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Existence of Entomopathogenic Fungi, *Beauveria Bassiana* as Endophyte in Cocoa (*Theobroma Cacao* L.) Seedlings

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Abstract

Beauveria bassiana is one of entomopathogenic fungi which is also known as biological control agent of cocoa pod borer and cocoa mirids (*Helopeltis* spp.). Considering that its effectiveness in fields is still not consistent, a research was conducted with the objective to study the possibility of *B. bassiana* to be established as a endophyte. The research has been conducted in Laboratory of Plant Protection, Indonesian Coffee and Cocoa Research Institute by inoculating cocoa seeds and cocoa seedlings with *B. bassiana* suspension. The trial was arranged by randomized complete block design with a factorial arrangement. The factor were spore concentration of *B. bassiana* (0; 2; and 4 g/ 10 L) and cocoa varieties (family of ICS 60, TSH858, and hybrid). Every combination treatment had four replications. The results showed that *B. bassiana* was established as an endophyte both by inoculation on cocoa seeds and seedlings. Percentage of existence of *B. bassiana* colonies as endophyte one month after seeds application was 93.3 % in ICS 60 for both concentration treatments, while in family of TSH 858 by 80 % and 86.7% respectively, in 2 g and 4 g per 10 L of *B. bassiana* spores concentration treament. The lowest percentage was in cocoa hybrids which had respectively, 66.7% and 50%. *B. bassiana* colonies existed as an endophyte found in root, stem and leaf of cocoa seedling up to 5 months after inoculation. While application through nursery by soil drenching, leaf spraying, and stem injection, it was found that *B. bassiana* colonies were found in the tissues of leaf, stem, and root until two months after application. Colonies of *B. bassiana* as endophyte still existed until six weeks after seedling was planted in the field.

Keywords: cocoa, *Beauveria bassiana*, endophytes

INTRODUCTION

Applying biological agents and botanical insecticides to control cocoa pod borers (CPB) and mirids, *Helopeltis*, on cocoa crop is starting to develop. It is driven by environmental issues emphasizing on sustainable agricultural patterns. Biological agents which have been widely used in cocoa cultivation are black ants, *Dolichoderus thoraxicus* (See & Khoo, 1996; Sulistyowati *et al*., 2012), *Beauveria bassiana* entomopathogenic fungi (Junianto & Sulistyowati, 2001) and *Paecilomyces fomosoroseus* (Sulistyowati *et al*., 2002), as well as egg parasitoid, *Trichogrammatoidea bactrae fumata* (Sulistyowati *et al*., 2009). A study by Junianto & Sulistyowati (2001) and Sulistyowati *et al.* (2002) showed that spraying *B. bassiana* isolate BBY-725 was able to suppress CPB attack between 54-60.5%. A weakness in exploiting entomopathogenic fungi in the field is that the lack of consistency in reducing CPB attack or other pests. It is due to various factors, including environmental factors such as temperature and humidity; entomopathogenic fungal factors such as production techniques, storage and application; or the interaction of those factors. To solve the problem, an attempt was suggested by establishing formulation of biological agents, namely by adding active ingredient compounds with entomopathogenic fungal spores or other applications, such as applying *B. bassiana* fungus as endophyte. Fungi which are taxonomically and biologically diverse but all share characteristic of colonization within plant tissues without causing any apparent damages to their hosts are called endophytes (Mejia *et al.*, 2008). As a biological agents endophyte may reduce plant damage caused by insect, nemathode, and pathogen through plant resistance induction, besides endophyte act as biological agents through antagonist competition interaction (Yulianti, 2013).

