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Fungal Endophytic *Beauveria bassiana* in Chinese Kale Against *Plutella xylostella* (L.) Larvae

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KEYWORDS

beauveria bassiana; endophytic fungi; entomopathogen; inoculation methods; *plutella xylostella*.

Abstract This study evaluated three methods of inoculation of *Beauveria bassiana* endophytes from Chinese kale and then infected into *Plutella xylostella* L to determine the mortality rate. The endophytic *B. bassiana* using three different inoculation methods including seeds-soaking, leaf-spraying, and soil-wetting. The Chinese kale seeds from Winsa variety, *B.bassiana* isolates derived from collection planthopper. The endophytic fungus was identified to be *B. bassiana* based on the analysis of colony morphology. The results of this study included the degree of colonization of Chinese kale plants and the mortality of *P. xylostella*. The average *B. bassiana* colonies in leaves were 13.89%, stems 2.77% and roots 5.55%. The results showed on leaves that the seeds-soaking method obtained higher colonization of *B. bassiana* rate when compared to leaf-spraying and soil-wetting. The highest percentage of *P. xylostella* mortality was generated from seeds-soaking (45%), soil-wetting (37.5%), leaf-spraying (35%). The *B. bassiana* fungus which caused the fastest LT_{50} with a seeds-soaking (409.48 hours), and then soil-wetting (679.15 hours), leaf-spraying (1090.21 hours). The positive effects of seeds-soaking of endophytic *B. bassiana* and mortality of *Plutella xylostell.*

Introduction

Chinese kale (*Brassica alboglabra* Bailey) is an original Chinese vegetable belonging to the Brassicaceae family (Sun *et al.*, 2010). The Chinese kale (Kailan, *Brassica oleracea* var. *alboglabra* Bailey) is vegetables collected from Malang, East Java, Indonesia (Limantara *et al.*, 2015). Production fluctuations in Chinese kale cultivation often occur. Pests of *P. xylostella* was a pest that harms Brassicaeae plants, besides Chinese kale these pests also attack cabbage, broccoli, cabbage, and *Brassica napus* (Batta, 2013). In the field, *P. xylostella* causes hollow leaves, at a high larval population level, leaving only the leaf bone. The attack of *P. xylostella* causes a loss of up to 83% and harms most farmers (Wang *et al.,* 2004). The alternative for controlling *P. xylostella* was by using microbial endophytic fungi.

The entomopathogenic fungus *Beauveria bassiana* has been endophytically introduced with success in different plant species and has shown activity against different pests (Vega, 2018). *Beauveria bassiana* is an entomopathogenic fungus that has a particularly broad host range, over 700 species of vertebrate (Glare *et al.,* 2012). Like other entomopathogenic fungi, *B. bassiana* conidia penetrate through the cuticle of host insects (Ortiz-urquiza & Luo, 2014). *Beauveria*

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bassiana is considered minimal effects on non-target organisms, including humans and animals (Gouli *et al.,* 2014).

The ability of endophytic fungi *B. bassiana* has a broad role for plants, one of which was an insect pathogen. In cabbage plants, *B. bassiana* can become endophytes and insect pathogens against *P. xylostella* (Zhang, 2014). Endophytic fungi can colonize plant parts naturally and by inoculation resulting from seed immersion, leaf spraying and soil watering with *B. bassiana* suspension. *Beauveria bassiana* proved to be successful in becoming endophytic by inoculation method such as cabbage plants broad bean (*Vicia faba*), green bean, and tomatoes (Akutse *et al.*, 2013; Qayyum *et al.*, 2015; Vidal & Jaber, 2015). The inoculation method used for *B. bassiana* becomes endophytic was seeds-soaking, leaf-spraying, soil-wetting. The seeds-soaking method can be used to make *B. bassiana* become endophytic in beans (Behie *et al.* 2015). In addition to seeds-soaking and leaf-spraying can also be used to make *B. bassiana* as endophytes in Rapeseed and bean plants (Batta, 2013; Parsa *et al.*, 2013). Soil-wetting method can also be used to make *B. bassiana* as endophytes in beans (Parsa *et al*., 2013). Effect positive of seed treatment of *Vicia faba* by endophytic *Beauveria bassiana* (Jaber & Enkerli, 2016). Therefore, colonization studies in Chinese kale with three methods of *B. bassiana* inoculation and mortality against *P. xylostella* were carried out to determine the effectiveness of the method. This study aimed to determine the most effective inoculation method to control *P. xylostella* pests.

