Olfactory response of *Cosmopolites sordidus* (Germar) is affected by volatiles emitted by banana plants inoculated with non-pathogenic *Fusarium oxysporum and Beauveria bassiana?*

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Abstract

Plants have direct strategies to overcome invaders. Their defence system is complex because of their immobility; they cannot escape invaders by moving. For example, plants can use mutualistic relationship with other organisms to increase their defence against herbivore arthropods. Fungi such as *Beauveria bassiana* (entomopathogen) and *Fusarium oxysporum* (endophyte) are found in association with the banana plant, which gives protection against the banana weevil *Cosmopolites sordidus*. We investigated the behaviour of these herbivores in the presence of volatiles produced by the host plant in association with endophytes and entomopathogens. Inoculation of *B. bassiana* and *F. oxysporum* in banana plants decrease the attraction of *C. sordidus* towards the banana plant. To test our hypothesis, we used olfactometer bioassays to analyze whether weevil responses were affected by volatile kairomones from banana plants treated with *B. bassiana* and *F. oxysporum*. Leaf tissue from plants treated with *B. bassiana* did not decrease attraction of *C. sordidus*, while treated pseudostem and corm did. Plants treated with the endophyte *F. oxysporum*, were less attractive to weevils than control banana plants. It is known that endophytes and entomopathogens reduce banana weevils' damage. Now, our data suggests that infestation with *B. bassiana* and *F. oxysporum* induce a change in the emission of volatiles in banana plants affecting the weevils behaviour.

Key words: Musa spp - banana, volatiles, herbivores, kairomones, indirect plant defence, endophytes, *Fusarium oxysporum, Beauveria bassiana*, banana weevil - *Cosmopolites sordidus*, Curculionidae,

Chapter 1

Introduction

Banana (*Musa* spp.) is an important food crop in East Africa. In addition to being a staple food for rural and urban consumers, banana is an important source of income for poor farmers (Purseglove, 1985; mentioned by Masanza, 2003). However, biotic and abiotic constrains are the main reason for the recent decline in the production. The most important diseases are: Fusarium wilt (*F. oxysporum, f. sp. cubense* Schlecht. emd. Synd. Hans.), Black Sigatoka (*Mycospharella fijensis* var. *difformis* Mulder & Strover) and bacterial wild caused by *Xanthomonas campestris pv. campestris* (Dowson) (Tushemereirwe and Waller, 1993). The main abiotic constraint in banana production is the poor soil fertility due to land shortage and inadequate labor of the fields (McIntyre *et al*., 2001). The major pest in most cooking bananas and plantain is the banana weevil *Cosmopolites sordidus* (Germar) (Gold *et al*., 1999 and Gold *et al*., 2001b). First, the female lays its eggs near the corm. Then the damage starts when the larval is tunnelling into the corm and pseudostem, which affects the uptake of nutrients and water, making the plants weak and unstable (Gold *et al*., 2001b). Yield losses cause by banana weevils damage can be up to 100% (Sengooba, 1986; mentioned by Tinzaara, 2003).

This recently decline of banana production in Uganda affects both farm incomes and food security (Bekunda, 1999). To increase yields the control of pests and diseases with an integrate pest management (IPM) is needed. The IPM approach should contain a methodological perception with: combination of cultural methods and reduction of the use of pesticides; integration of control methods in the farm-system and participation of farmers in the development of an IPM (van Huis and Meerman, 1997).

Banana is commonly propagated by suckers, which are often infected by *C. sordidus*. The use of tissue culture banana plantlets provides clean planting material which enables the production of vigorous and uniform banana plants (Mateille *et al*., 1994). However, some studies mention that micro-propagated plants are more susceptible because they are not just free of pest and diseases, but also free of beneficial microorganisms (Viaene *et al*., 2003).

Plants have shown in different studies interactions among tri-trophic levels to protect themselves from herbivores. For instance, it is well known that plants provide shelter and food to predators and parasitoids to use them as body guards, and produce herbivore-induced volatiles to inform natural enemies about herbivore presence (Sabelis *et al*., 1999 mentioned by Elliot *et al*., 2000). However, few studies have analyzed the effect of herbivore-induce plant volatiles on entomopathogens/endophytes behaviour or the effect these pathogens have on plant volatiles emission (Elliot *et al*., 2000). Nevertheless, it is well known that plants infected with endophytic fungi are chemically protected against herbivore consumption (Breen, 1994).

There is a mutualistic relationship among plants and fungi endophytes, while plants may gain protection from phytophagoes by toxins or deterrent effects of the alkaloids synthesized by the fungus, the endophyte get nutrients from their hosts (Breen, 1994). However, the association among microbes and plants has not been largely study on multitrophic interactions (Omacini *et al*., 2001). Now a day, the effect of fungal endophytes to protect plants against herbivores has become a relevant topic between ecologists (Saikkonen *et al*., 1998). There is evidence that fungal endophytes reduce herbivore insects via toxins as is the case of *Beauveria bassiana* that gives protection against the corn borer larvae (Saikkonen *et al*., 1998) and microbial populations in the rhizosphere protect the plant against phytopathogens (Elad *et al*., 1996 mentioned by Elliot *et al*., 2000).

In Uganda since 1993 the potential of the use of indigenous entomopathogenic and endophytic fungi have been studied (Allard *et al*., 1993; Nankinga, 1994; Paparu, 2005 and Akello *et al*., 2007). Promising biopesticide fungi like *Beauveria bassiana* and *Fusarium oxysporum* have been isolated from several areas of the country and researchers have been investigated their potential to control banana weevils (Nakinga *et al*., 1994; Dubois *et al*., 2004; Tinzaara, 2005; and Masanza, 2003). However, this technology is just used by a small group of farmers due to its expenses and low availability (Masanza, 2003).

