Endophytic Colonization and Plant Growth Promoting Effect by Entomopathogenic fungus, *Beauveria bassiana* to Red Chili (*Capsicum annuum* L.) with Different Inoculation Methods

IOP Conf. Series: Earth and Environmental Science **305** (2019) 012070 doi:10.1088/1755-1315/305/1/012070

Endophytic Colonization and Plant Growth Promoting Effect by Entomopathogenic fungus, *Beauveria bassiana* to Red Chili (*Capsicum annuum L.*) with Different Inoculation Methods

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Abstract. *Beauveria bassiana* (Bals.) Vuill is one of entomopathogenic fungus , that be able to endophytically colonize different plants. *Beauveria bassiana* can promote the growth of red chili following their endophytic establishment within plants through seed treatment, soil drenching and foliar spraying. The aims of this study were: (i) To get the best colonisation *B. bassiana* from different isolates and to differentiate inoculation methods, i.e. seed immersion, soil drenching and foliar spraying, for the endophytic inoculation of *B. bassiana* in red chili; and (ii) to assess the effect of *B.bassiana* to stimulate or trigger plant growth from isolate *Wheat, Coffea*, *Cacao* and *Leptocorisa acuta*. Our result showed that all of *B. bassiana* isolate were able to colonize seedling of chili and *B.bassiana* of wheat isolate was the best isolate giving spuring seedling or plant growth. From the three inoculation methodes, inoculation *B. bassiana* by foliar spraying was the highest colonisation. The highest colonization was in leaves, and the second were in stem. Root plant was the poorly colonized by *B. bassiana* fungus.

1. Introduction

Endophytes are microorganisms which colonize symptomless living plant tissue without causing any immediate, overt, negative effect on the plant [1]. Entomopathogenic fungi are commonly found in diverse habitats and are known to infect many different taxa of arthropods. These fungi have also been found as rhizosphere colonizers in the surrounding environtment of the host plant. In addition, recent evidence suggest that certain entomophatogenic fungus species have the potential to engage in fungus-plant interactions, as fungal endophytes or plant disease antagonists, without causing any immediate negative effect or even promoting growth of host plants [2]. However, although many entomopathogenic fungal endophytes might not be very abundant in most plant species, some taxa like *Beauveria bassiana* (Bals) Vuill. (Ascomycota: Hypocreales) have a wide range of plant hosts.

The occurence of entomophatogenic fungi as natural endophytes also indicates that these fungi have complex life cycles, which can be completed in soil, invertebrates, and plants. *B. bassiana* is an entomophatogenic fungus with worldwide distribution, which can live as a plant endophyte and usually does not cause visible damage to the host [3]. It has been naturally isolated from several plant species, and artificially introduced into many others, such as banana (*Musa paradisiaca* L.) [4], coffee (*Coffea arabica* L.) [5], sorghum (*Sorghum bicolor* Kuntze) [6], tobacco (*Nicotiana tabacum* L.), corn (*Zea mays* L.), wheat (*Triticum aestivum* L.) and soybeans (*Glicine max* L.) [7] by using various techniques. Colonization of plant tissues by *B. bassiana* has proved to provide protection against insect damage and inhibition of insect establishment and development [8,2]. No reports are yet available on the potential of *B.bassiana* to establish as an endophyte in chili, an important Solanaceae

crop in West Sumatera. Endophytes can actively or passively promote the plant growth through a variety of fitness the endophytic metabolites provide. Preliminary studies showed that *B.bassiana* colonizes chili, but the rate of colonization may depend on how the plant is exposed to the fungus. Therefore, the present work evaluated the effects of inoculation method and plant growth medium on colonization of chili by *B.bassiana* entomopathogenic and endophytic fungus.