Materials and Methods

Experimental design

The study was conducted in the Laboratory of Biological Control, Department Plant Pest and Disease, Faculty of Agriculture, University of Brawijaya, and Karangwidoro Village, Dau Sub-district, Malang District. The study was carried out from May to September 2016. Chinese kale seeds were obtained from Winsa variety. *Beauveria bassiana* isolates used in this study were obtained from Department Plant Pest and Disease, Faculty of Agriculture, University of Brawijaya, and *P. xylostella* larvae were obtained from the Indonesian Sweetener and Fiber Crops Research Institute (ISFCRI), Malang. This study was used a Randomized Block Design (RBD) consisting of six methods with four replications to obtain 24 plants. Planting was done three times so that the total plant used was 72 plants.

Inoculation of Beauveria bassiana as Endophytic Fungus

The inoculation of *B. bassiana* using three methods and one control, including seeds-soaking, leaf-spraying, and soil-wetting with a suspension of *B. bassiana* with a density of $1x10^8$ conidia/ml and for control using sterile distilled water. The leaf-spraying and soil-wetting method were done when the plants begin to emit true leaves for the first time or 14 days after planting. The seeds-soaking method was carried out before planting by soaking the Chinese kale seeds into 20 ml of conidia *B. bassiana* suspension with a density of $1x10^8$ conidia/ml for 30 minutes on a petri dish in Laminar Air Flow Cabinet (LAFC). For the control treatment, the seed was soaked with sterile water. The inoculation was carried out in the morning or evening aimed at maintaining virulence of *B. bassiana*.

The leaf-spraying method was carried out by spraying 20 ml/plant of conidia *B. bassiana* suspension with a density of $1x10^8$ conidia/ml and sterile water for the control methods. At the time of application, the surface of the polybag was closed using aluminum foil to prevent the conidia from going down to the ground. After the application of leaf-spraying was complete, aluminum foil was taken and

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Chinese kale plants were covered with clear plastic for 24 hours to get moist and good environmental conditions for fungal growth. In the control method, the leaves were sprayed using sterile distilled water. The inoculation was carried out in the morning or evening to maintain the virulence of *B. bassiana*. The soil-wetting method was carried out by wetting the soil using 20 ml of conidia *B. bassiana* suspension with a density of $1x10^8$ conidia/ml at the soil surface. In the control method of soil-wetting was using sterile distilled water. The inoculation was carried out in the morning or evening to maintain the virulence of *B. bassiana*.

The Endophytic B. bassiana Colonization Ability in Chinese Kale Plants

The evaluation was carried out twice, at 7 and 14 DAI (Days After Inoculation) of fungi (Batta, 2013). The evaluation was carried out by carefully removing the Chinese kale and then cleaning the Chinese kale plant using running water. Chinese kale plants are taken from the leaves, stems, and roots. From each plant, two leaves, two stems, and two roots were taken. The leaves used were two true leaves, cut 1x1 cm by three pieces/leaves, stems, and roots were used measuring 1x1 cm as many as three pieces to be isolated and identified endophytically macroscopically and microscopically. Samples sterilized in LAFC using 1% NaOCl, 70% alcohol, and rinsed 3 times using sterile water, each for 2 minutes and dried on sterile tissue. The outer edge of the sample was cut and removed to eliminate the sample affected by the disinfectant.

Samples of plant parts were isolated into PDA (Potato Dextrose Agar) media which had added 1 mg/l of chloramphenicol antibiotics to prevent bacterial contamination. Later, it was observed until *B. bassiana* colonies with characteristic solid mycelia appeared to be pale yellow cream on the edge. After that, colony purification was carried out to prevent contamination. Colonies that were successfully purified and incubated for 14 days, then carried out observations of macroscopic and microscopic sightings. The percentage of colonization was calculated by using the following formula bellow (Deguchi *et al*., 2017). Macroscopic observations included the shape and color of the colonies that grew on the media, while microscopic observations were carried out by observing conidia, hyphae, and conidiophores. The identification of the *B. bassiana* fungus was carried out according to Barnett & Hunter (1972).

