improving soil pore size distribution for better water holding capacity [23]. Leri (rice washing water waste) which was known contains high organic C (10.04%) expected in increasing microbial activity [24]. Improvement of physical properties of soil as a result of adding organic matter also may increase the water buffering, aggregation, permeability and aeration of the soil [25].

The combination of antagonistic fungi and appropriate organic media must be used in order to suppress the disease well. Disease suppression can be more effective by adding a small number of combinations of antagonistic fungi and organic media directly on the root than those applied to the soil [26].
4. Conclusion
The technology package, i.e. combination application of the Nano-BC and biofertilizer (cow manure plus RBTPG) at a dose of 300 g per plant could reduce the incidence and intensity of patchouli mosaic disease and induce plant height in var. Sidikalang at KP Laing, Solok, West Sumatra. In this treatment, the highest content of P\textsubscript{2}O\textsubscript{5} was available in the soil at 39.00 ppm. P\textsubscript{2}O\textsubscript{5} content in the control treatment ranged from 7.72 to 11.76 ppm. The efficacy rate ranges from 9.37 to 12.47%. However, further testing in different aspects of agro-ecosystems and the application of Nano-BC to the surrounding roots of patchouli plants are still needed.

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