Endophyte as biological control agents can be derived from bacteria, fungi, and actinomycetes. Endophytic fungi which can be grouped as biological control agents are *Fusarium solanii, Acremonium zeae, Vermicillium* sp., *Phomopsis cassiae, Muscodor albus, Periconia* sp., *Ampelomyces* sp., and *Neophodium lolii* (Gao *et al.*, 2010). According to Parsa *et al.* (2013), *B. bassiana* is an entomopathogenic fungus with the ability to endophytically colonize plants. As an endophyte, it may play a role to protect plants from pests and diseases. Meanwhile, it has not been investigated whether *B. bassiana* can survive as an endophyte in perennial crops, spread in the host's plant tissue and protect host plants against pests, such as cocoa pod borers or *Helopeltis*. To determine whether *B. bassiana* inoculated on seed or seedling stages of cocoa was able to maintain its existence as endophytic fungus in the field, a series of studies had been conducted in Laboratory of Crop Protection and in a greenhouse of Indonesian Coffee and Cocoa Research Institute.

MATERIALS AND METHODS

The study was conducted in a greenhouse and in Laboratory of Crop Protection, Indonesian Coffee and Cocoa Research Institute. Application of suspension of *B. bassiana* isolate Bb-725 was done in two ways, namely application at seed and seedling stages. The experiment was arranged according to factorial randomized complete block (RCB) design, where concentration treatment of *B. bassiana* spores, i.e. 0.2 g and 4 g per 10 L of water as the first factor and the seeds of cocoa genotypes of TSH 858 and ICS 60, as well as F1 hybrids as the second factor. The genotypes of TSH 858 and ICS 60 were obtained from those clones with open pollinated seeds. Each treatment plot consisted of 20 cocoa seeds and each treatment was replicated three times. The seed stadium application was conducted by soaking the cocoa seeds with the length of radicles about 1 cm into *B. bassiana* suspension for 15 minutes. The cocoa seeds were soaked and air dried, then planted in polybags with diameter of 12 cm containing mixed soil medium and fine sand (1:1) which had been sterilized by autoclaving at 15 psi; 121^{O}C for one hour. Application of *B. bassiana* at the seedling stage used factorial RCB design, with the concentration of *B. bassiana* spores; namely 0 g, 2 g, and 4 g per 10 L of water as the first factor while the second factor was methods of application, i.e. watering through soil, spraying leaves and injecting stem. Two months old cocoa seedlings were used.

Observation was directed to the occurrence of *B. bassiana* colony as endophytic fungi out from tissues of leaf, stem and root of the cocoa seedlings. The observation was conducted destructively every month by sampling the leaves, stems and roots and then washed the tissues using distilled water. The surface of the leaves, stems and roots of cocoa seedlings was sterilized by soaking them in 0.5% bleach solution, then in alcohol solution and sterile distilled water each for two minutes. After that, the leaves, stems and roots of cocoa were dried by sterile tissue paper. They were cut into 2 mm length, then the pieces each of leaf, stem, or root were placed on sterile agar medium. A Petri dish was filled with five pieces of leaf, stem or root. All these activities were done in laminar air flow. The agar medium on Petri dishes containing pieces of leaf, stem, and root of cocoa were incubated at the temperature of 25^{O}C. Observations on the colony growth of *B. bassiana* and fungal spores from leaf, stem or root tissue were carried out every day. The percentage of *B. bassiana* as endophyte was calculated from the ratio of colony number which appeared from the tissue compared to the planted tissues.

RESULTS AND DISCUSSION

Existence after seed application

Results showed that cocoa seeds treated with suspension of *B. bassiana* and planted in sterile soil media in a polybag could grow perfectly. There was no specific symptom indicating abnormalities compared to control (untreated cocoa seedlings). This study indicated that inoculation of *B. bassiana* on cocoa plants did not cause disease symptom.