$$\%\ \text{Colonization} = \frac{\text{Number of Fungi Growing}}{\text{Number of Plants Sample}}$$

Endophytic Efficacy of Beauveria bassiana Against P. xylostella

The effect of the observed endophytic *B. bassiana* on Chinese kale plants on *P. xylostella* was mortality. The method used to measure this value by taking 24 leaves of the Chinese kale plant results from three methods and the control plants that were picked from true leaves. Then placed on a jar (l= 25 cm, w = 25 cm, and h = 20 cm), the method used as diet for *P. xylostella*. Chinese kale plant ages 24 DAP (Day After Planting) were used to calculate mortality due to the highest intensity of *P. xylostella* attacks. Ten larvae of *P. xylostella* (2^{th} instar) were inserted with Chinese kale leaves in a jar. The percent mortality of *P. xylostella* was counted by using the following formula (Abbott, 1925):

$$\text{Percentage of Mortality} = \frac{\text{Dead Insects}}{\text{Living Insects}}$$

Data Analysis

The data were analyzed by the one-way-Analysis of Variance (ANOVA) at α=5%. To determine LT_{50} from the conidia fungi method of *B. bassiana* on *P. xylostell*a larvae were analyzed by using Probit analysis software (Chi, 1997).

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Results and Discussion

The Endophytic B.assiana Ability in Chinese kale Plants

The results showed that endophytic *B. bassiana* ability was evidenced by the growth of *B. bassiana* colonies which were located on the edges of the leaves, stems and roots of the Chinese kale plant grown on PDA media have white features, are round in shape and have smooth, regular texture on the edges of leaves, stems and roots (Figure 1A). The texture of the *B. bassiana* fungus on the surface has a rather rough colony and the colonies grow rather tightly (Figure 1B). Whereas microscopically the endophytic *B. bassiana* fungus has the characteristic of insulated hyphae, hyaline color with a width of 1.10 µm, hyaline-colored conidia in a round to oval shape with a diameter of 1.50 µm and conidia assemblages clustered on conidophores (Figure 1C). From the results of research on *B. bassiana* inoculated in Chinese kale plants, the mortality of *P. xylostella* from all methods reached an average of 39.17% and 0.00% control (Table 1).

Various studies have shown that the efficacy of *Beauveria bassiana* can be improved by the inoculation method which results in the highest percentage of colonization. Endophytic fungus was a microorganism that can colonize the roots, stems, and leaves of plants. *Beauveria bassiana* mycelia was insulated, single-celled conidia are rather round oval-shaped like eggs, and conidiophores grow zigzag (Barnett & Hunter, 1972). The conidia of *B. bassiana* in the Chinese kale plant can kill *P. xylostella* larvae which indicate the potential for endophytic success. According to Correa-Cuadros *et al*. (2014), *Plutella xylostella* (Lepidoptera: Plutellidae) larva was killed by *B.bassiana* Bb9205 with white mycelium. The *B. bassiana* fungi became endophytic in cabbage plants (*Brassica olaroceae*) as evidenced by the death of *P. xylostella* (Vidal & R Jaber, 2015). The mortality of *P. xylostella* was caused by larvae eating Chinese kale leaves containing endophytic *B. bassiana.*

Figure 1. A. Endophytic of *B. assiana* at the edge of leaves, stems, roots on PDA medium, B. Macroscopic morphology of *B. Bassiana* colony at 14 days, and C. Microscopic morphology with 400x magnification (1) conidia (2) conidiophores, and (3) hyphae.

Table 1. The average percentage of *P. xylostella* larvae due to *B. bassiana* fungi from all methods

Inoculation Method	∑ Mortality (%)
Control	0.00
All treatment	39.17

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Beauveria bassiana Colonization in Chinese Kale Plants

Colonization of *B. bassiana* in Chinese kale plants gets a different percentage on each plant tissue. The average *B. bassiana* colonies that grow in leaves were 13.89%, which stems 2.77%, and roots 5.55% (Table 2). The three different methods of inoculation showed that the seeds-soaking method obtained a higher colonization rate when compared to leaf-spraying and soil-wetting (Table 3).

