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Comparative efficacy of endophytic versus foliar application of the entomopathogenic fungus *Beauveria bassiana* against the crucifer diamondback moth larvae for sustainable cabbage protection

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ABSTRACT

In recent investigations, *Beauveria bassiana* has been reported to be pathogenic to insect, endophytically colonize cabbage plants and may, therefore, be an alternative to chemical control of the diamondback moth, *Plutella xylostella*. In this study, the comparative efficacy of endophytic and foliar application of *B. bassiana* was assessed on different larval instars of *P. xylostella*. Cabbage plants were inoculated with *B. bassiana* conidia using seed coating and root soaking methods. Six weeks after plant endophytic inoculation, larval instars 2, 3 and 4 of *P. xylostella* were released on endophytic plant leaves without fungal spraying, on non-endophytic leaves sprayed immediately with fungal formulation and on untreated control leaves. Our results indicate that the average mortality rates of larval instars 2, 3 and 4 were significantly higher ($P < 0.0001$) on plants colonized by the endophyte than non-inoculated, untreated control plant. Similarly, significant differences were obtained between the foliar spraying of the fungus (corrected mortalities $\geq 96.72\%$) and the endophytic applications (corrected mortalities $\leq 57.61\%$). In endophytic treatments, larval mortality rates varied with larval age. Further investigations are needed to elucidate the cellular and molecular mechanisms of *B. bassiana* endophytism in cabbage.

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Keywords: Fungal endophyte, foliar application, cabbage plant, *Plutella xylostella*, larval mortality, cadaver sporulation.

INTRODUCTION

Cabbage, *Brassica oleracea* L. (Brassicaceae) is one of the most common vegetable crops grown worldwide and is becoming an economically important crop in Benin and in other West African countries (Godonou et al., 2009; Komlan et al., 2014).

The major constraints to cabbage production are insect pests that severely limit its marketable yields (Gnago et al., 2010; Mondedji et al., 2014). The diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) is one of the most devastating and pesticide-resistant insect pests of cabbage (Agboyi et al.,

2016). It has a short life cycle; it produces a very high number of individuals per generation and has multiple generations per year (more than 20 generations in the tropics). *Plutella xylostella* is a key pest of cabbage plants in Africa (Mondedji, 2010); therefore, its management is essential for maintaining the high levels of productivity required to meet the demand. However, the approaches used to increase crops production, rely mainly on chemical pesticides (Sane et al., 2018), with the major side-effect of inducing pesticide resistance in *P. xylostella*, thereby leading to the vicious circle of more frequent applications (Gnago et al., 2010; James et al., 2010; Tidjani et al., 2018). In Benin, vegetable growers apply two different synthetic insecticides including bifenthrin and deltamethrin for *P. xylostella* control on cabbage plants, with up to 19 applications during the three months of crop growth prior to harvest (James et al., 2010). Nevertheless, some growers also use endosulfan, a banned chemical insecticide usually sprayed on cotton plants, to protect their cabbage crop, without caring for of the intoxication risk to humans.

Hence, a more sustainable pest control approach is called for, which should be based, as much as possible, on the utilization and preservation of natural enemies (Tamò et al., 2012; Liu et al., 2014). In that respect, biological control methods have been developed against *P. xylostella* and were based mainly on the use of hymenopteran parasitoids such as *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) and *Diadegma semiclausum* Hellen (Hymenoptera: Ichneumonidae) (Kfir, 2005). Previously, *Bacillus thuringiensis* (Bt) formulations have been used against *P. xylostella* for many years but their repeated and indiscriminate applications have quickly led to the appearance of resistance (Cheema et al., 2011).

Similarly, water suspensions of *B. bassiana* conidia were sprayed at the dose rate of 1 kg per hectare to control *P. xylostella*, but applications must be repeated several times during the cabbage production cycle of nearly 3 months (Godonou et al., 2009). Moreover, the cost of 1 kg of *B. bassiana* conidia powder ranges between 300 and 400 US\$ (Heviefo,

unpublished data), making this control option totally unaffordable for subsistence farmers.