Therefore, an artificial, effective and economically feasible inoculation technique of mutualistic endophytes into banana tissue culture plantlets is important for the effectiveness of the biological control strategy in an IPM (Sikora and Schuster, 1998 and Dubois *et al*., 2006).

Problem statement and justification

Banana weevil is one of the main reasons that limit the production of Bananas in East Africa. An IPM strategy is a novel method for pest and disease control, and more sustainable and less harmful to the environment (Gold *et al*., 1999). Among the biological control strategies that can be apply against *C. sordidus* is the use of endophytic and entomopathogen microorganisms (Griesbach *et al*., 1996).

Endophytes have an incredible capacity to colonize internal host tissues, which make them relevant for biological control. This can reduce the use of chemical control against banana weevils, which indeed is not a wise option due to the high cost and limited availability, its variable effectiveness, and the negative side effects on human and environmental health (Cortesero *et al*., 2000).

The novel fungi *F. oxysporum* has been deeply studied for its potential use as an endophyte (Hallmann and Sikora, 1994; Griesbach, 2000; Niere, 2001 and Paparu *et al*., 2004). Artificial inoculation technique of mutualistic endophytes into banana tissue culture plantlets could be an important strategy to control *C. sordidus*. However, this technique lacks of a reliable and cheap inoculation (Azevedo *et al*., 2000).

Microbial control may be a possibility to minimize the damage caused by *C. sordidus* in banana plantations. Tinzaara (2005) to control banana weevils integrated pheromone trapping with the entomopathogenic fungy *B. bassiana*. He found that weevils' mortality due to pathogen infection was higher in plots where aggregation pheromone was used in combination with the entomopathogen than in those plots with out the pheromone.

Fungal endophytes have mutualistic relationships with their hosts. Various *Fusarium* spp have been re-isolated from banana plants, and some of them have shown a potential option as biological control agents (Dubois *et al*., 2006). Dubois *et al.* (2004), through in vitro experiments, found that spore suspensions of the endophyte *Fusarium oxysporum* strains V2w2 and III4w1 resulted in 50.3 % and 43.7 % mortality of banana weevil eggs, respectively. Jongebloed *et al*. (1992), found that infection of tomato plants with the vascular fungus *F. oxysporum* f.sp. *lycopersici* induce defence response or at least a change in tomato host quality that also affects the oviposition of spider mites with a reduction of 20-25% in the oviposition mites performance.

We investigated whether the entomopathogen *B. bassiana* and the endophyte *F. oxysporum* would have an effect on the defence system of the East African highland bananas against *C. sordidus*.

General objective

The objective of this study was to assess the attraction behaviour of *C. sordidus* towards material of banana plants treated with the entomopathogen *B. bassiana* and the non-pathogenic endophyte *F. oxysporum*. It is assumed that the banana plants treated with the fungi emit a different blend of volatile organic compounds (VOCs) than control plants.

Specific objectives

1. To analyze the behaviour of the banana weevil when exposed to odours from pieces of banana plants inoculated with *Beauveria bassiana*.

2. To analyze the behaviour of the banana weevil when exposed to volatiles from intact banana plants inoculated with *Fusarium oxysporum*.

Hypothesis

1. We hypothesize that inoculation of *B. bassiana* and *F. oxysporum* of banana plants have an effect on the behaviour of the banana weevil *C. sordidus* towards

the banana plant. For instance, Strobel (2006) found a novel endophyte *Muscodor albus* that emits a mixture of volatile compounds that vary in quality and quantity and can be lethal to a wide range of plant pathogenic fungi and bacteria and also has been effective against nematodes and insects.

Chapter 2

Literature review

The banana weevil in East Africa

Banana weevil - *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae), is the main pest in the production of bananas, causing yield loses up to 42% in East Africa. (Gold *et al*., 2004b).

Biology and life cycle

The adults live in the soil. They are active during the night and display positive hygrotropism, thigmotactism and gregariousness (Uzakah, 1995, mentioned by Gold *et al*., 2001). During the day the weevils prefer to stay in sheltered and isolated places and are not commonly visible by casual observers. Banana weevils are characterized by extended periods of sedentarisms and limited mobility. In one experiment weevils were released for six months and the results showed that some moved 60m while others less than 5m (Whalley, 1957; mentioned by Tinzaara, 2005). *Cosmopolitus sordidus* has a ‘k’ selected life cycle (Pianka, 1970, mentioned by Gold *et al*., 2001). The life span of this weevil is extremely long, up to four years. The fecundity is low; Gold *et al*. (2001)

reported 1-3 eggs/week. The female lay eggs either in the pseudostem or in leaf sheaths at the base of the banana mat (Abera *et al*., 2000). After hatching, the larvae start tunnelling inside the corm. They damage the vascular system decrease nutrient uptake, and affect the stability of the plant (Gold *et al*., 2001).

The dispersal of *C. sordidus* is commonly by infested plant material (Gold *et al*., 1999b). In the field, adult weevils can be found in banana mulch, crop residues of corms, pseudostems, and old fallen leaves. They prefer a humid habitat (under the soil surface). They are closely associated with the banana mat (Gold *et al*., 2004a).

