

Evaluating the Toxicity of Secondary Metabolites of Endophytic Bacteria from *Jatropha Curcas* L. to Suppress *Meloidogyne* spp. *in Vitro*

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Abstract: Endophytic bacteria isolated from internal tissue of surface sterilized *Jatropha curcas* L. leaves, stem, fruits and roots. Collected from field plots produced secondary metabolites in nutrition broth media (NB) that were highly toxic to *Meloidogyne* spp. especially strains SJS54 followed by RJS175 were highly active among 23 strains producing secondary metabolite toxic to Root-knot nematodes. The mechanism of action of the toxic metabolites produced by the non-pathogenic strains SJS54 and RJS175 with proven biological control of *Meloidogyne* spp in *Syracuse glass experiments* was investigated. These metabolites reduced *Meloidogyne* spp mobility within 6-12 h of exposure. After 6 h, approximately $\pm 50\%$ of juveniles was inactivated. But with exposure of 12 h 80-90% of the juveniles were dead. The SJS54 and RJS175 toxicity were highly effective towards migratory parasites. Most of nematodes have been shown body wall degradation caused by hydrolytic enzymes produced by endophytic bacteria, and the highest effect for body wall was caused by LJS69 and SJS54.

Keywords: Toxicity, secondary metabolite, exposure, hydrolytic enzymes, migratory.

1. Introduction

The damage by plant parasitic nematodes decreases 40% yield of essential and worldwide economic crops with an expected yearly yield loss of 12.3% for 20 life sustaining crops and 10.7% for the 20 crops that not considered to be life sustaining. In addition, these nematodes can be involved synergistically with other plant pathogens and cause yield losses of vegetables around 5%-34% yield losses in vegetables in tropical areas. The wide host range of plant species is susceptible to attack by plant parasitic nematodes. About 2000 plants can be infected by root-knot nematodes and they cause around 5% of worldwide harvest losses [20]. The root-knot nematodes (*Meloidogyne* spp.) are generally stationary endoparasites with more than 70 recently described and identified.

The development of root-knot galls that depletes the plant's photosynthetic activities are more often is mostly caused by second-stage juveniles (J2) of Root-knot nematode. Second-stage juveniles (J2) start the invasion by root penetration, and then hatch in soil from eggs stored in egg masses that have been laid by the females on the infected roots [11]. Many nematicides have ability to control the root-knot nematode in vegetables including eggplant, but their use is expensive and poses hazards to humans and the environment [14]. Due to the problems of pesticide impacts and residuals, nematologists have found natural enemies with modes of action similar to those used in the control of soil-borne diseases. *Rhizobium* spp., have a beneficial effect on plants including biological control of soil-borne pathogens, induce systematic resistance to plant against pathogen and improvement of nutrient uptake of plant [20]. Non-chemical

means control such as biological and cultural methods might are being good substitutes for chemical use and provide satisfactory control of root-knots in vegetables and other crops [14]. Extensive research has been directed on the utilization of mutual bacterial and endophytes as biological agents to control the plant-parasitic nematodes. A different species of plant-parasitic nematodes have been focused for biological control by endophytes, for instance, the root-knot nematode, *Meloidogyne incognita*, the Reni form nematode, *Rotylenchulus Reniform*, the cyst nematode, *Globodera pallida* and the tunneling nematode, *Radopholus similis*. The consequences of research facility, nursery and field consider related to the significance of these endophytes for the natural control of nematodes on these harvests has been checked on somewhere else [18].

Jatropha curcas L. is a woody perennial, drought-tolerant shrub is widely distributed in tropical and subtropical regions. *Jatropha* seeds contain a high level of triacylglyceride with a fatty composition well-suited for biodiesel production. *Jatropha* is resistant to drought, able to thrive on marginal land under climate and soil conditions that are unsuitable for food crop plantation. *Jatropha* helps to control soil erosion, and detoxify polluted soil [19]. The seeds are also a source of a high toxic toxalbumin, curcinn or Jatrophin [1].

This study was aimed to study the toxicity and the effect of exposure time for nematodes mortality during 6-12 hours' exposure using secondary metabolite of some endophytic bacteria isolated from *Jatropha curcas*. L to suppress Root-knot nematodes *Meloidogyne* spp extracted from infected tomato roots under *in vitro* conditions.