The interest to endophytic microorganisms increased only during the last 20 years, when their capacity to protect their host-plants against insect pests and pathogens has been demonstrated (Vega et al., 2008). Mutualistic associations between plants and endophytic fungi are very common in natural and agricultural ecosystems (Rasmussen et al., 2012). By living in symbiosis with their host-plants, endophytic fungi can produce metabolic products that negatively impact herbivorous organisms including arthropod pests (Benkhelil et al., 2004). Indeed, the presence of endophytic microorganisms in their respective host-plants resulted in the reduction of insect attacks (Azevedo et al., 2000). Isolates of *B. bassiana* are known to produce numerous secondary metabolites such as beauvericin, beauverolides, bassianolides, oosporein, cyclosporine A and oxalic acid with antibacterial, antifungal, cytotoxic and insecticidal activities (Ownley et al., 2010). Apart from its direct pathogenic activity on *P. xylostella* (Godonou et al., 2009), *B. bassiana* has recently been reported as being able to endophytically colonize cabbage plant (Heviefo et al., 2017), thereby opening new opportunities for using this organism in a novel biocontrol approach. In the present study, we assessed the pathogenicity of *B. bassiana* applied as foliar spray and an endophyte through seed coating and root soaking to the diamondback moth, *P. xylostella*.

MATERIALS AND METHODS

Study sites

The experiments were conducted in laboratory and screen house at the Benin station of the International Institute of Tropical Agriculture (IITA) located at the North of Cotonou (N 06°25'15.6", E 002°19'41.1" and Alt. 20 m). The experimental conditions were set at 25 ± 1 °C and 75% - 90% RH in the laboratory. In the screen house, temperatures fluctuated between 23.3 ± 0.2 °C and 33.8 ± 0.3 °C whereas relative humidity varied from $70.5 \pm 1.5\%$ to $97 \pm 0.2\%$.

Fungal isolate

The *B. bassiana* isolate used in all the experiments was Bb11, isolated from specimens of the cereal stemborer *Sesamia calamistis* Hampton (Lepidoptera: Noctuidae) collected at the village of Penessoulou (Northern Benin). This strain was selected based on results of field spray trials that demonstrated its virulence against *P. xylostella* and *Psara basal* W. (Lepidoptera: Pyralidae). (James et al., 2006; Godonou et al., 2009). Production of the dry conidia started first with a fermentation phase in a liquid medium composed of 20 g of sugar, 20 g of brewery yeast and one litre of tap water during three days on rotary shaker to produce blastospores. The blastospore solution was used to inoculate rice grains in plastic balloons to induce the development of conidia. The conidia were subsequently extracted from the rice grains and dried indoors on a tray following the procedure described by Heviefo et al. (2018). A standard germination test was carried out and viability of conidia (percentage of germination) was calculated using the following formula developed by LUBILOS, 1996:

$$V = [a / (a + b)] \times 100,$$

where a was the number of germinated spores, b the number of non-germinated spores, V the viability of conidia.

Insect colonies

Adults and larvae of *P. xylostella* were collected from different cabbage production fields near Cotonou in southern Benin. Larvae were reared on cabbage plants in mesh cages in a laboratory at IITA-Benin. Adult insects were fed with honey solution (10%) and were allowed to oviposit on cabbage leaves in mesh cages where embryonic and larval development occurred.

Cabbage variety

The cabbage variety used in the experiments was K-K Cross premium, Hybrid imported from Japan. The seeds were purchased in local seed stores, Bénin semences (Abomey-calavi) and Gbgamey (Cotonou).