Observation on the presence of *B. bassiana* as endophyte carried in tissues of leaf, stem, and root of cocoa seedling confirmed that one month after treatment, growth of *B. bassiana* colonies can be derived from pieces of leaf, stem, and root of cocoa seedlings grown in agar medium, at concentrations of 2 and 4 g of spores per 10 L. Whereas in control treatment, it did not appear that *B. bassiana* grew on agar medium. Based on *B. bassiana* existence percentage as endophyte, it showed that higher the concentration of *B. bassiana* spores was, higher the percentage of *B. bassiana* colony occurrence as endophytic fungus would be. The highest percentage of colony occurrence of *B. bassiana* as endophyte in one month after treatment was found in the leaf tissue of ICS 60 genotype by 93.3%, while TSH 858 genotype was 80% and 86.7% respectively for concentration treatment of *B. bassiana* spores of 2 g/10 L and 4 g/10 L of water. The lowest percentages of 66.7% and 50% were found in hybrid for both concentrations.

Previous study by Posada & Vega (2005) showed that inoculation on cocoa seeds produced colonies of *B. bassiana* as endophyte. One month after inoculation, endophyte percentage of *B. bassiana* CS16-1 isolated from leaves was 24.3% and then increased to 37.6% in two months after inoculation. Colonization of *B. bassiana* CS16-1 was found in cocoa stem one month after inoculation up to 56.4% and then increased to 68.9% in the next observation. Meanwhile in root, colonization of *B. bassiana* one month after inoculation was 4.3% and 81.4% in two months after inoculation. Observations of *B. bassiana* endophytic existence was conducted for five months and its spores were still found on the tissues of leaf, stem, and root of cocoa plants with very low percentage. Discovery of *B. bassiana* fungal colonies grew out of leaf, stem and root tissues of cocoa proved that *B. bassiana* can act as endophyte, since they can enter the tissue of cocoa plants without causing disease symptoms in the plant. Furthermore, the existence of *B. bassiana* colony in those plant tissues showed that as endophyte, *B. bassiana* is moble throughout the plant tissues.