2. Material and Methods

2.1 Isolations of Endophytic Bacteria

Each sample (1 g) was homogenized in sterile pestle and mortar using 12.5 mm potassium phosphate buffer (pH 7.0). Serial dilutions of the homogenate up to (10^{-10}) PPB. Dilutions of all the homogenate samples have been placed separately on *Tryptic soy agar* (TSA), with three replications. The plates were incubated at 28 °C for 1 to 3 days to allow growth of endophytic bacteria. Single colonies were further sub-cultured in respective media, homogenized sample of endophytic bacteria have been selected randomly, purified, and grouped on the basis of phenotypic characteristics, e.g., colony morphology, colony color, cell shape, motility, growth rate, then have been assayed to Gram grouping test and biochemical tests [3].

2.2 Nematodes Extraction from Infected tomato Root

The root-knot nematode females, second stage juvenile (j2) and egg-masses of *Meloidogyne* spp have been extracted from infected tomato roots that were collected from the (Cipanas - west java -Indonesia) , a single egg mass culture of this nematode has been established and reproduced into tomato seedlings in a greenhouse condition at $30\pm 5^{\circ}\text{C}$. For morphological characteristics, we used the adult females, for nematode species identification following female perennial pattern [2]. For hatching second stage juvenile of *Meloidogyne* spp. Eggs were separated from egg masses with sodium hypochlorite (0.5%, 1 min). Then J2 has been obtained by placing the eggs in sterile distilled water [10].

2.3 Toxicity of Secondary Metabolite of Bacteria towards Nematodes in Vitro

Testing bacteria that are antagonistic against nematode had been described by [17]. Endophytic bacteria that have been isolated from *Jatropha curcas* roots, stem, fruits and leaves, selected isolates were tested to control nematodes larvae's of *Meloidogyne* spp, in other hand, endophytic bacteria that have been selected as a good promoter for eggplant growth were placed on *tryptic soy agar* (TSA) media for 48 h in room temperature, then the pure culture moved onto *tryptic soy broth* (TSB), bacteria was shaken for 48 h. Secondary metabolism of endophytic bacteria obtained by centrifuging the bacterial suspension at 6500 RPM, then was filtered by filter paper and finally filtered with Millipore. Then the bacterial metabolism filtrate saved into Syracuse, then adding 25 second stage juvenile (j2) of *Meloidogyne* spp. and incubated into the room temperature for 12 h. Observation has been performed to mortality numbers of nematode *Meloidogyne* spp. larva every 6 h and 12h after treatment using stereoscope [13]. For the mortality percentage were used the formula (1) described by [13].

$$\text{nematodes mortality formula} = \frac{\text{number of dead nematodes}}{\text{All tested nematodes}} \times 100\% \quad (1)$$

2.4 Proteolysis Activity Test

Proteolytic test was conducted by using the skim milk agar media (SMA) is the TSA medium supplemented with 8g skim milk. Isolates were cultured into skim milk agar, and incubated for 24 h at a temperature of 37 °C. Proteolytic activity of bacteria grow on media SMA was determined. Proteolytic index calculated by measuring the diameter of clear area and the diameter of bacterial colonies. The calculation of index was the recording the diameter of proteolytic clear halo compared to diameter of bacterial colonies [4].

2.5 Chitinolytic Activity

Chitin lytic activity on the Petri dish, bacterial cell has been streaked on semi minimal medium, a mixture of SM and *Nutrient broth* (3:1) supplemented with colloidal chitin (0.2%) and solidified with 1.5% agar. The plates were incubated at 30 °C for 72 to 96 h until the clear halo had seen around the colonies [12]-[5].

3. Results and Discussion

3.1 Production of protease and chitinase enzymes.

Twenty three single isolates of endophyte bacteria from *Jatropha curcas* L. have been used to test their biochemical properties. The test results showed that among the 23 single isolates tested, 9 isolates (39.19%) were able to hydrolyse the protein and 3 isolates (13.04%) is able to hydrolyse chitin Such as described in figure (1). The ability of bacteria from *Jatropha curcas* to produce proteolysis enzymes appear when they tested on TSA media supplemented with skimmed milk there were 9 strains have shown their capability to show up clear zone around the bacteria colonies, and this result is rely corresponded with the results of [8] when they examined the strains from *Jacaranda decurrens* to produce proteolysis enzymes. [24] Suggested that the better bio-control agent micro-organism should be isolated from the plant part such as rhizophore of a specific crop may be better adapted to that plant and may give better performance to eliminate the pathogens or disease causal than organisms originally isolated from other plant species. Moreover, bio-control agent to control pathogens should have several mechanisms, such as the ability antibiosis, hydrolysis of chitin, proteins, and lipids; production of cyanide (Hydrogen Cyanide / HCN), dissolving phosphate (P), and fixation of nitrogen (N₂) [9].