Host preference

The herbivore *C. sordidus* uses plant kairomones to locate host plants. Cuille (1950) showed the 'chemotropism' of banana weevil in a series of experiments using lures and olfactometers. Sumani (1997) stated that both male and female weevils regularly are more attracted to pseudostem than to corm material, but oviposition is larger on the corm. This observation suggests that olfactory cues and chemoreception are important in host location and host acceptance (Sumani, 1997 and Tinzaara, 2005). In terms of trapping, corm material tends to be more attractive than pseudostem; but less available (Gold *et al*., 2002). In addition, the weevil prefers corm volatiles emanating from plants with fruits than those from young or adult plants (Cerda *et al*., 1999) and they respond in an additive way to the combination of fermented plant tissue and the aggregation pheromone (Tinzaara, 2005). Budenberg *et al.* (1993) suggested that weevils' orientation is towards food sources rather than oviposition sites.

Control options

Cosmopolites sordidus is a difficult pest to control. The immature stages feed and develop inside the plant, which make them inaccessible to pesticides and natural enemies. The main strategy to control weevils' damage is targeting the adults (Gold *et al*., 2001a).

Cultural control

Use of clean planting material, selection of cropping systems, management of crop residues and trapping are some of the cultural control options. The use of clean planting material can reduce initial infestations and delay the pest build-up. The problem with using suckers as planting propagules in new plantations is that they are often infested with weevil eggs or larvae (Gold *et al*., 2001a).

Nowadays, weevils are controlled by crop sanitation, mulching and by using split pseudostems or corm traps (Gold *et al*., 1993). Traps made out of corm material are generally more attractive to banana weevils than those made from pseudostems. Four to ten times more weevils can be found in corm than in pseudostem traps. In terms of material availability the use of pseudostems is a better option than using corms. Moreover, weevils are more attracted to traps made from the lower part of the pseudostem (e.g. 30–70 cm above the collar) than those from higher parts of the plant (Mestre and Rhino, 1997). However, there is a lot of controversy about this strategy. This is due to the unavailability of trap material, the low efficacy of the system, and labour intensiveness. The strategy is an expensive solution for small holders (Gold *et al*., 2003).

Enhancing mass trapping by the application of infochemicals, but including aggregation pheromones and kairomones lures and combining it with other compatible control methods (e.g. entomopathogens) may be the key to develop an effective method to control the banana weevil. In this case, the use of the pheromone instead of pseudostems trapping implies more effectiveness and less labour (Alpizar *et al*., 1999). The aim is to control insect species by capturing a large proportion of the adults, in order to decrease the population for the next generation (Birch and Haynes, 1982).

Alpizar *et al*. (1999) mention that in Latin America, especially in Costa Rica the use of pheromones for mass trapping is an easy and efficient strategy to control *C. sordidus*. Experimentally corm damage has been reduced by more than 60% in 4-5 months in banana plantations in Costa Rica. On the other hand, the application of this technique in East Africa did not give similar encouraging results as those in Costa Rica (Tinzaara *et al*., 2000). This shows how trapping efficiency is influenced by environmental factors, weevil biology and trap intensity, density and placement. Trapping with pheromones is an expensive control strategy due to the lack of its manufacturing and availability in East Africa (Tinzaara, 2005).

Mass trapping could be a key component of a set of pest management practices. Implementation of only traps may not result in suppression of the pest population. A number of factors are involved in the effectiveness of pheromone traps. For instance, pheromone dose and release rate, colour and trap designs, as well as environmental conditions, all together are factors involved in the capture efficiency (Sappington and Spurgeon, 2000).

Chemical control

Since 1907, pesticides have been used to control banana weevils, by immersing suckers in Bordeaux mixture. Several synthetic insecticides have been tested to control banana weevils (Gravier, 1907, cited by Gold *et al.*, 2004). Currently, in East Africa the use of chemical pesticides remains one of the main methods to control *C. sordidus* by drenching suckers and applying it to planting holes, the base of the mat and pseudostem traps. However, pesticides are costly, and consequently they are not an affordable and feasible solution for small farmers (Gold *et al.*, 1993). Moreover, chemical control leads to development of weevil resistance, environmental concerns and mammalian toxicity (Gold *et al.*, 1999).

Infochemicals and Biological control

Plants when under attack can not run away from invaders. Their defence is based on chemical and morphological strategies. Indeed, plants are mediators between entomophagous arthropods and herbivores. Nowadays, the improvements in genetic engineering have made it possible to manipulate chemically and morphologically crop plants to enhance pest and disease control in agro-ecosystems (Cortesero *et al.*, 2000).

Infochemicals

Plant volatiles play an important role in a multitrophic context such as plant-carnivore and plant-herbivore interactions (Dicke, 1999 and Dicke, 2000). Host plant selection by arthropods may be affected by infochemicals from herbivores and natural enemies (Grostal and Dicke, 1999). For instance, the cell damage caused by herbivores feeding on a plant, will result in a release of volatiles from the wound site. The information will be used by natural enemies to locate the herbivore being their prey or host. The message could help to enhance the foraging strategies efficiency, host utilisation and reproductive capacity of natural enemies (Dicke *et al.*, 1990 and Bottrell *et al.*, 1998). Insects have a diverse range of sensory organs and capacities to check the environment. They perceive constantly information from abiotic and biotic factors, and have the ability to catch and integrate relevant signals to make a choice (Vet and Dicke, 1992 and Dicke *et al.*, 2003).

