BIOCONTROL MECHANISMS OF ENDOPHYTIC *BEAUVERIA BASSIANA* IN THREE TOMATO (*LYCOPERSUM ESCULENTUM*) VARIETIES

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Abstract
***Beauveria bassiana* has gained considerable attention as biological control agent for most agricultural pests, however environmental variability has affected its efficacy and persistence. There is need to improve the delivery mechanism for *B. bassiana*, such as it's use as an endophyte for protection from harsh environmental conditions. Endophytic fungi vary in their mode of action for insect and disease control. A clear understanding of the mode of action involved in biological control of *Tetranychus evansi* by endophytic *Beauveria bassiana* is important for successful application in the field. *B. bassiana* is known to synthesize hydrolytic enzymes such as lipase and protease which assist in degrading of the insect cuticle components. *B. bassiana* isolate IC 35 colonized tomato varieties Cal J, Kilele and Anna F1 in the screen house. The objective of this study was to determine the production of extracellular enzymes such as lipase and protease by endophytic *B. bassiana* isolate IC 35 on solid medium. To test the production of lipases, endophytic *B. bassiana* IC 35 from the three tomato varieties was grown for a-week on Sabourand Dextrose Agar and point inoculated on medium containing sorbitan monolaurate. To assess the protease activity, IC 35 was point inoculated on gelatin medium. The control plates lacked enzyme–specific substrates. All petri dishes were incubated for one week under laboratory conditions of 27^0c and 70% Relative Humidity. The diameters of the clear zone of inhibition and of fungal colonies were measured. Control plates had no clear zone since no enzymes were produced in them. Maximum lipase value of 2.15 cm and protease values of 2.2 cm activities were recorded on day 5 and there were significant difference among the tomato varieties for the treatments and control. These enzymes are important since they correspond to the main chemical integuments of the insect body, hence assist in breaking it down for effective biological control.**

Keywords: Entomopathogenic fungi, Biocontrol, Protease, Lipase, clear zone

Introduction

Beauveria bassiana is a natural fungal endophyte in corn (*Zea mays*), cotton (*Gossypium hir sutum*) and jimson weed (*Datura stramonium*). Endophytic isolates from these crops have been isolated using plating techniques on selective media (Doberski and Tribe, 1980). In horticultural crops such as tomato (Powell et al., 2009), the fungus becomes established as an artificial

endophyte for controlling insect pests and plant diseases (Posada and Vega, 2006). *B. bassiana* has also been reported as an endophyte in opium poppy seeds (Quesada-Moraga et al., 2006) and cotton (Gurulingappa et al., 2011).

The term endophyte refers to the colonization of plants by micro-organisms that live most of their life inside plant tissues without causing

disease symptoms (Carroll, 1988). Endophyte protection of plants lies in the synthesis of defensive alkaloids and other toxic compounds (Faeth and Fagan, 2002), the induction or priming of resistance-related genes and enhancing the genetic (Conrath et al., 2006) and biochemical diversity of their host plants(Akello, 2012). These processes are determined by a combination of environmental conditions, plant and endophyte genetic factors (Vega et al., 2008). Alkaloids released by endophyte-infected plants have the capacity to deter insect feeding or reduce insect performance (Vega, 2008). The alkaloids also promote growth and enhance the absorption of nitrogen and phosphorus from the soil (Faeth and Fagan, 2002; Vega et al., 2009). Direct parasitism can also occur as a mechanism of control through the hydrolytic activity of extracellular enzymes such as protease and lipase which penetrate the plant cell wall and aid to hydrolyze the epidermis of the insect body (Burke and Cairney, 2002; Ownley et al., 2010; Petrini et al., 1992; Smith et al., 1981).