The difference in the percentage of colonization of *B. bassiana* in the leaves, stems, and roots of the Chinese kale plant was thought to be influenced by the three inoculation methods used. The *B. bassiana* can enter plants and form colonies in plant tissue influenced by the inoculation method used (Vidal & Jaber, 2015). In addition to the influence of the inoculation method used, the inoculation of the *B. bassiana* fungus also requires time to spread into plant tissues (Vidal & Jaber, 2015). The percentage of *B. bassiana* fungi colonies at 7 dai with weed-soaking in the leaves, leaf-spraying, and soil-wetting were rooted.

The spread of *B. bassiana* inoculated with different methods was not evenly distributed in the Chinese kale plant due to the absorption of conidia *B. bassiana* suspension into the plant tissue. Conidial suspension *B. bassiana* enters seeds-soaking, leaf-spraying through leaf stomata and roots with soil-wetting. The number, length, and growth rate of the germination tube or conidia movement are influenced by the number of plant extracts and the density of saprophytic microflora on the surface of the plant (Agrios, 2005). Inoculation of *B. bassiana* suspension using seed-soaking, conidia will enter the imbibition process simultaneously in diffusion, and penetrate the seed wall (Rodriguez *et al.*, 2009). Inoculation of suspension of *B. bassiana* was using leaf spraying, conidia will stick to the surface of the leaf, then enter through the stomata (Jaber, 2015; Posada *et al.*, 2007). While soil-wetting, conidia can be attached to the root surface through the root feathers along with water to the xylem vessels to spread to the stem and leaves (Agrios, 2005). Distribution of *B. bassiana* in plants through vascular tissue, xylem (Landa *et al.*, 2013; Wagner & Lewis, 2000).

The spread of *B. bassiana* fungus which was not evenly distributed in the Chinese kale plant in addition to the absorption of conidia suspension is also thought to be caused by environmental factors around the plant and also internal factors in plant tissue. The number, length, and growth rate of sprout tubes or conidial movements are influenced by the physical environment such as temperature and type humidity (Agrios, 2005). The study also supports that *B. bassiana* can grow and move through vascular tissue in corn plants (Wagner & Lewis, 2000). Many factors in plants and fungi, including fungal physiology, plant species, plant age, exposure to UV rays, temperature, and plant conditions which could influence the presence of endophytic fungi in plants (Behie et al., 2015).

Decreasing or inhibiting *B. bassiana* in Chinese kale plants at 14 dai was caused by temperature and humidity factors which were not suitable in the greenhouse. The temperature in the greenhouse reached 28-30.5°C and humidity was 90%, while the maximum development of *B. bassiana* with temperature 23-25°C and humidity 92%. The decrease in *B. bassiana* colonies in cabbage plants can be caused by high temperatures which are not in accordance with fungal growth (Agrios, 2005). Solar radiation can reduce fungal virulence due to DNA damage that causes mutations (Braga *et al.*, 2015).

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Table 2. The average percentage of *B.assiana* endophytes in the Chinese kale plant parts

Plant Parts	∑ Colony of *B. bassiana* (%)
Leaf	13.89
Stem	2.77
Root	5.55

Table 3. Colonization of *B. bassiana* fungi in the Chinese kale plant section

Inoculation Method	Colonization of *B. bassiana* (%)		
	Leaf	Stem	Root
	$\dot{x} \pm SE^{ns}$	$\dot{x} \pm SE^{ns}$	$\dot{x} \pm SE^{ns}$
Seeds-soaking (control)	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Leaf-spraying (control)	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Soil-wetting (control)	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
Seeds-soaking	3.85 ± 1.88	1.98 ± 1.27	1.98 ± 1.27
Leaf-spraying	3.26 ± 1.47	0.7 ± 0.0	0.7 ± 0.0
Soil-wetting	0.7 ± 0.0	0.7 ± 0.0	1.98 ± 1.27

Notes: ns = not significantly different.