Plants differ in genetic and phenotypic factors as well as variation in quantity and quality of infochemical products. Arthropods have the ability to distinguish odours from different plant cultivars even from closely related ones (Visser, 1986 and Vet and Dicke, 1992 Shimoda *et al.*, 2002). Banana weevils are able to differentiate among banana cultivars. This banana weevil seems to be more attracted to plant volatiles from the susceptible AAA cooking banana than the AB resistant cultivar (Budenberg *et al.*, 1993). Plant infochemicals may affect the performance of herbivore arthropods directly or indirectly through multitrophic relationships (Vet and Dicke, 1992; Dicke, 1999 and Dicke *et al.*, 2004). Direct chemical defences include production of toxins, repellents, deterrents and digestibility reducers; while indirect chemical defences involve the attraction of natural enemies in response to herbivore attack. Plant volatiles can influence foraging, survival, abundance, development and success of natural enemies and herbivores into the ecosystem (Dicke, 1999 and Cortesero *et al*., 2000).

Kairomones

Herbivorous insects are known to use kairomones (chemicals produced by direct or indirect plant defence) to locate their host plant (Visser, 1986). There are several studies with coleopteran species that have shown attraction to host plant volatiles. For instance, palm weevils are attracted to odours from pseudostem and sugar cane stalk in the field (Giblin-Davis *et al*., 1994); Colorado potato beetle is attracted to undamaged potato plants volatiles (Visser, 1986). However, an increased attraction is shown when plants are damaged mechanically or by herbivores (Bolter *et al.*, 1997).

The use of kairomones in pheromone-baited traps has reduced successfully the damage caused by *C. sordidus* in banana plantations in Costa Rica (Gold *et al*., 2002 and Cerda *et al*., 1999). However, some field studies conducted in Uganda have shown that this trapping system is not an optimal solution to control weevils in East Africa (Gold *et al*., 2002). Factors such as cropping system, environmental conditions, management of trapping and residues material may affect the efficiency of the pheromone. In addition, importing pheromones is costly, which make it a difficult option for small holders.

Price *et al*. (1980), mention that in multitrophic interactions plant infochemicals play an indispensable role in communication between plants and insects. Plant kairomones are an essential and interactive component of herbivores behaviour in agroecosystems. For instance, Tinzaara (2005) mentions that plant kairomones can be exploited in plant-breeding to enhance plant resistance. Agelopoulos *et al*. (1999) state that non-host plant chemicals can dilute or mask chemical signals from host plants which can confuse the herbivores' foraging behaviour. This would be a sustainable strategy non-toxic to humans and non-target arthropods.

Biological control

Biological control explodes the action of a natural enemy complex (parasitoids, predators, pathogens) which results in a suppression of the pest density population to a lower level than would occur in their absence. The use of exotic and endemic predators and parasitoids, secondary host associations, and microbial control agents are some of the studies that have been done to control banana weevils (Gold *et al.*, 2001a).

Biological control programs against *C. sordidus* have gained momentum. The use of natural enemies to control banana weevils may be enhanced by infochemicals but this control strategy has not yet been widely exploited. Application of infochemicals in pest control is impossible without knowledge of the behaviour induced by the chemicals. Infochemicals can be used in pest management either by exploiting the natural enemy response or by manipulating the source of the infochemical, which could lead either in attraction of the predators or parasitoids or in repellence or arrestment of the weevils, respectively (Dicke *et al.*, 1990).

Microbial control is currently gaining importance as an option in IPM of the banana weevil. Basically, research has tested some organisms with high potential to control *C. sordidus* including entomopathogenic fungi like *B. bassiana* and *Metarhizium anisopliae*, entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis* spp.)

and endophytes like non-pathogenic *Fusarium* spp. Entomopathogenic agents are used to control adult weevils, while endophytes kill the immature stages. Several strains of entomopathogens have shown excellent results controlling weevils in the laboratory and semi-field studies. However, more studies should be done in order to find an efficient and economically viable mass production and deliver system of the microbial control agent in order that the fungi will efficiently reach the target pest (Gold *et al*., 2001 and Dubois *et al*, 2004 and Tinzaara, 2005).

Laboratory studies reported weevil mortality up to 100% by *B. bassiana* applications (Nankinga, 1994). However, in the field some problems are presented due to the lack of an easy and cheap application method, also a big constrain is that the entomopathogen targets the adult and not the damaging weevil stage (larvae). Indeed, more research and studies need to be done to development a better system to apply *B. bassiana* against banana weevils under field conditions.

Entomopathogen - Beauveria bassiana

Entomopathogenic fungi have been tested against banana weevils since the 1970s (Ayala & Monzon, 1977; Delattre and Jean-Bart, 1978). The use of entomopathogens offers an alternative management strategy to control insect pests. For instance, the potential of the entomopathogenic fungus *B. bassiana* has gained a lot of attention as biological control agent for *C. sordidus* (Ferron, 1981). This fungus has the potential to grow, multiply and persist on the weevil. In addition, infected weevils will move and spread the pathogen throughout the pest's habitat.

Banana growers that applied *B. bassiana* to control newly planted suckers have obtained a decrease in the population of *C. sordidus* (Nankinga, 1999). These results showed the effectiveness of this method to reduce banana weevil populations (Nankinga and Moore, 2000). However, one limitation of the technique is the lack of an efficient delivery system of the entomopathogen in the field (Nankinga, 1999).