The use of *B. bassiana* as endophyte in the management of insect pests and diseases in plants is beneficial since it requires little inoculum and can overcome environmental constraints, hence it can stay in the plant for a longer period of time (Akello et al., 2007). Endophytic isolates of *B. bassiana* occur naturally in plants such as corn, cotton and jimson weed (Doberski and Tribe, 1980), however successful introduction has occurred in plants such as maize (Wagner and Lewis, 2000),cacao (Posada and Vega, 2006), date palm (Gómez-Vidal et al., 2009), opium poppy (Quesada-Moraga et al., 2006), banana (Akello et al., 2007), coffee (Posada et al., 2007), sorghum (Tefera and Vidal, 2009), jute (Biswas et al., 2012), bean (Mutune et al., 2016), grapevine (Jaber, 2015), radiate pine (Brownbridge et al., 2012), wheat, cotton, bean, pumpkin and tomato (Gurulingappa et al., 2010). However, the effect of endophytic *B. bassiana* on tomatoes is yet to be determined.

Mechanisms through which entomopathogens can control pests and diseases in plants include; antibiosis, competition for niches and nutrients, parasitism/predation, and induction of a plant defense response (Vega et al., 2009). *Beauveria bassiana* produces a variety of relatively low molecular weight secondary metabolites (Fravel, 1988), some of which have antibiotic properties while others aid in virulence. The secondary metabolites are also known as toxins and include lytic agents, enzymes, volatile compounds and antibiotics (Fravel, 1988), although not all are produced by endophytic *B. bassiana* isolates (Strasser et al., 2000).

Penetration of insect cuticles by *B. bassiana* is a result of mechanical forces combined with the action of enzymes proteases, chitinases, lipases and lipoxygenases which break down the cuticle, and also provide nutrients to the fungus (Mustafa and Kaur, 2009). A positive correlation between the presence of extracellular enzymes and pathogenicity has been reported (Bidochka and Khachatourians, 1994).

Enzyme lipase production depends on the fungal isolate strain and the composition of the growth media which includes the nitrogen to carbon ratio. Cultivation conditions such as temperature and agitation may also influence its production (Burke and Cairney, 2002). Lipase is involved in the breakdown of lipids in organisms as well as their transfer from one organism to another, as well as nutrients release (Shukla and Gupta, 2007). In addition lipases also improve the adhesion of the spores to the epicuticle, aiding the fatty acids and alkenes degradation in the cuticle waxy surface. Insects secrete ebelactone B as a defense mechanism, a compound that inhibits

the infection by the entomopathogenic fungi (Supakdamrongkul et al., 2010).

Proteases are important in the metabolism of most organisms. Plant cell wall components such as cellulose and carbohydrate polymers are held together by protein linkages, hence these enzymes play a significant role in aiding the fungi to invade the host plant (Sreedhar et al., 1999). Pathogenicity and virulence are related to protease production, since they are most important within the infective process. After the lipases breakdown the epicuticle breaks down by the lipases, the fungus usually produces large quantities of protease, which degrades the protein material in the insect body so that it serves as nutrients for entomopathogenic fungi (Wang et al., 2002). Research on new strategies have been done to improve the entomopathogenic fungi virulence through genetic engineering with protease recombinants since without this enzyme no infection can occur (Mustafa and Kaur, 2009; Wang et al., 2002).

In most studies conducted with endophytic *B. bassiana*, no adverse or ill effects have been reported to result from the association between the fungus and the host plant (Akello et al., 2009). The objective of this study was therefore to determine the production of extracellular enzymes such as lipase and protease by endophytic *B. bassiana* isolates on solid medium from Cal J, Kilele and Anna tomato varieties.

Materials and Methods

Culture of fungal mycelia

This study was conducted at Jomo Kenyatta University of Agriculture and Technology laboratories, Nairobi, Kenya. *Beauveria bassiana* isolates were obtained from the ICIPE's Arthropod Germplasm Centre, Duduville, Nairobi, Kenya. The isolates were cultured on Sabouraud Dextrose Agar (SDA) medium which contains glucose, peptone and agar amended with 0.05 g antibiotic chloramphenical to minimize bacterial contamination and incubated for 21 days at 27°C (Inglis et al., 1996). Conidia were gently scrapped from fungal cultures and suspended in 10 ml sterile distilled water in 20-ml universal bottle containing 0.01% Tween-20 and glass beads. The conidial suspension was vortexed for 5 minutes to produce a homogenous conidial suspension. From the stock solution, a concentration of 1×10^9 conidia ml^{-1} was prepared by counting the spores and calculating to get exact concentration since the standard concentration of 1×10^8 from preliminary studies did not establish well in the tomato varieties. The viability of conidia was assessed before any bioassay by spread-plating 0.1 mL of 3×10^6 conidia mL^{-1} which is the standard used for germination counts on to 90-mm Petri dishes containing SDA (Goettel and Inglis, 1997). The plates were incubated at 27 ± 2 °C and were examined after 16-20 hours under the compound microscope (× 40 magnifications). Conidia were considered as germinated when the germ tube was twice the diameter of the conidium (Goettel and Inglis, 1997). The experiment was replicated four times over time. In viability tests, >90% of conidia of all the isolates germinated.