Determination of the endophytic virulence of *B. bassiana* on *P. xylostella*

These experiments were conducted in a screen house at 23.3 ± 0.2 °C to 33.8 ± 0.3 °C and $70.5 \pm 1.5\%$ to $97 \pm 0.2\%$ HR. Endophytic colonisation of the cabbage plants by *B. bassiana* was induced by either seed coating and root soaking (Heviefo et al., 2017). The virulence of the endophytic *B. bassiana* on larval instars 2, 3 or 4 of *P. xylostella* was tested separately in six different treatments as follows:

Treatment 1 (Endo 1): 10 g of cabbage seeds were coated with 4 g of *B. bassiana* conidia powder and 6 ml of tap water mixed with Tween 80 (Polyoxyethylene (20) sorbitan mono-oleate) was added to allow spores to become hydrophilic when they adhered. Tween 80 usually facilitates the homogenization of imperfect fungi in water formulations (LUBILOS, 1996). The Coated seeds were sown but seedlings were not transplanted after emergence;

Treatment 2 (Endo 2): cabbage seeds coated as in treatment 1 but seedlings were transplanted into fresh pots;

Treatment 3 (Endo 3): cabbage seeds coated as treatment 1 but seedling roots dipped in *B. bassiana* conidia powder prior to transplanting;

Treatment 4 (Endo 4): cabbage seeds not coated, but simply immersed in Tween-water before sowing. After growth, seedlings roots were dipped in *B. bassiana* conidia powder prior to transplantation;

Treatment 5 (foliar spray): cabbage seeds not coated before sowing and roots were not dipped in *B. bassiana* conidia powder; however, plants were sprayed with a *B. bassiana* formulation (10^8 conidia ml⁻¹ of Tween-water), using a hand-sprayer, immediately before releasing *P. xylostella* larvae.

Treatment 6 (control): cabbage seeds were not coated; but simply immersed in Tween-water before sowing; roots were not dipped in *B. bassiana* conidia powder; plants were sprayed with Tween-water.

For each treatment including the control, three cabbage seedlings were sown

individually in plastic pots (5 l capacity) containing 4 kg of compost obtained from IITA-Benin's vegetable garden. On each cabbage plant (i.e. seedling), 20 larvae of the same instar were deposited on the leaves after checking that stems and leaves were well colonized by the endophyte *B. bassiana* following the detection and re-isolation methods described by Heviefo et al. (2017), six weeks after inoculation. Each potted plant was enclosed in a fine mesh cage to avoid larval escape. Insect mortality or pupation were observed and recorded daily. Larval cadavers were put in plastic Petri dishes (9 cm diameter) containing a humidified sterilized paper towel and incubated at 25 ± 1 °C for fungal sporulation while pupae were kept at 26 ± 1 °C for adult emergence. The experiment was replicated three times under the same conditions. Larval instars 2, 3 and 4 were separately used.

Natural cumulative percentages of larval mortalities data were corrected using Abbott's formula (Abbott, 1925) and were used for comparison among treatments:

$$Cm = \left(1 - \frac{nTr}{nCo}\right) \times 100$$

Cm is corrected mortality,
nTr is number of surviving treated larvae after treatment,
nCo is number of surviving larvae in the control treatment.

Data analysis

Larval mortality rate was expressed as the percentage of dead larvae within the total number of larvae deposited on the plant. The sporulation rate was expressed as the percentage of larval cadavers from which fungus emerged out of the total number of cadavers.

Larval mortality or sporulation rates were compared among treatments using Analysis of Variance with SAS software, version 9.2. Cumulative mortality and sporulation rates were transformed using Arcsine square root values before statistical analyses. When Anova revealed significant differences, treatment larval mortality or

sporulation means were separated using the Student-Newman-Keuls (SNK) multi-range test.

RESULTS

Larval mortality

The temporal trends of the corrected cumulative mortalities caused by *B. bassiana* in the different treatments are presented in Figures 1-3. For all larval instars, mortalities started two days after larval deposit and increased throughout the duration of the experiments. However, mortality rates increased faster for the foliar application, which induced the highest overall mortality rates throughout the experiments as compared to the endophytic inoculation (Figures 1-3). The mortality rates of larval instars 2, 3 and 4 were significantly higher ($df = 4$; $P < 0.001$) on plants colonized by the endophyte than non-inoculated, untreated control plant.