Endophytes-Fusarium oxysporum

Research now focuses on alternative management methods, such as the use of fungal endophytes. Fungal endophytes have mutualistic relationships with their hosts and some act as antagonists to pests and diseases. Plants endophytes colonize internal tissues for at least a part of their life cycle. They do not produce disease symptoms but give protection against biotic and abiotic factors harmful to the host plants (Gold *et al.*, 2003). Their mode of protection seems to be involved with; the production of metabolites inside the plant that act as repellents, toxins or feeding deterrents and their efficiency is correlated with plant physiological and ecological factors (Breen, 1994).

Although, *B. bassiana* is not commonly reported as a natural endophyte, it has investigated recently as an artificial endophyte controlling insect pests and plant diseases (Posada and Vega, 2005 and Akello *et al*, 2007). *Beauveria bassiana* has been reported as an endophyte in *Zea mays* (Bin and Lewis, 1991, cited by Akello *et al*., 2007), potato (Jones, 1994, cited by Akello *et al*., 2007), and tomato (Leckie, 2002 cited by Akello *et al*., 2007). Recently endophytic *B. bassiana* has been isolated from coffee plants in Colombia (Posada and Vega, 2005); and from banana plants in Uganda (Akello *et al*., 2007). Lately, Akello *et al*. (2007) discovered that inoculation with *B. bassiana* can develop an endophytic relationship with tissue culture banana plants by dipping the roots in a conidial suspension. The main benefit for agriculture of such endophytic associations is that it targets pests and diseases that are hidden inside plant tissues, as is the case of the banana weevil eggs and larvae. Furthermore, little inoculum would be required reducing application costs and *B. bassiana* would be protected from those environmental constraints that limit its efficacy under field conditions (Dubois *et al.*, 2006a).

Other fungal species, such as non-pathogenic *F. oxysporum* (Ascomycota: Hypocreales) have been documented to be a natural endophyte in banana plants (Griesbach, 2000). Many non-pathogenic strains of *F. oxysporum* can colonize easily the plant rhizosphere and roots without inducing disease symptoms (Dubois *et al.*, 2006a). *In vitro* screening experiments have shown that spore suspensions of *F. oxysporum* strain V2w2 caused 50.3% mortality of banana weevil eggs (Dubois *et al.* 2004b).

Banana weevils are a difficult pest to control. Several techniques have been used to reduce weevil damage, including cultural control, chemical insecticides, pheromone-baited traps and biocontrol agents. Recently research has described the potential benefit of the mycofumigation of certain endophytic fungus to control herbivores. There is limited evidence indicating that endophytes could have an influence on herbivore population dynamics. Nevertheless, Abrahamson and Weis (1997 mentioned by Elliot *et al.*, 2000), suggested that previous defences in the plant before herbivore-plant interactions will increases the reduction of herbivore attack because will have less herbivores to prevent. Therefore, more research is necessary to understand the role and ecological function of endophytes (Athman, 2006).

In this paper we studied how banana plant endophytic relationships with *B. bassiana* and *F. oxysporum* affect volatile emission and influence the behaviour of *C. sordidus* females.

Chapter 3

The response of *Cosmopolites sordidus* to volatiles from banana plants inoculated with *Beauveria bassiana* and *Fusarium oxysporum*

Olfactometer bioassays will be conducted to determine host preference by the banana weevil.

Materials and Methods

Site Description

Laboratory studies were conducted from August till October 2007 at the International Institute of Tropical Agriculture (IITA) Sendusu Farm (0°32’N, 32°35’E, 1260 m.a.s.l.). It is located 28 km northeast Kampala, Uganda. The site has two rainy seasons (March to May and September to November) with an average precipitation of 1180 mm/year. Average daily temperatures range between 16 and 29 ºC and relative humidity in the dark room range from 60 to 80%.

Dark room

The bioassays were conducted in a dark room (5 × 3 m). The windows and the door of the room were tightly sealed with black polythene sheets. Temperature in the room was between 22-28 °C. The room had a red light (Philips bulb, 40 W, 2 m from the observation surface) that was switched on during all experiments to facilitate observations without disturbing insect's behaviour (Tinzaara, 2005). Bioassays were conducted between 9:00 till 17:00 h. An electric fan (40 cm stand fan, Evernal1, 50 W) was always on during bioassays to provide aeration.

Banana plants

Tissue culture banana plants (variety Kibuzi; *Musa* spp., genome group AAA-EA) used in the experiments were produced at the International Institute of Tropical Agriculture (IITA), Sendusu Farm in Uganda. Plants were micro-propagated by shoot-tip culture. The roots were cleaned up with tap water and plants were kept inside a humidity chamber (19 - 32 ºC, 70% r.h., L12:D12 photoperiod) for 4 weeks in 300 ml cups containing 250 ml nutrient solution to enhance root development. The nutrient solution was made with Polyfeed 1 g/l (Haifa Chemicals, Haifa, Israel). It was changed weekly to ensure stable nutrient availability to the plants.

Weevil trap catches and identification

Adult banana weevils were collected from banana fields at IITA, Sendusu farm using pseudostem traps. The traps, obtained by longitudinally cutting fresh harvested banana plants, were laid near the base of a banana mat. Weevil sex was determined by using the curvature of the last abdominal segment (Roth and Willis, 1963 mentioned by Gold *et al.*, 2004) and the punctation on the rostrum (Longoria, 1968 mentioned by Gold *et al.*, 2004). Weevils were kept in the dark room for at least 15 h prior to the behavioural experiment. The reason was to enhance the mobility of the insect.