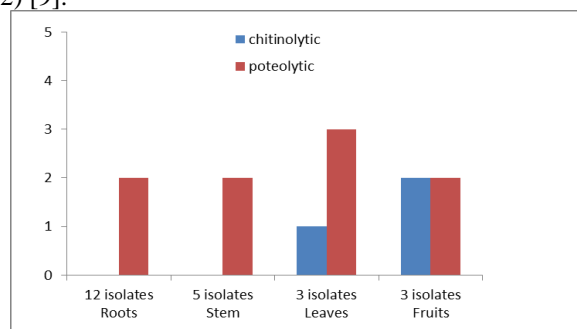


Figure 1: The number of bacterial isolates produce hydrolytic enzymes biases on the plant part.

[22] Mentioned that some of the *Bacillus cereus* endophytic strains are able to hydrolyse colloidal chitin after 96 h on agar (SM) supplemented with colloidal chitin as the sole carbon supply. Sector of clearing have been located around the developing micro-organism, suggest the presence of *chitinolytic* activity within the area around bacteria colony into the selective medium. This final result absolutely agreed with our result from endophyte strains isolated from *Jatropha curcas*, 3 strains had been shown their capacity to produce *chitinase* enzyme become proved in the selective medium as described above inside the methodology.

Many endophytic bacteria invade plant tissues using mechanisms similar to pathogens, the use of hydrolytic enzymes, or herbal (e.g. Stomata) or synthetic (wound) openings; but, their population density is usually lower than pathogens. Maximum are now not diagnosed with the aid of the vegetation as potential pathogens. Endophyte bacteria depend heavily on nutrients furnished by the host plant. For that reason, variables affecting plant nutrition also have an effect on the endophyte communities [26]. According to this

concept, we considered that why the number of endophyte bacteria was a little affected in *Jatropha curcas* by biotic and abiotic factors and the importance of the hydrolytic enzymes help the endophyte bacteria to colonize the plant tissue

3.2 Toxicity of Secondary Metabolite of Bacteria Towards Nematodes in Vitro

Observation of 6 hours and 12 hours after treatment, the treatment by SJS54 supernatant has given highest effect 27% and 97.51.4% respectively. However, the isolates RJS175 caused the mortality rate was about 93.4% after 6 hours and increased to 95.4% 12 hours after treatment compared with the control. Data have been shown in table (1). moreover, Nematode mortality caused by strain RJS175 could be associated with the ability of this strain to produce *hydrogen cyanide* (HCN) toxic to *Meloidogyne* spp and can produce extracellular protease enzymes which can degrade nematode body wall that consisting of chitin and protein and were also able to solubilizing phosphate.

Table 1: The result of mortality percentage for nematodes due the exposures for bacteria supernatant, and the result of chitinase and protease assays

| strains code | 6-(HAT) mortality% | 12-(HAT) Mortality% | chitinase | protease |
|--------------|--------------------|---------------------|-----------|----------|
| RJS175 | 93.4 | 95.4 | - | +++ |
| RJS176 | 66.4 | 89.2 | - | - |
| RJS177 | 51.9 | 60.2 | - | - |
| RJS178 | 14.5 | 68.5 | - | - |
| RJS179 | 12.4 | 74.7 | - | - |
| RJS181 | 70.5 | 93.4 | - | - |
| RJS184 | 80.9 | 85.1 | - | - |
| RJS185 | 58.1 | 78.8 | - | - |
| RJS186 | 56.0 | 72.6 | - | ++ |
| RJS188 | 49.8 | 64.3 | - | - |
| RJS189 | 66.4 | 76.8 | - | - |
| RJS161 | 51.9 | 85.1 | - | - |
| FJS16 | 70.5 | 80.9 | ++ | ++ |
| FJS17 | 27.0 | 45.6 | ++ | ++ |
| FJS37 | 34.5 | 57.5 | - | - |
| SJS54 | 27.0 | 97.5 | - | +++ |
| SJS57 | 31.1 | 93.4 | - | ++ |
| SJS60 | 72.6 | 85.1 | - | - |
| LJS67 | 78.8 | 80.9 | - | + |
| LJS69 | 27.0 | 80.9 | ++ | +++ |
| LJS70 | 68.5 | 91.3 | - | +++ |
| FJS23 | 33.2 | 76.8 | - | - |
| FJS24 | 29.0 | 62.2 | - | - |
| Control | 0 | 35.3 | - | - |

The mortality rate 6 and 12 hours after treatment (HAT). +) is positive result; ++ moderate positive result; +++ is strongly positive result for test; -) negative result for test.