Efficacy of B. bassiana Fungus against P. xylostella Larvae

The results show that *P. xylostella* larvae due to *B. bassiana* were different in the three inoculation methods including seeds-soaking, leaf-spraying, and soil-wetting. The highest percentage of *P. xylostella* mortality was generated from 45% with seeds-soaking method (Table 4). Based on the observation of *P. xylostella* larvae that died because *B. bassiana* has the characteristics of a dry, brownish and hardened body, and after incubation on a tissue soaked using sterile distilled aquatic larvae *P. xylostella* overgrown with white hyphae covering the larva's body surface (Figure 2). The value of LT_{50} was the time needed to cause 50% death in the insects tested. The *B. bassiana* fungus which caused the fastest LT_{50} with a seeds-soaking method of 409.48 hours. While the longest death of *P. xylostella* LT_{50} resulted from leaf-spraying method of 1090.21. This shows that the higher the conidia *B.bassiana* colonization on Chinese kale leaves, the faster the value of LT_{50} (Table 5). *Plutella xylostella* larvae have died up to 168 hours in seeds-soaking method, leaf-spraying, and soil-wetting (Figure 3).

In the mortality of *P. xylostella* larvae which did not show hyphae in the larva's body due to the influence of the toxin produced by *B. bassiana*. The Beauvericin, a cyclohexadepsipeptide ionophore from the entomopathogen *Beauveria bassiana*, shows antibiotic, antifungal, insecticidal, and cancer cell antiproliferative and antihaptotactic (cell motility inhibitory) activity in vitro (Xu *et al.*, 2008). On the observation of *P. xylostella* larvae which did not experience death and change into pupae and imago, it was thought that the larval development was delayed. *B. bassiana* which was included in plant tissue can be as antibiosis so that it not only directly affects the mortality of larvae but indirectly affects the development of larvae to become pupa and imago. According to Bing and Lewis (1993), endophytic *B. bassiana* can be toxic to insects by producing deterrent

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feeding or antibiotics. The mortality rate was influenced by the way inoculation of the larvae. The inoculation method has an effect on the level of colonization on the part of the plant. Endophyte fungus *B. assiana* makes direct contact with the surface of the skin of *P. xylostella* larvae and then forms an appressorium that penetrates the cell wall and then enters the digestive tract.