Seed inoculation and colonization

Seeds of Cal-J, Kilele F1 and Anna F1 hybrid were surface-sterilized in 70% ethanol for 1 min and then in 1.5 % sodium hypochlorite solution for 3 min. They were then washed three times with sterile distilled water and blotted with sterilized paper towels to remove the excess water. The last rinse water was plated out by spreading on a plate with SDA to assess the effectiveness of the surface sterilization procedure. Inoculation was carried out by soaking seeds in conidial suspension (10 ml) of each isolate at the concentration of 1×10^9 conidia ml^{-1} for 2 hours. Mixtures were hand stirred at a 30 min interval until the seed

were uniformly coated (Powell et al., 2009). Control seeds were soaked in sterile distilled water 0.01% Tween-20 for 2 hours. The seeds were then removed and placed in pots filled with sterilized soil. There were 8 individual plants for each of the three tomato varieties and their respective controls. The trays containing the seedlings were grown in the growth chamber at 25 ± 2 ^{0}C, 70% RH and a photoperiod 12:12 L: D for 4 weeks. Sampling for colonization of plant parts was done for tomato seedlings after every two weeks by uprooting entire plants for each variety and treatment.

To determine the colonization of tomato plants by *B. bassiana*, plants were carefully removed from the pots 2 weeks after inoculation and were washed with tap water and surface-disinfected by submersing them in 70% ethanol for 15 s, followed by 3 min in 1.85% sodium hypochlorite and rinsed 3 times in sterile distilled water (Elena et al., 2011). To determine the effectiveness of the disinfecting process the final rinse water was plated on SDA as previously described. Plants were dried on sterile paper towels and tissues from leaves, shoots and roots were cut into 4 mm^2 sections with a sterile scalpel and about 5 of them were placed on Petri dishes containing SDA medium. Four plates were cultured for each tissue (roots, shoots and leaves) and for each treatment including the control. For each plant part, percent colonization was calculated as number of sections exhibiting *B. bassiana* outgrowth per total number of sections (Fisher and Petrini, 1987).

$$\%\, colonization = \frac{Number\ of\ pieces\ exhibiting\ B.bassiana\ outgrowth}{Total\ number\ of\ pieces\ plated} \times 100$$

Endophytic *B. bassiana* isolate IC 35 established on the three tomato varieties and was cultured on Sabouraud Dextrose Agar (sigma) amended with chloramphenicol and malt extract agar based on isolation medium developed by Elena *et al*. (2011). IC 35 was evaluated for extracellular secretion patterns of lipases and proteases. The use of solid media for the detection of a wide array of extracellular enzymes produced by fungi was carried out as per the method described by Hankin and Anagnostakis (1975).

To test lipase production, IC 35 isolate from 1-week-old cultures on SDA was point-inoculated on medium containing a lipid (Tween 20) as the primary source of carbon. The medium components was as follows: peptone - 1 g, yeast extract - 0.1 g, agar 18 g, Tween 20 - 10 mL (autoclaved separately from the rest of the medium), distilled water - 990 mL, the pH was adjusted to 6.0 before autoclaving and pouring. A positive test was the occurrence of precipitated fatty acid crystals around the colony (Gessner, 1980). The plates were measured after 6 and 8 days.

The medium used to detect protease enzyme activity contained gelatin as the protein substrate (Hankin and Anagnostakis, 1975). The medium consisted of nutrient agar plus 0.4% gelatin at pH 6. An 8% solution of gelatin in water was sterilized separately and added to the nutrient agar at the rate of 5 mL per 100 mL of medium. After incubation, plates were flooded with an aqueous saturated solution of mercuric chloride which precipitates protein. A clear zone around colonies indicated the presence of protease. The plates were measured after 6 and 8 days.