While the strain SJS54 was able to produce the proteolysis enzyme and can fixing nitrogen. Some reports have been recorded the endophyte ability of some bacteria as bio-control agents to suppress the various plant soil-borne pathogens and nematodes. These endophyte bacteria have been recorded have a possible role of growth inhibition by the bacterial antagonists, either as an endophyte or exogenous colonizer was attributed to the production of diffusible and volatile metabolites [21]. See figure 2. Otherwise, this antagonistic bacteria *Pseudomonas* spp are

strongly capable to produce volatile compounds such as HCN and salicylic acid [7]-[15]. however *Bacillus* spp strains was found Able to produce IAA production and *Bacillus thuringiensis* (Bt) produces one or more Para sporal crystal inclusions (Cry or d-endotoxins) and reported that although ome Also Cry proteins are toxic to nematodes [13]. In research [25]. stated that the culture filtrate of endophytic bacteria *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Stenotrophomonas* and *Enterobacter* showed nematocidal effect between 38% to 98%. Several in vitro studies

demonstrate that isolates of endophytic bacteria can inhibit the development of nematodes and hatching [16].

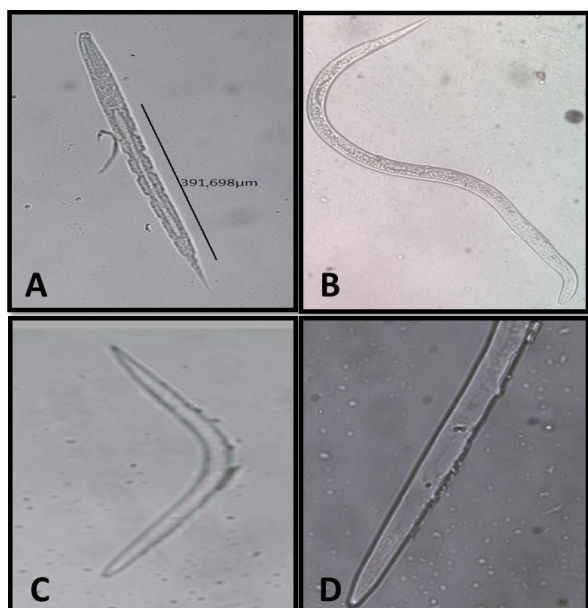


Figure 2: the action of the proteases and chitinase hydrolytic enzymes LJS69, SJS54 and culture supernatant of Endophytic bacteria. RJS175 against Root-Knot. (a) The nematode cuticles were degraded and their bodies destroyed after 24 h in the LJS69 treatments. (b) The cuticles of nematodes in the control were not affected within 24 h. (c) The cuticles of nematodes in the RJS175 treatment were also degraded within 24 h. (d) the nematode cuticles were degraded after 24 h in the secondary metabolite treatments by SJS54.

The former results have been mentioned that the *Bacillus* strains have the potential to inhibit the plant disease casuals and can be as a growth promoter. And it's well known their ability to produce some beneficial compounds for the plant such as nitrogen fixation, phytohormones production (IAA), siderophore, hydrolytic enzymes, and antimicrobials. *Bacillus subtilis* and *B. megaterium* are most common *Bacillus* spp studied and they have been shown direct antagonism against plant nematodes [23]. Currently, there are some Bio-control products composed from rhizobacteria to be used for pests and disease control some of this commercial Bio-control products contain some of *Bacillus* Spp. [13]. The native population of *Bacillus* occurs in the soil, Some research findings they reported the mode of action of *Bacillus* spp are related with for examples they eliminate the plant pests and pathogens through producing antimicrobial substances, also can enhance the plant defense system to the pathogens (induce the systemic resistance ISR) and plant health and growth [6].

4. Conclusion

The research finding that the endophytic bacteria are powerful tools to control the plant disease casuals especially nematodes. The result of nematodes mortality 6 and 12 hours after treatment (HAT) due to the secondary metabolite of endophytic bacteria that have been recorded their ability to

produce chitinase and protease hydrolytic enzymes were very effective to control nematodes *in vitro*. Among 23 tested isolates the isolate SJS54 has shown highest mortality rate after 12 hours. The proteolytic and chitinolytic bacterial supernatant of LJS69, SJS54 and RJS175 have been shown the good result to degrade and destroy the nematodes body wall due to hydrolytic enzymes. These bacteria's might have more modes of actions and should be formulating as commercial products in the future to be more available and useable by the simple farmers as Bio-nematicidal.

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