2. Materials and Method

2.1. Fungal inoculum preparation

The fungal strain used was *B.bassiana* from cacao, coffeae, wheat and *Leptocorisa acuta* isolates, obtained from the culture collection of Biological Control Laboratory, Faculty of Agriculture, Universitas Andalas. *B.bassiana* fungus was cultured on Sabouraud dextrose agar medium supplemented with yeast extract (SDAY) (10 g peptone, 20 g dextrose, 5g yeast extract and 15 g agar, 1 L distilled water) and antibiotics (0,1 g penicillin, 0,2 g streptomycin and 0.05g chlortetracycline^{-1} SDAY) in 55 mm diameter petri dishes, incubated for two weeks in the laboratory. Conidia were harvested by gently scraping them off the surface of the dried medium using a sterile scalpel blade. Conidia were determined by dissolving 0.1 g conidial powder in 10 ml sterile deionized water containing 0.01 % Tween 80 in a sterile 500 ml bottle. After vortexing for one minute, serial dilutions were made, and the conidial concentration was determined using an improved Neubauer haemocytometer. The conidial concentration for each treatment was adjusted to $1x10^8$ conidia ml^{-1}. Germination test of conidia was done before inoculating in the plants.

2.2. Inoculation methods

All isolates of *B.bassiana* fungus was inoculated by three different methods: seed immersion, soil drenching and foliar spraying. Fifteen plants were used per inoculation. For seed immersion treatment, 50 g of seeds were immersed into 10 ml of *B.bassiana* conidial suspension of various consentrations for 6 hours. After that the inoculated seeds were dried on sterile tissue paper for 30 min and they were sown in 15 cm diameter pots. Seeds soaked in sterile distilled water containing 0.01 % Tween 80 were used as control. For control seeds were immersed with sterile water. The foliar spray inoculation method was performed with a hand sprayer to inoculate each seedling with 10 ml conidial suspension of various concentrations at fifteen days after emergence of seedlings.

The spray was directed mainly to the leaves but also incidentally coated the stems. To avoid conidial runoff to the soil, the soil top of each pot was covered with alumunium foils. For the soil drench inoculation method, 10 ml conidial suspension concentrations were applied around the root zone of each seedling. In the control, sterile distilled water was applied in the same way as mentioned above. After inoculation, each plant was covered with a plastic bag for 24 hours to maintain a high level of humidity.

2.3. Evaluation for presence of B.bassiana in chili tissues

The recovery of *B.bassiana* as endophytic fungi was evaluated by culture methods seven weeks of post inoculation. Stems were cut off (about 5 cm above the stem base) from roots using a sterile blade. The leaves were randomly selected from the middle section of the seedling. Similarly, two parts of the stem were sampled, one towards the middle of the plant and the second one closer to the soil surface. The leaves were cut into 1 cm^2 sections, sterilized in a laminar airflow cabinet by dipping in 75% ethanol for 2 min. The tissues were dried on sterile paper towels and placed in 55 mm petri dishes containing SDAY. The medium was supplemented with antibiotics to prevent bacterial contaminanation. A total of 15 plants and 75 plants tissue sub samples were evaluated for each treatment during the course of one inoculation period. The petri dishes were incubated for seven days at 25 ± 2^0 C, in the laboratory, after which all plant samples were visually examined for fungal outgrowth. *B.bassiana* colony was characterized as based on white dense mycelia, becoming cream to pale yellow at the edge [9]. Percentage colonization was calculated as number of samples exhibiting *B.bassiana* outgrowth per total number of samples, results are expressed as the percentage of plants positive for the presence of *B.bassiana* after inoculation.

3. Results and Discussion

3.1. Colonization of B.bassiana as endophyte

This study demonstrates that *B.bassiana* can be established as an endophyte in chili leaves, stems and roots, by inoculating leaves, seeds, or soil. All fungal isolates of *B.bassiana* were used and success to colonize chili plants. All inoculation methods were effective in introducing *B.bassiana* into the plant, although with different levels of efficiency. When the data are combined for all inoculation methods, the total of highest percent of plants was in foliar spraying inoculation for leaf colonization and the second was in soil drenching inoculation for stem colonization, and root was lower colonized by soil drenching and foliar spraying, data was shown on Table 3.

In application using foliar inoculation method, highest colonization of 80 % was observed in leaf wheat isolate followed by 40 % in stem samples and 13.3 % in root. For soil drenching methods, was 66,7 % in stem of wheat isolate, 53,3 % in leaf and 13,3 % in root samples respectively. In seed immersion methods were the lowest percentage of colonization in leaf and stem of wheat isolate. For all treatment inoculation methods, root colonization of seed immersion application was the highly colonized with 33,3% in *L.acuta* isolate. There was not isolated from any of the samples from control plants. In soil drenching inoculation methods, percent colonization of *B.bassiana* was higher in stem, than in leaves, whereas in foliar application methods, the percentage of colonization was higher in leaf than in stem. Three weeks after applications of *B.bassiana* on plant, all fungal isolates were successfully recovered from the stem and leaves interior of chili plants, clearly indicating that chili can serve as a suitable host for *B.bassiana* endophyte.