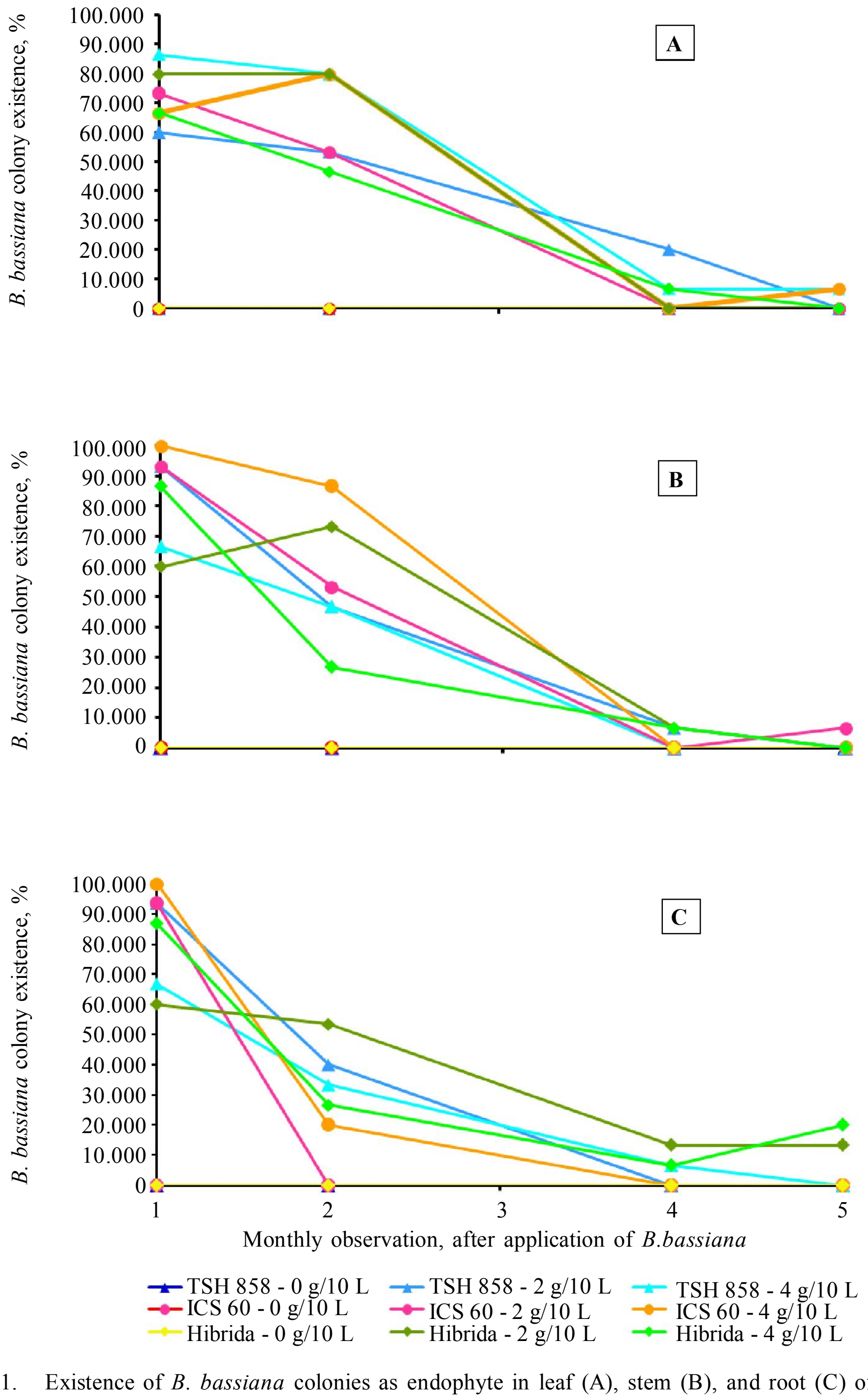

Figure 1. Existence of *B. bassiana* colonies as endophyte in leaf (A), stem (B), and root (C) of cocoa inoculated by *B. bassiana* on seed

Figure 2. Existence of *B. bassiana* as endophyte on cocoa leaves (top) and roots (bottom), two months after inoculation

Existence after seedling application

Based on this research result on the existence of *B. bassiana* as endophyte after the fungus inoculation on cocoa seedlings carried out by leaf spraying, stem injection, and root watering, it explained that there was no significant difference between of *B. bassiana* spores concentration treatment, technique of application, and also no interaction between the two treatment factors. Observation on growth of *B. bassiana* colonies in tissues of leaf, stem, and root on agar medium after one month of application confirmed that *B. bassiana* fungal colonies were established from the cocoa tissues with the percentage between 4.4-35.7% on leaf; 2.2 to 15.5% on stem, and between 0-12.2% on root. Based on the analysis of variance, there was no significant difference between the treatments. From Figure 3, it can be seen that the method of treating plantlet by soil watering produced *B. bassiana* as endophyte in tissues of leaf, stem and root. The existence of *B. bassiana* as endophyte was often found on leaf, especially after application method of soil watering and leaf spraying. While on the root, *B. bassiana* as endophyte were only found in the soil watering application treatment. The results of this study were similar with study conducted by Porsa *et al.* (2013) who found that *B. bassiana* fungus was able to endophytically spread in *Phaseolus vulgaris* plant in response to *B. bassiana* inoculation.

Both methods of inoculation namely leaf spraying and soil watering produced more than 80% endophytic colonization by *B. bassiana* on treated plants. However, the extent of colonization spreading highly depended on parts of plant evaluated and on inoculation method. The best response toward inoculation was from leaf through spraying; while roots only responded to soil watering treatment. Stem responded to both leaf spraying and soil watering. *B. bassiana* was not detected in any part of control plant. A study by Russo *et al.* (2015) demonstrated the effectiveness of three inoculation methods (foliar spray, seed soaking, and root soaking) to establish *B. bassiana* as entomopathogenic fungus in tobacco, corn, wheat and soybeans. Leaf colonization by *B. bassiana* was assessed on day 7, 14, 21 and 28 after inoculation. There was a significant difference ($p < 0.001$) in endophytic colonization among the inoculation techniques tested.