As presented in Table 1, fungal endophytic treatments caused larval corrected mortality ranging from 38.27 to 57.61% in larval instar 2, 30.58 to 40.65% in larval instar 3, and 16.67 to 26.67% in larval instar 4; while conidia applied in water formulation (i.e. foliar spray) induced 100% mortality in larval instar 2, 98.78% in larval instar 3 and 96.72% in larval instar 4.

The foliar application of fungus caused significantly higher mortality of all larval instars tested ($df = 4$; $P < 0.001\%$) (Table 1) than endophytical application. Endophytical treatment 3 (Endo 3) of *B. bassiana* induced significantly higher corrected mortality to larval instars 2 (57.61%) compared to larval instar 3 (40.65%) and instar 4 (18.38 %) ($df = 2$; $F = 75.25$; $P < 0.0001$). Natural mortalities of 8.8%, 10.5% and 3.3% were also observed respectively for larval instar 2, 3 and 4 in the untreated control and were used to correct treatments mortalities.

Endophyte applied in Treatment 3 (Endo 3: seeds coated and roots soaked) caused significantly higher mortality to *P. xylostella* larval instar 2 ($57.61 \pm 2.15\%$) than Treatment 1 (Endo 1: seeds coated simply and not replanted) ($38.27 \pm 3.93\%$) ($df = 4$; $F = 8.24$; $P < 0.0001$). Corrected mortalities of the second

instar (Table 1) did not differ significantly among Endo 1 ($38.27 \pm 3.93\%$), Endo 2 ($42.77 \pm 4.81\%$) and Endo 4 ($45.33 \pm 5.52\%$) ($df = 3$; $F = 3.51$; $P < 0.09$). As for larval instar 3, corrected mortality rates did not differ significantly among all endophytic treatments six days after application ($df = 3$; $F = 1.22$; $P < 0.38$) (Table 1). Similarly, larval stage 4 corrected mortality values were not significantly different among endophytic treatments ($df = 3$; $F = 1.30$; $P < 0.36$). With respect to the influence of larval age, the corrected mortality rates varied with larval instar, where larval instar 4 recorded the lowest corrected mortality rates (Table 1). In the treatment 3 (Endo 3), younger instars displayed higher corrected mortalities than elder ones ($df = 2$; $F = 75.25$ $P < 0.0001$).

Fungal sporulation

In the foliar applications of *B. bassiana*, mean sporulation rates ranged from $93.33 \pm$

5.44% to $94.70 \pm 2.32\%$; while the mean sporulation rates ranged from $7.58 \pm 3.36\%$ to $32.66 \pm 13.63\%$ in the endophytic treatments (Table 2). No sporulations were observed on dead larvae in the untreated control. Foliar applications of *B. bassiana* induced significantly higher mycosis (sporulation) rate than endophytic treatments ($df = 5$; $P < 0.0001$). In contrast, there were no significant differences (statistically) in larval sporulation among endophytic treatments (seed coating and/ or root soaking) ($df = 3$; $F = 0.25$; $P > 0.85$). However, mean sporulation rates were generally higher on larval instar 3 than instar 2, while larval instar 4 had the lowest sporulation rate (Table 2). Referring to the influence of larval age in cadaver sporulation, there were no significant differences among larval instars ($df = 2$; $P > 0.084$) (Table 2).