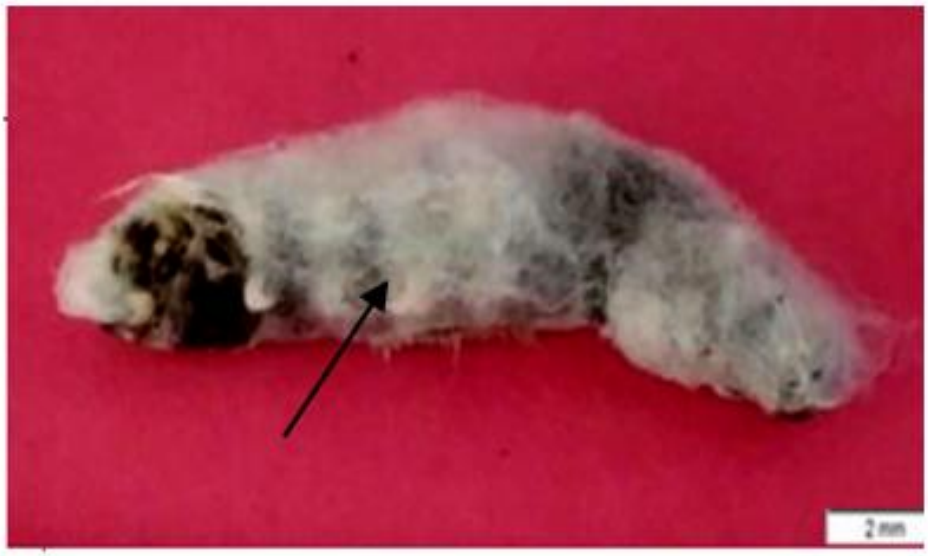

Figure 2. Hyphae of *B. bassiana* in the body of *P.xylostella* larvae

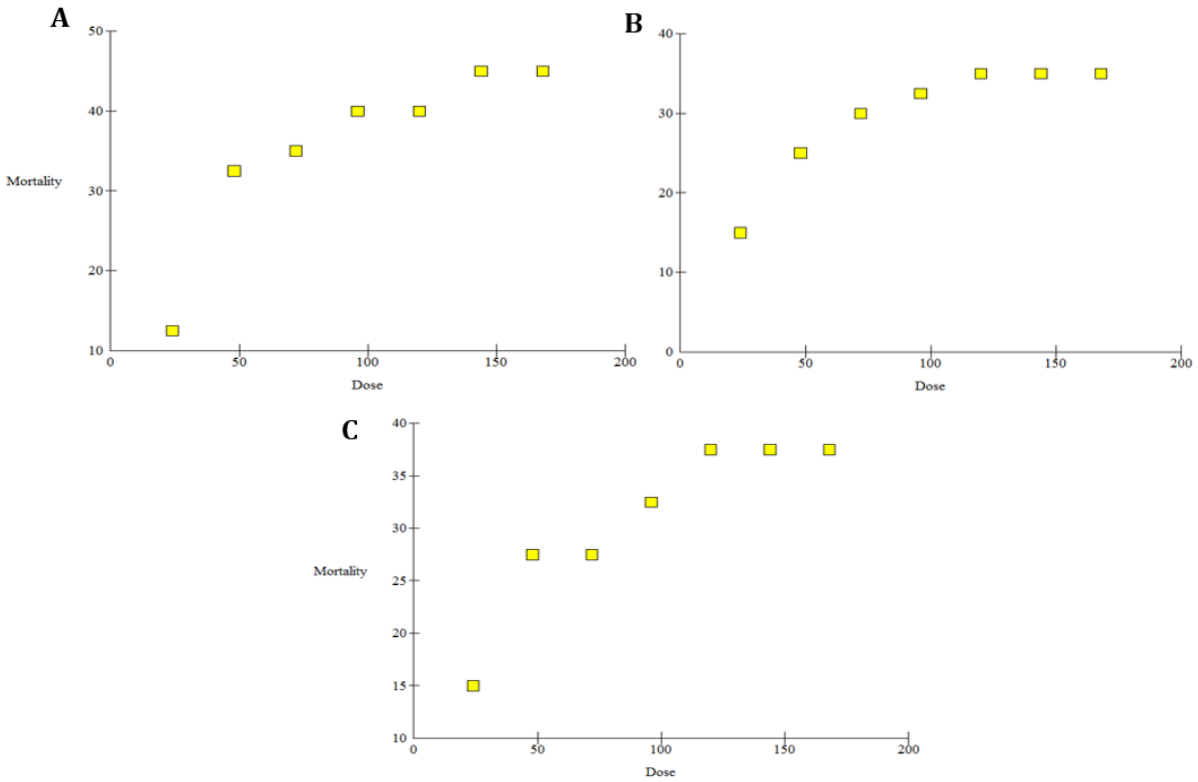

Figure 3. LT_{50} of *B. bassiana* against *P. xylostella* larvae in different inoculation method, A. Seeds-soaking, B. Leaf-spraying, and C. Soil-wetting.

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Table 4. The average percentage of *P. xylostella* larvae caused by *B. bassiana* fungi at different levels of inoculation method

Plant Parts	Mortality (%)
Seeds-soaking (control)	0.00 a
Leaf-spraying (control)	0.00 a
Soil-wetting (control)	0.00 a
Seeds-soaking	45.00 c
Leaf-spraying	35.00 b
Soil-wetting	37.50 b

Note: the numbers followed by the same letter notation, means that they are not significantly different from the Duncan test (p = 0.05); the data were transformed using the arcsin formula (√ (x + 0.5)) for statistical analysis

Table 5. LT_{50} of *B. bassiana* fungus against *P.xylostella* larvae in different inoculation method

Inoculation Method	Regression Equation	SE	LT_{50} (hour)	95 % Probit Limit	
				On	Under
Seeds-soaking	Y = 2.86 + 0.84 x	0.58	409.48	287.14	806.46
Leaf-spraying	Y = 2.53 + 0.81 x	0.74	1090.21	499.94	10415.89
Soil-wetting	Y = 2.29 + 0.96 x	0.72	679.15	346.83	6440.96

Conclusion

Beauveria bassiana has succeeded in becoming endophytes in Chinese kale plants. The highest percentage of endophytic *B. bassiana* colonization was obtained from the results of the inoculation of seeds-soaking by 13.87% and the lowest from leaf-spraying 5.55% and soil-wetting 2.77%. The highest percentage of *P.xylostella* mortality was obtained from the seeds-soaking method by 45% with LT_{50} 409.48 and the lowest yield from leaf-spraying 37.5% with LT_{50} 1090.21 and soil-wetting at 35% with LT_{50} 679.15.

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