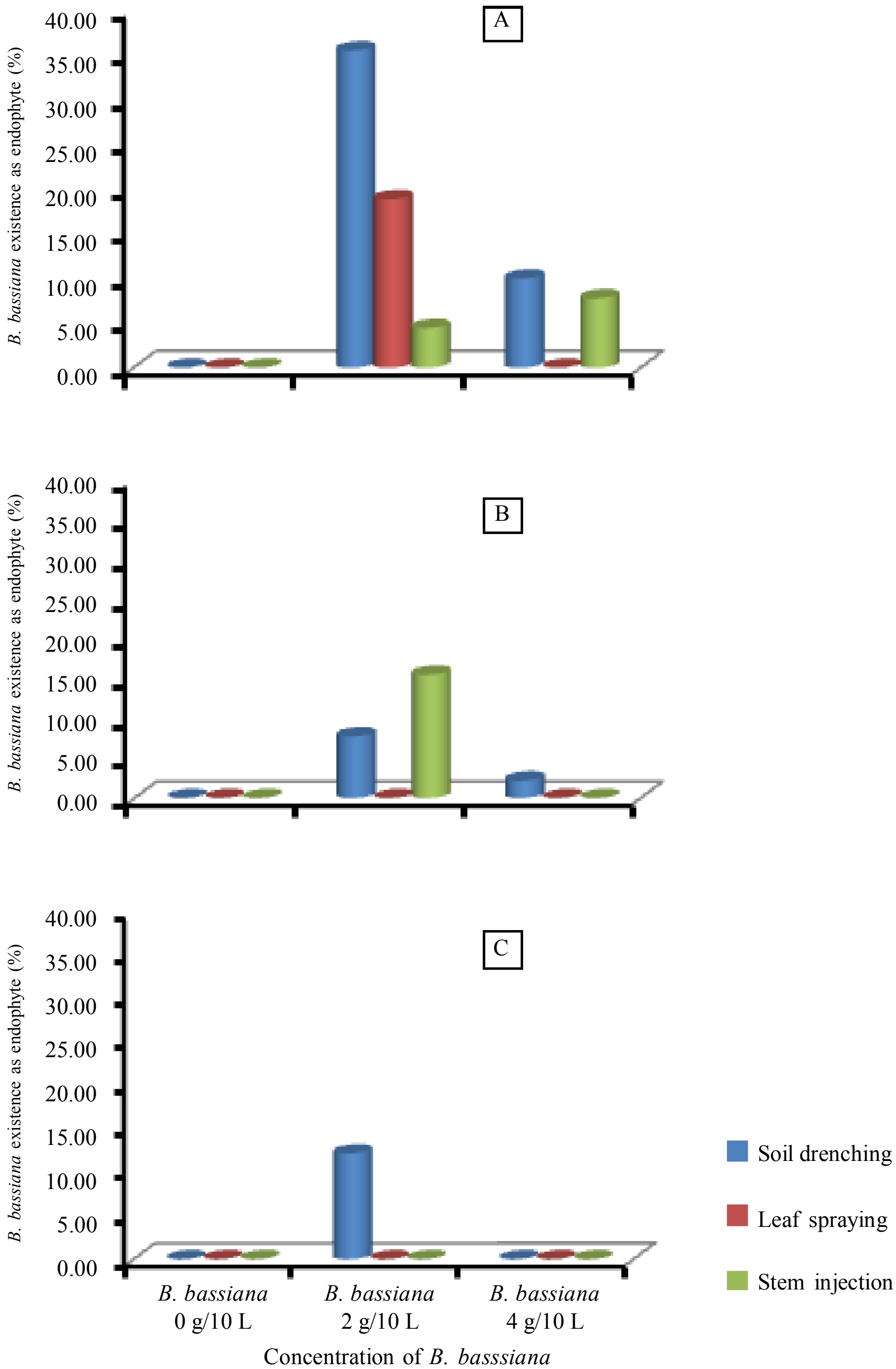

Figure 3. Occurrence of *B. bassiana* as endophyte in leaf (A), stem (B), and root (C) after application by leaf spraying, stem injection, and soil drenching on cocoa nurseries