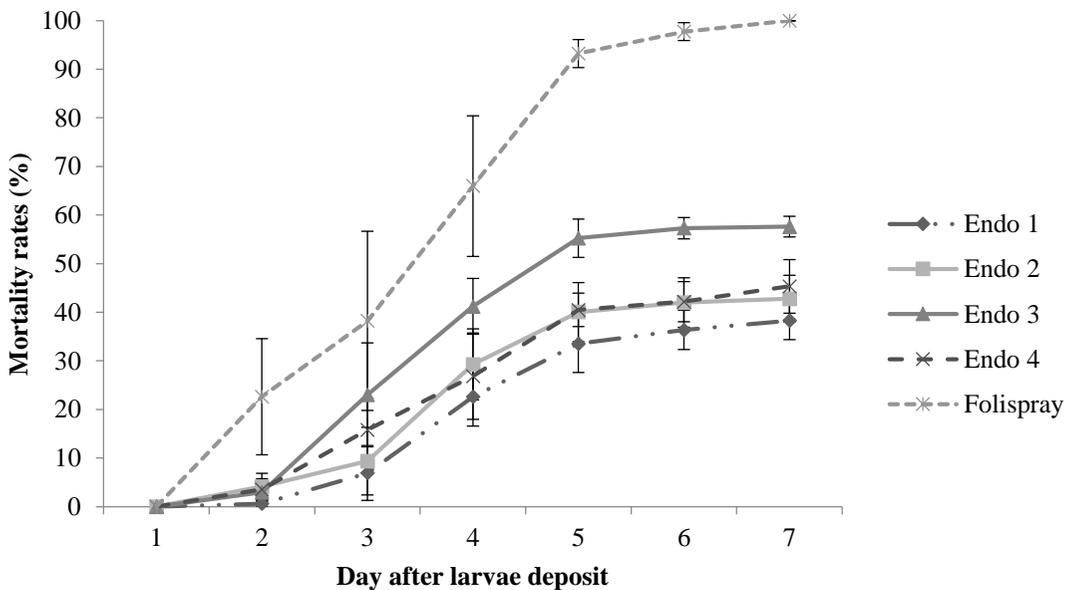


Figure 1: Temporal evolution of corrected mortality rates of *P. xylostella* larval instar 2 deposited on endophytic and foliar application plants.

(Untreated control mortality rates were used to correct mortality rates of treatments).

Endo 1: seeds coated and sown; seedlings not transplanted after emergence; Endo 2: seeds coated and seedlings transplanted; Endo 3: seeds coated and seedling roots dipped in *B. bassiana* conidia powder prior to transplantation; Endo 4: seeds not coated; seedlings roots dipped in *B. bassiana* conidia powder prior to transplantation; Folispray (Foliar spray): seeds not coated and roots not dipped in *B. bassiana* conidia powder; plants sprayed with a *B. bassiana* formulation (10^8 spores.ml⁻¹) before larvae deposit.