As an entomopathogenic fungi, *B.bassiana* fom cacao, coffeae, wheat and *L.acuta* isolates has been reported as an endophyte in various plants by different methods of inoculation such as foliar sprays, radical dressing, root and rhizome immersion, seed coating and soil drenching [10]. By giving high consentration of inoculum, it may increase colonization and persistence of endophytic *B.bassiana* through translocation inside plant tissues. The lack of any visual symptoms on the seedlings also indicates that *B.bassiana* can colonize this plant without causing any detrimental effects to the host. Differenti *B.bassiana* colonization on plant parts was demonstrated in corn and cocoa [5]. In corn, the fungus was more frequently isolated from the internode below the primary ear and less frequently from the leaf collar at the primary ear. In cocoa, colonization rates in roots were higher than those in stems and leaves.

3.2. Endophytes fungal isolated from chili plant

All fungal isolates were successfully recovered from plants. In conclusion, the result of this study indicated that the entomopathogenic fungi *B.bassiana* can form an endophytic relationship with chili plants through foliar spraying and soil drenching inoculation methods as the best methods for the delivery of *B.bassiana* into the chili plants. In addition. *B.bassiana* inoculum most likely remains stable in the chili plant. *B.bassiana* becoming an endophyte, introduces the possibility that the fungus might naturally recycle in the chili ecosystem. However, the level of of colonization seemed to be substantially affected by plant growth medium on plant colonization by the fungus which has not been previously considered. Successful colonization of many plant species following inoculation with *B.bassiana* has been reported previously [4,5,8,11,12,13]. Endophytic colonization by *B.bassiana* however, depend on the inoculation method, fungal isolates, and plant species. For example, the highest post-inoculation recovery of *B.bassiana* occurred after direct injection in coffee [5], dipping plants in conidial suspension in banana tissue culture [4], foliar application in opium poppy [13] and maize [11], and seed coating in tomato [8]. In the current study, *B.bassiana* colonization differed among the plant parts. Leaves and stems were colonized to a greater extent than roots. The colonization of the different plant parts indicate that the fungus moves within the plant system.

The reason for higher colonization of leaves and stems is not clear but could reflect differences in microbial and physiological characteristics in different plant parts. Endophytic fungi exhibited tissue

specificity because they are adapted to particular conditions present in a given plant part [14, 15]. The success of artificial inoculation of *B.bassiana* as an endophyte into chili plant determines many future works. It should focus and optimize the long term establishment of *B.bassiana* on chili plant and inoculated plants will be evaluated for its virulence and resistence against major pest of chili such aphids and mites.

3.3. Plant growth

The effect of the fungus on plant growth was determined by measuring plant height, and total leaves each treatment at six weeks or 42 days after inoculation of the plants with the fungus. Plant height was measured from the soil surface to the tip of the stem and for leaves by calculating each leaf pair, every leaf that has opened perfectly in each plant sample in strands. Data about the height of chili plant can be seen on Table 1.

Table.1. The effect of Inoculation methods on chili height

Isolates	The average of chili height (cm)		
	Soil Drenching	Seed Immersion	Foliar Spraying
Cacao	28.30^{b}	27.20^{bc}	29.40^{b}
Coffee	27.60^{b}	25.90^{c}	28.20^{b}
Wheat	31.80^{a}	30.10^{a}	32.50^{a}
L.acuta	30.80^{a}	28.60^{ab}	32.10^{a}
Control	24.30^{c}	24.30^{d}	24.30^{c}

The plant growth data were subjected to one way ANOVA using the SPSS version 16. Significant differences between means were determined with the Student-Newman-Keuls test (P= 0.05). Plant growth was affected by the different inoculations methods. Aplication of *B.bassiana* to leaves, seeds, or soil were significantly different in plant height. All treatment showed, were significantly different between inoculation and control. Wheat isolate was the highest for plant height and followed *L.acuta* isolate. Wheat isolate was the superior strain in triggering plant growth. Endophytes can actively or passively promote the plant growth through a variety of mechanisms, as endophytic metabolites provide a variety of fitness to host plants enhanced by increasing plant resistance to biotic and abiotic stresses, as well as enhance plant growth.