Ten Petri dishes were used for the isolate and enzymes; five with medium amended with the substrate and five without the substrate (controls) according to the protocol described by Hankin and Anagnostakis, 1975. The medium in the control plates comprised of each of the above-mentioned ingredients

except the enzyme–specific substrate. All petri dishes will be incubated for 1 week under laboratory conditions. Cultures were examined daily for the presence of a clear zone (halo) and the precipitation of fatty acids crystals around the fungal colony. The diameters of the clear zone was measured in cm and precipitation of fatty acids crystals were measured in cm, and the difference between these areas and the fungal colony calculated to provide an estimate of the levels of enzyme production (Alves et al., 2002).

Data Analysis

For the enzyme production assays, results were expressed as mean ± standard error (SE). Comparison of means was made using the Student's t-test. The difference was considered significant when $P < 0.05$.

Results and Discussion

There were significant differences between the treatments and controls among the isolates for lipase and protease enzymes production in all the tested tomato varieties of Cal J, Kilele and Anna (p=0.05) (Table 1). The level of protease activity as demonstrated by clear zone was highest in Kilele variety compared to Anna and Cal J which were almost similar. There were no significant differences among the varieties (p=0.05) (Table 1).

Our study demonstrated that endophytic *B. bassiana* ICIPE 35 on solid media could produce both lipases (Figure 1) and proteases (Figure 2) in all the tested tomato varieties of Cal J, Kilele and Anna. The maximum clear zone was observed in day 5. No clear zones were observed in their respective controls. The fungus *Beauveria bassiana* has been known globally to control insect pests and diseases. Spray applications have been successfully used but limitations of environmental conditions of high temperature and moisture have limited their wider application (Akello et al., 2007). There is therefore need to improve delivery mechanisms such as the use of endophytes. The endophytic growth of *Beauveria bassiana has* been documented in tomato, the authors demonstrated also that the endophytic colonization of tomato plant tissues by this fungus, led to reduced larval feeding of 3rd-instar *Helicoverpa zea* (Powell et al., 2009*).*

Table 1: Enzyme activity of three tomato varieties

Varieties	**Protease**	**Lipase**
Kilele IC 35	2.0 ± 0.03[a]	2.0 ± 0.00[a]
Kilele control	0.0 ± 0.00[b]	0.0 ± 0.00[b]
Cal J IC 35	2.0 ± 0.00 [a]	2.0 ± 0.00 [a]
Cal J control	0.0 ± 0.00[b]	0.0 ± 0.00[b]
Anna IC 35	1.5 ± 0.50[a]	2.0 ± 0.00[a]
Anna control	0.0 ± 0.00[b]	0.0 ± 0.00[b]
P	0.68	0.96

[a]Means (±SE) followed by the same letter within a column are not significantly different at $P < 0.05$ (Student t-test).

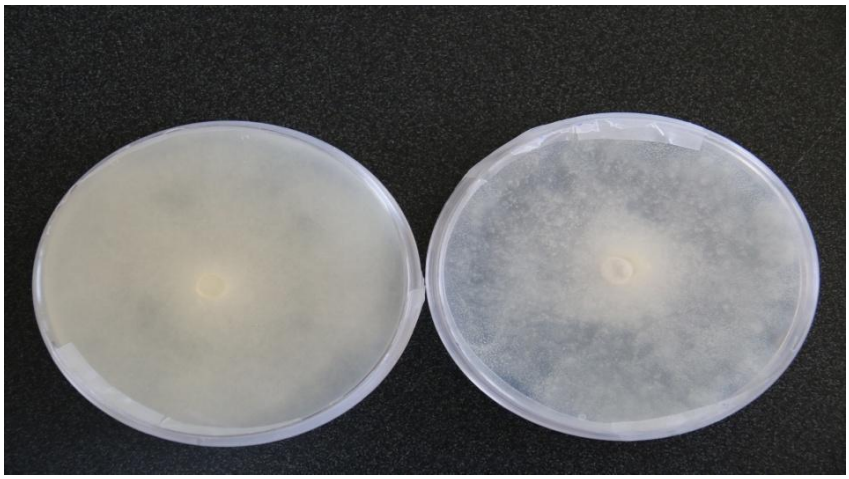

Figure 1. Lipase enzyme activity of tomato varieties on solid medium, control (Left) no precipitation and treatment (Right) with precipitation of fatty acids crystals around the fungal colony.