Two-choice olfactometer assay

Olfactometer

An olfactometer similar to the one employed by Lofgren *et al.* (1983) and Cordova-Yamauchi *et al.* (1998) was used (Figure 1-odour sources). The apparatus consists of a Petri dish (10 cm in diameter) with a lit. Two holes were made at the sides of the dish to fit the arms of the olfactometer. These arms were connected to the Erlenmeyer that contained the plant sources (40-50 g 1 x 1 x 1 cm pseudostem or corm fresh tissue: leaves segments: 10 x 10 cm) or clean air. A wet filter paper was placed on the bottom of the Petri dish at the start if each test. After testing 6 individuals the arms of the olfactometer as well as the odour sources were replaced to avoid asymmetry in the set up. Volatiles entered the arena by diffusion from the plant parts in the Erlenmeyer.

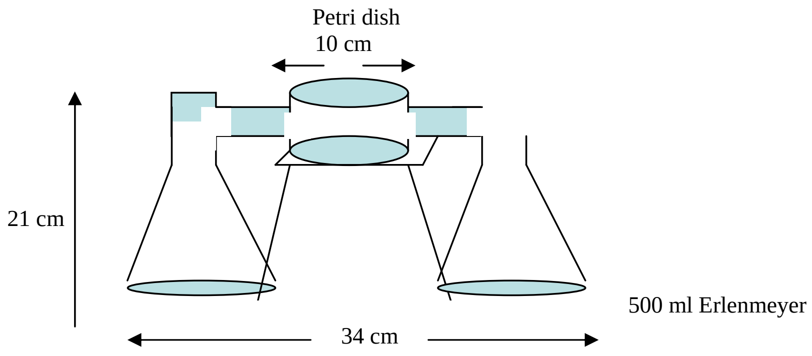

Figure 1. Two-choice olfactometer assay apparatus used for testing banana weevil response to host plants volatiles in the laboratory.

A single female weevil was used once and then discarded. Six female weevils were individually tested per experimental set with one odour source. Before each experimental set the apparatus was washed with ethanol (70%) and water. Each female was observed for 15 minutes. It was considered to have responded when it entered one arm of the olfactometer, or when at the end of 15 minutes the female was within less than 1 cm from the entry point of the arms (Fig. 1). When during 2 minutes since the start of the experiment, the weevils that did not show any movement were discarded. The first experiment was repeated during 6 days (n=30), while the rest of the experiments were repeated for 10 days (n=50).

The olfactometer used to analyze the response elicited from intact banana plants consisted of two plastic containers (10 cm length). The containers were connected by a bridge made by plastic was (10 cm length). No airflow was generated. On the top of the

Comment [m1]: WHY did you use two different olfactometers?? Because for *F. oxysporum* I used the whole plant (small plant size to take plant material) Also, Dennis will use the plants afterwards to do the olfactometer tests with the different parts (corm, pseudostems and leaves).

container a removable walking arena (3.5 cm high x 12 cm diameter) made with a plastic rim and gauze (mesh 0.6 mm) was placed (Figure 2). A wet filter paper was placed on the plastic bridge and was changed after every tested weevil. Volatiles could enter the arena by the diffusion from the plant material in the bottles. After 6 female weevils were tested, the bottles were cleaned with ethanol (70%), and the position of the bottles alternated, and the intact plants or plant parts were replaced with fresh ones.

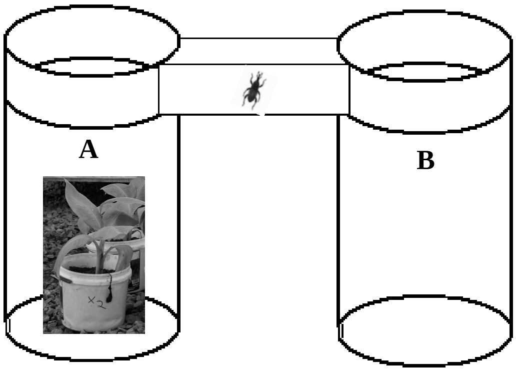

Figure 2. Two-choice olfactometer assay apparatus used for testing banana weevil response to intact banana plants volatiles in the laboratory inoculated with *Fusarium oxysporum*.

Endophyte

Fusarium oxysporum

Inoculum

Fusarium oxysporum strain V5w2 was obtained from the fungal collection at the Laboratory of Microbiology of IITA-Sendusu. The fungus was preserved at 4°C in glass test tubes (15 cm length and 13 mm diameter) containing sand-loam-sterile soil mixture (3:3:3). The fungus was growing on potato dextrose agar (PDA) media (4 g potato infusion, 20 g dextrose, and 34 g agar/l distilled water) supplemented with 30 g/l $KClO_3$ and genotyped as the nitrate-nonutilizing (nit 3) strain. Puhalla (1985) and Zamani *et al.* (2004) found that chlorate resistance in nitrate-nonutilizing 9nit fungi facilitates their selective re-isolation from mixed cultures in media with potassium chlorate (19 g SNA/l). The medium was autoclaved at 121 °C for 20 min followed by the addition of the antibiotics: 0.1 g/l penicillin G, 0.2 g /l streptomycin sulphate, 0.05 g/l chlortetracycline, and 0.25 g/l chlorophenical. Small quantities of soil containing the endophytic mycelium were sprinkled in 55 mm Petri dishes. The strain was left to grow and sporulate in the laboratory (~25°C) for one week.

In previous experiments with plant inoculation, spore density was counted (2 g of the solid substrate/ 98 ml of distilled water, and passing the suspension through a 212 um laboratory test sieve to remove big solid particles). The spore suspension was mixed and loaded on to a haemocytometer for spore counting. The spore concentration was adjusted to1.5 x 10^8 spores/g.