Many endophytes are capable of solubilizing phosphate, enhancing uptake of phosphorus (P), fixing nitrogen, producing siderophores, and other the plant hormones such as auxin, abscisic acids, ethylene, gibberellins, and indole acetic acid (IAA), which are important for the plant growth regulations [16-21]. Gibberelin acid (GA) is a potent phytohormone, which regulates the plant growth. Fungal endophyte, *Cladosporium sphaerospermum* from Glycine max plant produced GA3 and GA4. It induced plant growth in rice and soybean [22]. *Fusarium tricinctum* and *A. alternata* also produced derivatives of indole acetic acid that enhanced plant growth [23]. Data for the number of chili leaves between all inoculation methods can be seen on Table 2, while data percentage of colonization on chili by differences inoculation methods can be seen on Table 3.

Table. 2. The effect of inoculation methods on number of chili leaves

Isolates	The average of number of chili leaves		
	Soil Drenching	Seed Immersion	Foliar spraying
Cacao	16.00^{c}	13.00^{c}	19.00^{bc}
Coffee	16.00^{c}	13.00^{c}	18.00^{c}
Wheat	20.00^{a}	18.00^{a}	24.00^{a}
L.acuta	18.00^{b}	16.00^{b}	22.00^{ab}
Control	10.00^{d}	10.00^{d}	10.00^{d}

All fungal isolates had significant effect with different inoculation between treatments and control plants. Endophytic fungi can enter plant tissue through seeds or seeds horizontally. Endophytic fungi can naturally be in the seeds on plants, promoting the plant growth along with other endophytic microorganisms. Recovery of endophytic fungi will confirm its presence on tested plants. The tested plants tested were chili aged 6 weeks after inoculation to see colonization percentage as shown in Table 3.

Table 3. The effect of differences inoculation methods on chili colonisation

Isolat	Percentation of colonization (%)								
	Soil drenching			Seed immersion			Foliar spraying		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Cacao	6.7^{a}	26.7^{b}	26.7^{b}	6.7^{b}	26.7^{a}	20.0^{a}	13.3^{a}	33.3^{a}	26.7^{c}
Coffeae	6.7^{a}	33.3^{b}	26.7^{b}	13.3^{ab}	26.7^{a}	26.7^{a}	6.7^{a}	26.7^{a}	33.3^{c}
Wheat	13.3^{a}	66.7^{a}	53.3^{a}	26.7^{a}	33.3^{a}	33.3^{a}	13.3^{a}	40.0^{a}	80.0^{a}
L. acuta	6.7^{a}	53.3^{a}	33.3^{b}	33.3^{ab}	40.0^{a}	26.7^{a}	13.3^{a}	40.0^{a}	60.0^{b}
Control	0^{b}	0^{c}	0^{c}	0^{a}	0^{b}	0^{b}	0^{a}	0^{b}	0^{d}

The result indicates a corelation between the number of plant leaves on the percentage of colonization, where the highest number of leaves founded in wheat isolate. Endophytic fungi can be transmitted horizontally and be able to colonize roots, stems and leaves which may increase root biomass and root shoots [24]. Infection with some endophytic fungi can increase growth, tolerance to plant stressors and to induce resistance to plant pathogens and herbivore insect of chili [25].

4. Conclusions

Application of *Beauveria bassiana* fungus using different methods on chili were shown to colonize all part of plants. All isolates were found establishing as endophytic fungi and were recovered from wheat which designated as the best isolate promoting plant growth. Inoculation of *B.bassiana* using foliar spraying produced the highest colonization on leaves, while the second colonization was on stem using soil drenching and roots were the less colonized by *B.bassiana* endophytic fungus using seed immersion method.

Acknowledgment

This project was full financially supported by Prof. Dr. Trizelia, MS as promotor through laboratory access in Universitas Andalas.

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