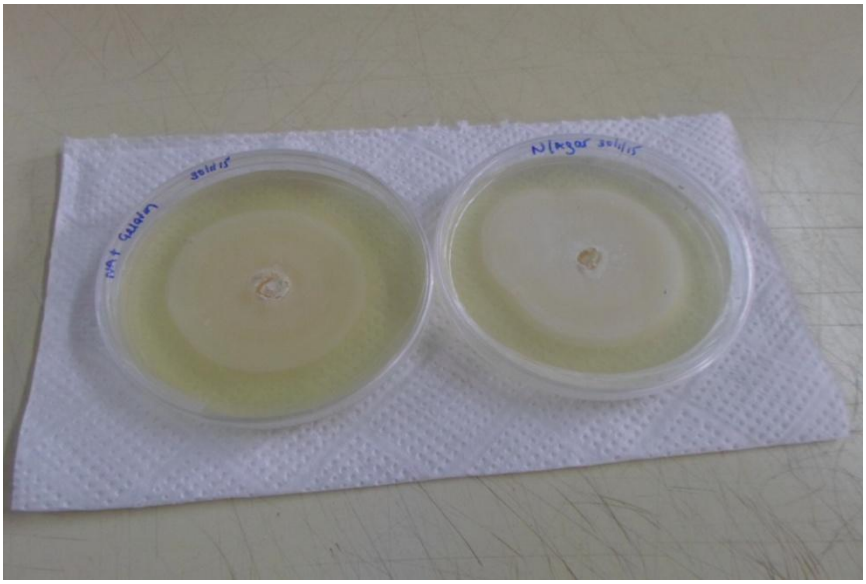

Figure 2. Protease enzyme activity of tomato varieties on solid medium, treatment (Left) with a clear zone around the fungal colony and control (Right) no clear zone around the fungal colony.

Entomopathogenic fungi produce a variety of cuticle degrading enzymes. Extracellular enzymes produced by *Beauveria bassiana* are believed to play a key role in cuticle hydrolysis, these enzymes include proteases and lipases (Hallsworth and Magan, 1996). Studies have shown that lack of these enzymes in some strains of *B. bassiana* may delay the infection process in certain insect species (Bidochka and Khachatourians, 1990).

Solid media enzyme experiments detect enzyme production, released from the mycelium, and activity in the medium following production. The lack of positive results could mean that either the enzyme is not produced, or that it is produced and not released from the mycelium, or that it is produced and released, but the medium may inhibit its detection.

According to (Silva et al., 2005) the enzyme lipase plays a very important role in the insect

infection process by entomopathogenic fungus such as fungus *Beauveria bassiana* and *Metarhizium anisopliae*. Lipases are essential for catalyze the hydrolysis of ester bonds in lipids which constitute part of the insect body (Belcarz et al., 2005). Protease enzyme plays an important role in killing the insect since it can degrade the fatty particles in the insect body (Wang et al., 2002).

Lipases and proteases are important enzymes in pathogenesis since they attack the plasmalemma after the degradation of cell wall by proteases. Proteases play a key role in the penetration process and a wide range has been identified e.g. trypsin, chymotrypsin (Bidochka and Khachatourians, 1990).

Conclusion

These results reveal that endophytic IC 35 produces extracellular enzymes which may play a key role in the degradation of the insect cuticle. Further studies are needed in the screen house to confirm the management of red spider mites in tomato.

Acknowledgments

The author is thankful to NACOSTI for funding this work through their postgraduate study programme, ICIPE Duduville campus, Arthropod Pathology Unit (APU) for providing the *Beauveria bassiana* isolates that were used in the study. Dr. Paulin Nana University of Dschang for reviewing this work and Edoh Kukon for statistical analysis.

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