Existence in cocoa plantation

Observation on cocoa seedlings inoculated with spores of *B. bassiana* when the seedlings planted in the field, showed that *B. bassiana* mycelium grew out from cocoa root tissues (Figure 4). One month after application, it showed that *B. bassiana* colonies grew on agar medium which derived from leaf, stem, and root of cocoa seedling. Meanwhile, in control treatment, there was no *B. bassiana* fungal colony grew on that agar medium instead of other fungi or bacteria. Result of the spore appearance percentage showed that *B. bassiana* spores from the leaf tissue after one-week in the field (Figure 6A) indicated that the hybrid cocoa seedlings of TSH 858 genotype showed the highest percentage (53.3%) compared with hybrids of ICS 60 genotype and other hybrids, namely 46.7% and 20.0%, respectively. *B. bassiana* fungus still acted as endophyte up to 8 week after application, although with lower percentage (0-6.7%). The presence of *B. bassiana* as endophyte in roots one week after sowing the seedlings in the field indicated that the average percentage of *B. bassiana* spore occurrence from the root tissue was lower than in leaves from THS 858, ICS 60 genotype and hybrid were 13.3%, 20.0% and 6.7%, respectively.

Five weeks after planting, spores of *B. bassiana* fungus on leaf tissue were found with very low percentages for TSH 858, ICS 60, and hybrid with 6.7%, 13.3%, and 6.7%, respectively. This result indicated that *B. bassiana* as endophytic fungi could stay until the plant grow in the field. A verification was still required the extent to which *B. bassiana* as the endophytic cocoa could increase plant resistance against major pests which attack cocoa leaves. Besides, it needs to be clarified whether *B. bassiana* as endophyte could enter cocoa pods. If it gets into cocoa pods, it will be highly prospective to control cocoa pest such as *Helopeltis* and cocoa pod borers. A study by Jaber & Salem (2014) showed

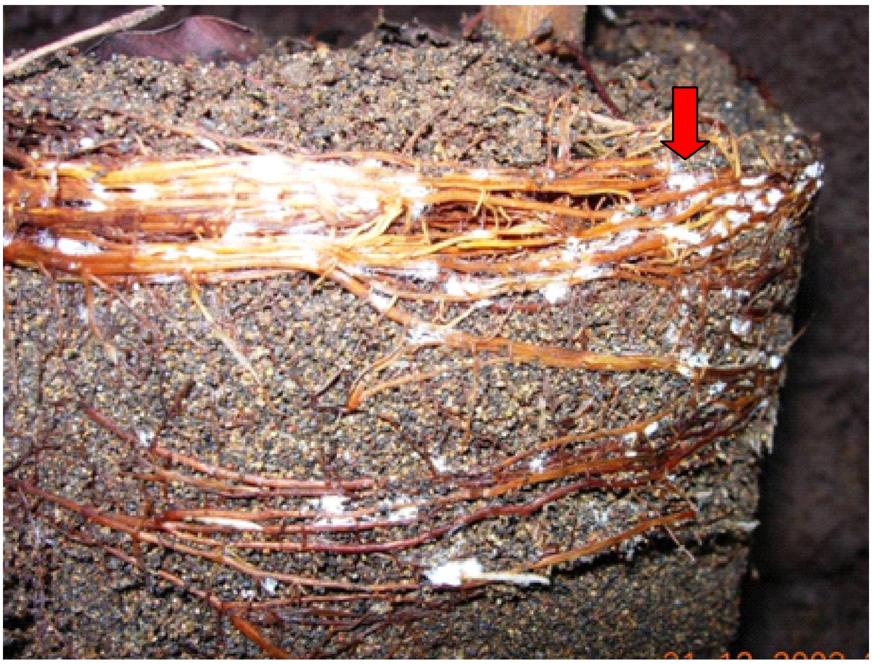

Figure 4. Mycelium of *B. bassiana* on root of seedling which has been inoculated by *B. bassiana* through cocoa seed