Plant inoculation with *F. oxysporum*

The banana plants were removed from the nutrient solution and divided for uniformity and size in two groups. The root-dipping technique was used to inoculate plants with *F.*

oxysporum (Latin and Snell, 1986; Paparu *et al*., 2006). Plants were planted in 5 l plastic pots containing steam-sterilized compost soil.

Entomopathogen

Beauveria bassiana

The Ugandan *B. bassiana* strain G41 was used for this study due to its; high virulence (92–97% mortality) against *C. sordidus*, high sporulation (4.49×10^{10} conidia/ml) and its origin of isolation from soil in banana fields (Nankinga, 1994, 1999). The strain was obtained from the Ugandan National Banana Research Programme at the Kawanda Agricultural Research Institute. The strain was stored on silica gel (21–24 °C and 55–78% R.H) in 55 mm diameter Petri dishes. Silica gel ingredients were: Sabouraud dextrose agar medium supplemented, yeast extract (SDAY) (10 g peptone, 20 g dextrose, 5 g yeast extract and 15 g agar/l distilled water) and antibiotics (0.1 g penicillin, 0.2 g streptomycin and 0.05 g chlortetracycline/l SDAY). The Petri dishes were incubated for three weeks in the laboratory (22 - 30 °C and a photoperiod of 12:12 h). Banana plants were treated with a dose of *B. bassiana* conidia of 1.5×10^{10} for 2 h (Akello *et al*., 2007)

Inoculum

The fungal strain used was *B. bassiana* G41, obtained from the Kawanda Agricultural Research Institute of the National Agricultural Research Organization (NARO), Kawanda, Uganda. The fungus isolated from soil samples of banana plantations, were stored on silica gel at 21-24°C. This strain was selected based on its high plant colonization rate, sporulation ability, and pathogenicity against the banana weevil, *C. sordidus* (Nankinga, 1999; Akello *et al*., 2007). The fungus was revitalized by sprinkling silica gel containing spores and mycelia on Sabouraud dextrose agar medium supplemented with yeast extract (SDAY) (200 g glucose, 20 g/l peptone, 5 g/l yeast extract and 15 g/l agar) and antibiotics (0.1 g/l penicillin, 0.2 g/l streptomycin and 0.05 g/l chlortetracycline SDAY) in Petri dishes of 55 mm diameter. The spores and mycelia were incubated in the laboratory (~25°C and a natural photoperiod of ~12:12 h L: D) for 21 days.

Inoculum preparation

Conidia obtained from the stock were inoculated on SDAY in 90 mm diameter Petri dishes and then incubated in the laboratory for 21 days. Petri dishes were subsequently air dried in a laminar air flow cabinet for 24 h. Conidia were harvested by scraping them off the surface of the dried medium and sieving them (sieve aperture of 150 µm). They were collected on sterile aluminium foil. The harvested conidia were dissolved in 500 ml sterile 0.01% Tween-80 in a sterile 180 × 70 mm bottle. After vortexing, serial dilutions of × 0.1 and × 0.01 were made and conidial density determined using an improved Neubauer haemocytometer. Conidial density was adjusted to 1.5×10^{10} conidia/ml in 300 ml (Akello *et al*., 2007)

Plant inoculation

B. bassiana was produced in the laboratory and the conidial density standardized to 1.5×10^{10} conidia/ml in 300 ml sterile 0.01% Tween-80. Plants were removed from the nutrient solution and randomly assigned to the various treatments. Plants were dipped in a 300 ml

B. bassiana suspension of 1.5 × 10^{10} conidia/ml density for 2 h. Plants for the control treatments were dipped in 300 ml 0.01% sterile Tween-80 for the same period of time. The plants were planted in 2 l plastic pots containing sterilized soil. The soil was sieved, poured on a metallic tray and covered with a black polythene sheet. The tray containing soil was connected to a water steamer and the soil steam-sterilized for 1 h. The plants were kept in a screenhouse for three months at 17-35°C and watered daily.

Two-choice olfactometer assays

Female weevils' response to banana tissue and *B. bassiana* and their interaction was tested using the following comparisons:

1- **Clean air vs Pseudostem**
2- **Clean air vs Pseudostem + *B. bassiana***
3- **Clean air vs Corm**
4- **Clean air vs Corm + *B. bassiana***
5- **Clean air vs Leaf**
6- **Clean air vs Leaf + *B. bassiana***
7- **Pseudostem vs Pseudostems + *B. bassiana***
8- **Leaf vs Leaf + *B. bassiana***
9- **Corm vs Corm + *B. bassiana***
10- **Pseudostem vs Corm**
11- **Pseudostem + *B. bassiana* vs Corm + *B. bassiana***

Female weevils' response to banana intact plants and the non-pathogenic *F. oxysporum* and their interaction was tested using the following comparisons:

1- **Clean air vs Control plant**
2- **Clean air vs Plant + *F oxysporum***
3- **Control plant vs Plant + *F. oxysporum***

Statistical analysis

In all the experiments the data of weevils' preference to the odour sources for one of the stimuli tested during olfactometer bioassays were analysed using a $\chi 2$-test (distribution of expected values 50:50).

Results

Testing the equilibrium of the olfactometer

Table 1. Number of weevils responding to clean air and banana corm odour sources in a two choice olfactometer test in the laboratory in Sendusu, Uganda.