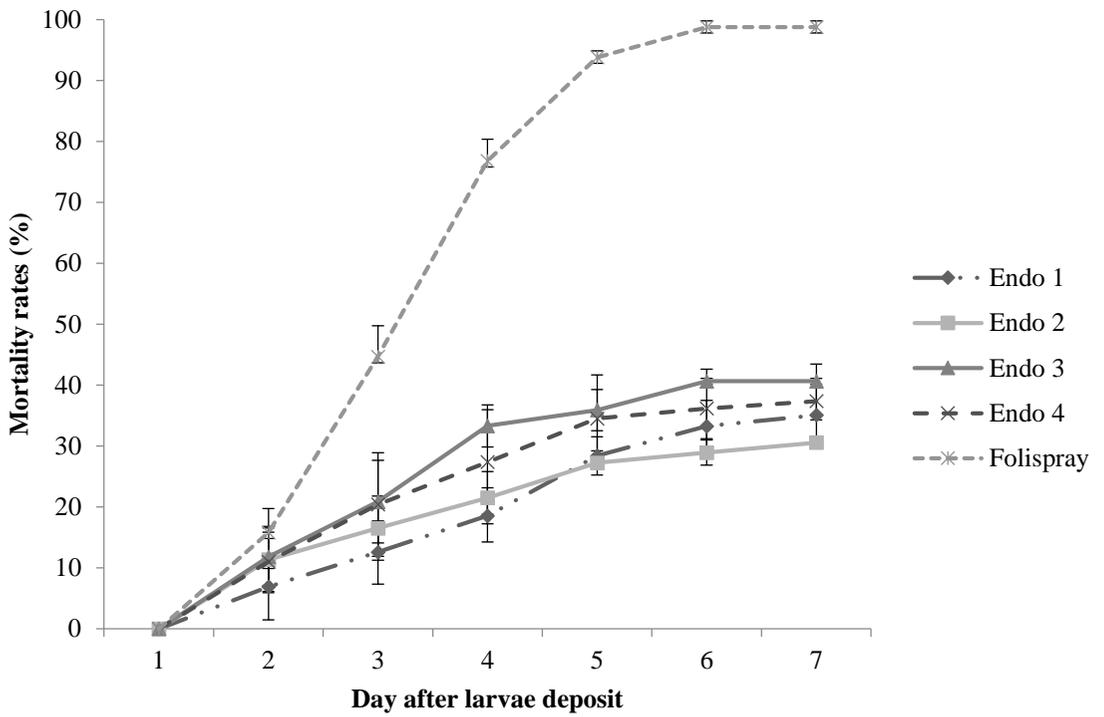


Figure 2: Temporal evolution of corrected mortality rates of *P. xylostella* larval instar 3 deposited on endophytic and foliar application plants.

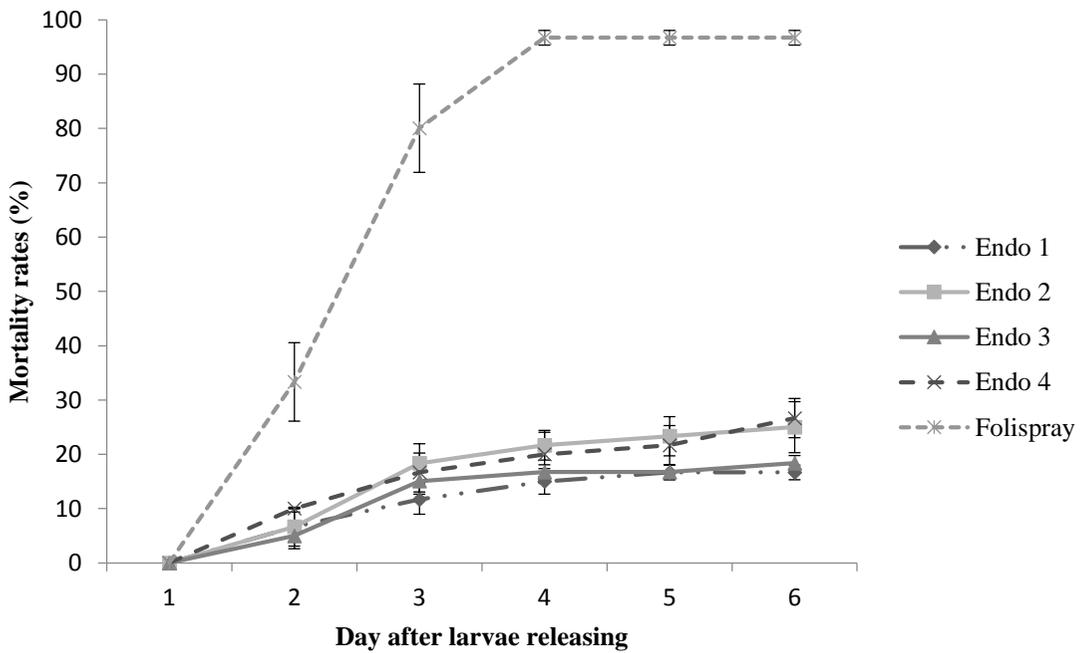


Figure 3: Temporal evolution of corrected mortality rates of *P. xylostella* larval instar 4 deposited on endophytic and foliar application plants.

Table 1: Corrected average mortality rates of *P. xylostella* larvae six days after deposit on treated plant.