Figure 5. Colonies of *B. bassiana* as endophyte appeared on leaf (a) and root (b) of cocoa seedlings, 2 weeks after inoculation on the plant in the field

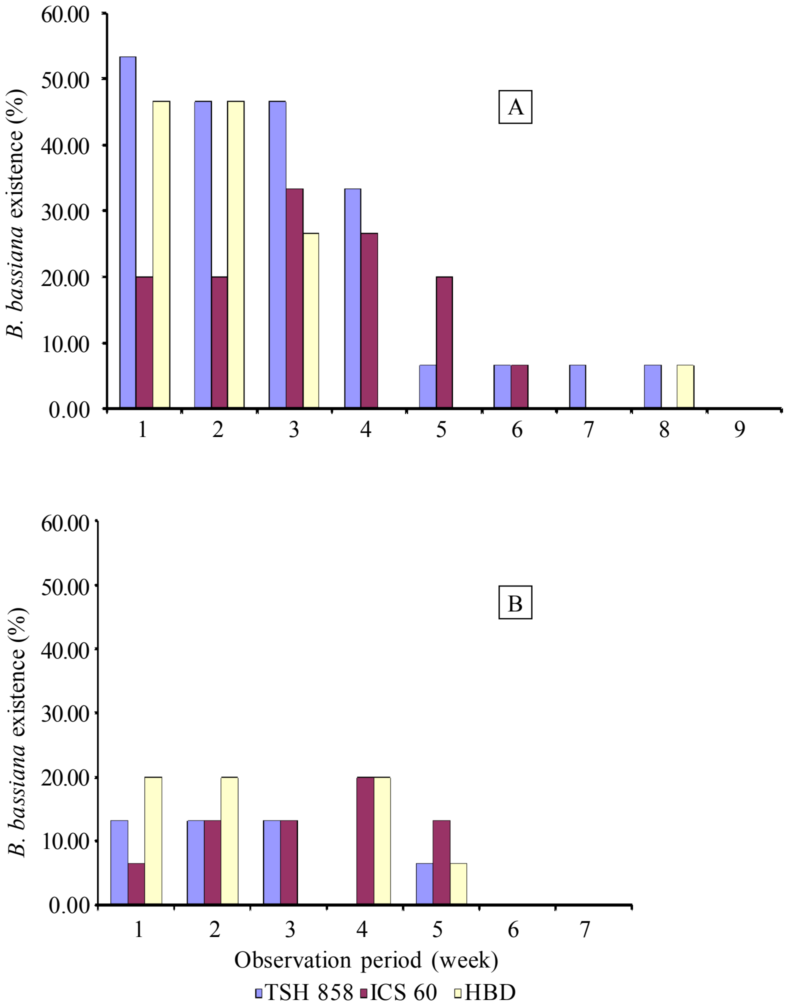

Figure 6. Percentage of existency of *B. bassiana* colonies as endophyte appeared in leaf (A) and root (B) of cocoa seedlings planted in the field

that *Beauveria bassiana* as endophyte potentially provide a protection to zucchini yellow mosaic virus (ZYMV) on cucurbit crops. The percentage of plants showing ZYMV symptoms and severity on cucurbit plants inoculated with *B. bassiana* was much lower than in control plants.

CONCLUSION

B. bassiana as entomopathogenic fungus may serve as endophytic fungus on cocoa plants. Colonies of *B. bassiana* as endophytic fungus are found in the tissue of leaf, stem and root of cocoa seedling inoculated with *B. bassiana* at seed stage. *B. bassiana* colonies as endophytic fungus can also be found in leaf and root tissues of cocoa seedling inoculated with *B. bassiana* suspensions through root watering, leaf spraying and stem injecting. Colonies of *B. bassiana* as endophytic fungus appear out of root and leaf of cocoa seedling inoculated with *B. bassiana* and still present in the field.

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