<i>Treatments</i>	<i>Larval instars</i>				
	<i>L 2</i>	<i>L 3</i>	<i>L 4</i>	<i>F</i>	<i>P</i>
Endo 1 (E 1)	38.27 ± 3.93cA	35.08 ± 4.51bA	16.67 ± 1.36bB	8.41	< 0.018
Endo 2 (E 2)	42.77 ± 4.81bcA	30.58 ± 0.76bA	25.00 ± 4.71bA	4.06	>0.077
Endo 3 (E 3)	57.61 ± 2.15bA	40.65 ± 0.48bB	18.38 ± 1.38bC	75.25	< 0.0001
Endo 4 (E 4)	45.33 ± 5.52bcA	37.38 ± 6.10bA	26.67 ± 3.60bA	3.53	>0.097
foliar spray	100.00 ± 0.00aA	98.78 ± 0.99aA	96.72 ± 1.34aA	1.98	>0.218
<i>F</i>	21.22	22.15	16.32		
<i>P</i>	< 0.001	< 0.002	< 0.0015		

Means followed by the same letters a, b, c in the same column are not significantly different using Student-Newman-Keuls (SNK) at *P* < 0.05.

Means followed by the same letters A, B, C in the same row are not significantly different using Student-Newman-Keuls (SNK) at *P* < 0.05.

* Untreated control mortality rates were used to correct mortality rates of treatments

Endo 1: seeds coated and sown but seedlings not transplanted after emergence; Endo 2: seeds coated and seedlings transplanted; Endo 3: seeds coated and seedling roots dipped in *B. bassiana* conidia powder prior to transplantation; Endo 4: seeds not coated; seedlings roots dipped in *B. bassiana* conidia powder prior to transplantation; Foliar spray: seeds not coated and roots not dipped in *B. bassiana* conidia powder; plants sprayed with a *B. bassiana* formulation (10⁸ spores.ml⁻¹) before larvae deposit.

Table 2: Average sporulation rate of *P. xylostella* larval cadavers at the end of the trials.

<i>Treatments</i>	<i>Larval instars</i>				
	<i>L 2</i>	<i>L 3</i>	<i>L 4</i>	<i>F</i>	<i>P</i>
Endo 1 (E 1)	7.58 ± 3.36bA	14.24 ± 8.57bA	0.00 ± 0.00bA	3.85	>0.084
Endo 2 (E 2)	8.88 ± 6.21bA	15.56 ± 7.83bA	0.00 ± 0.00bA	1.59	>0.279
Endo 3 (E 3)	20.66 ± 12.60 bA	32.66 ± 13.63 baA	16.67 ± 13.61 bA	0.30	>0.75
Endo 4 (E 4)	15.86 ± 10.35bB	25.45 ± 9.23b	18.76 ± 0.00 b	0.49	>0.64
foliar spray	93.89 ± 2.76aA	94.70 ± 2.32aA	93.33 ± 5.44aA	0.03	>0.97
Untrea. control	0.00 ± 0.00bA	0.00 ± 0.00bA	0.00 ± 0.00bA		
<i>F</i>	7.37	5.18	15.41		
<i>P</i>	< 0.0023	< 0.0092	< 0.0001		

Means followed by the same letters a, b, c in the same column are not significantly different using Student Newman Keuls (SNK) at *P* < 0.05.

Means followed by the same letters A, B, C in the same row are not significantly different using Student Newman Keuls (SNK) at *P* < 0.05.

DISCUSSION

Our results confirmed that the isolate Bb11 of *B. bassiana* used for these experiments is pathogenic to *P. xylostella* larvae. The high mortality rates obtained by foliar applications are similar to those reported by several authors who have tested *B. bassiana* on various insects (Gindin et al., 2006; Godonou et al., 2009; James et al., 2010; Fancelli et al., 2013; Lopez et al., 2014; Toffa et al., 2014). Also, the corrected mortality rate ($57.61 \pm 2.15\%$) obtained with the endophytic inoculation in our study is similar to those reported by Batta (2013) in endophytic *M. anisopliae* applied against larvae of *P. xylostella* infesting *Brassica napus* plants, and comparable to results by Vidal and Jaber (2015) using endophytic *B. bassiana* in *B. napus* and *Vicia faba* against *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). In this study, the range of larval mortalities was similar to the range reported by Valda et al. (2003).

The high larval mortality and sporulation rates observed in foliar applications can be explained by the high number of fungal conidia that must have been in direct contact with the cuticle of the larvae and hence germinated and invaded their haemocoel, thereby inducing mycosis (Butt et al., 2016). In the endophytic colonization treatments, however, we assumed that *B. bassiana* was translocated systemically through the plant tissues (Raja et al., 2016) and that larval infection occurred through ingestion of its hyphae in the palisade parenchyma of cabbage leaf tissues (Heviefo et al., 2017), with no direct exposure to conidia. Subsequently, the ingested ramified hyphae would have developed in the haemocoel of the infected larvae thereafter producing spores and toxins (Ownley et al., 2010) that induced lower levels of mortality and sporulation compared with the foliar applications, as also reported by Butt et al. (2016) and (Ortiz-Urquiza et al., 2010) and could explain why we only observed $57.61 \pm 2.15\%$ corrected larval mortality rate in the endophytic treatment (Endo 3) instead of 100% mortality induced by foliar application, as also reported by Butt et al. (2016).

The mortality rates recorded with the endophytic treatment 3 (Endo 3) showed significant variation among *P. xylostella* larval instars, whereby younger larvae were more susceptible than the elder ones. This is in support to the results by Douro-Kpindou et al. (2012) on *H. armigera* and Toffa et al. (2014) on *M. vitrata*. It appears that immature stages of *P. xylostella* are able to boost their immune systems with age thereby increasing their capacities of detoxification (Butt et al., 2016). Seed coating + root soaking (treatment 3) yielded the best endophytic colonization of cabbage leaves (Heviefo et al., 2017) with the highest mortality of *P. xylostella* larvae.

To explain the lower sporulation rates recorded with endophytic application of *B. bassiana*, we can speculate that fungal hyphae must grow, kill the host larva, ramify, colonize the haemocoel cavity of the cadaver and penetrate through the external larvae cuticle before producing conidia, thereby inducing mortality of the host, as observed by Butt et al. (2016). In addition, with this complex process, the fungus must overcome the internal larval resistance before achieving its development within the host-insect. It, therefore, contrasts with the direct spraying of conidia suspensions where many more fungal spores can adhere to the cuticle of the host and germinate in a shorter time. This mechanism could also explain why larval instar 4 is resistant to endophytical treatments but still very vulnerable to epiphytical treatments of *B. bassiana*. Irrespective of the application method, larval instar 3 displayed the highest sporulation rates, certainly due to its relatively better nutritional properties (compare with instars 2 and 4), for *B. bassiana* development, as reported in other studies (Sapna and Anushree, 2013).

In spite of all the inconveniences linked with the relatively lower mortality of the hosts, endophytic fungus applications might be more friendly with the ecosystem stability—especially, with regard to non-target effects on beneficial-arthropods than direct spraying of conidial formulations (Azevedo et al., 2000). While we now have an empirical evidence that endophytic *B. bassiana* constitutes a valuable

approach in *P. xylostella* management, the metabolic pathways underlying the exact mechanisms of action of endophytism still remain to be elucidated.

Conclusion

From this study, it can be concluded that the isolate Bb 11 of *B. bassiana* is pathogenic to *P. xylostella* larvae. In endophytic treatments, larval mortality rates varied with larval age. *B. bassiana* can be used as well as endophyte as foliar application for the management of *P. xylostella* pest. The results from this study suggest that seed coating + root soaking (treatment 3) yielded the beneficial mortality of *P. xylostella* larvae for plant protection. But the foliar application of *B. bassiana* decimates all larval instars in contrast of endophytic applications. Then, *B. bassiana* endophytic applications might be more friendly with the ecosystem stability.

COMPETING INTERESTS

All authors declare that they have no competing interests with this manuscript.

AUTHORS' CONTRIBUTIONS

GAH was the principal investigator and prepared the manuscript. IAG, SWN and MT supervised the work, guided, structured and corrected the manuscript; BDD helped for trials monitoring and earlier drafts writing.

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