Comparison odour sources (A/B)	***C. sordidus* ♀♀**		
	A	**B**	**No response**
Clean air/ Clean air	10	9	11
Corm/ Corm	8	12	10

A total of 30 weevils were tested per comparison test set. The response of the herbivores to the two odour sources did not show a significant difference (P > 0.05 $\chi 2$ – test).

Cosmopolites sordidus females responded equally to clean air vs clean air and to corm tissue vs corm tissue (P > 0.05). The data indicated that the equipment did not show asymmetrical aspects in the responses evaluated of female weevils to banana corm tissue and clean air (Table 1).

Cosmopolites sordidus response to volatile cues from banana corm, pseudostem and leaf tissue of plants treated with *Beauveria bassiana.*

In two-choice olfactometer bioassays the female weevils showed clear preferences for odours from control plants over clean air (Fig. 1). Significantly more weevils moved to the side of the apparatus with the control plant material (corm; pseudostem and leaf tissue) than to the one with clean air ($P = 0.001$, $P < 0.001$ and $P < 0.01$ respectively). On the other hand, weevils chose the side of the olfactometer with clean air when corm and pseudostem tissue from banana plants treated with *B. bassiana* were offered at the other side ($P < 0.05$ and $P = 0.01$ respectively). Meanwhile, no significant effect was obtained when leaf tissue from banana plants treated with *B. bassiana* were offered over clean air ($P > 0.05$) (Fig. 1).

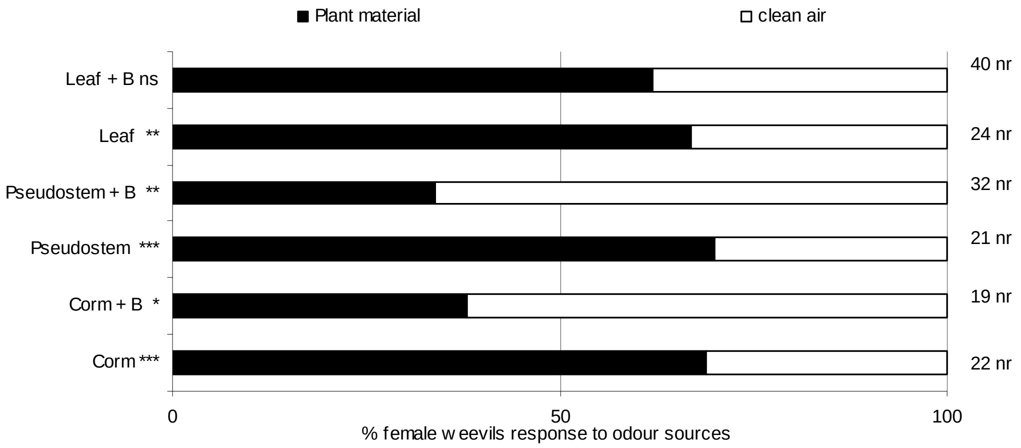

Figure 1. Response of female banana weevil *C. sordidus* to volatiles from banana corm; pseudostem and leaf tissue from plants treated with and without *B. bassiana* (+B) versus clean air in an olfactometer test. The percentage of responding individuals of each treatment per comparison set, choosing one odour source or the other is given. Number of tested females, n = 100. Asterisks indicate significant differences between test and control within the same treatment. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant, $\chi2$ – test, and nr = number of no respond weevils.

No significant differences were obtained when weevils had to choose between banana leaves with *B. bassiana* and leaves without the fungus. In contrast, significantly more female weevils showed a strong preference to the side of the olfactometer with corm or pseudostem tissue from control plants than to the side with corm or pseudostem tissue from plants treated with *B. bassiana* ($P = 0.03$ and $P = 0.04$ respectively) (Fig. 2).

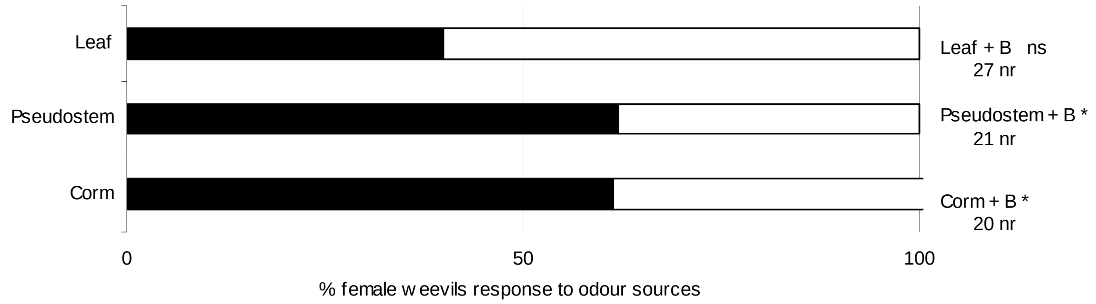

Figure 2. Response of the banana weevils females *C. sordidus* to volatiles from banana corm; pseudostem and leaf tissue from plants treated with *B. bassiana* (+B) versus control corresponding plant material in an olfactometer test. The percentage of responding individuals of each treatment per comparison set, choosing one odour source or the other is given. Number of tested female, n = 100. Asterisks indicate significant differences between test and control within the same treatment. * $P \leq 0.05$, ns = not significant, $\chi2$ – test and nr = number of no respond weevils.

The presence or absence of *B. bassiana* in the plants did not make any significant differences in the inclination of the behaviour of the weevil when corms against pseudostems from banana plants with and without *B. bassiana* were offered. ($P = 0.5$ and $P = 0.2$, respectively) (Fig. 3).

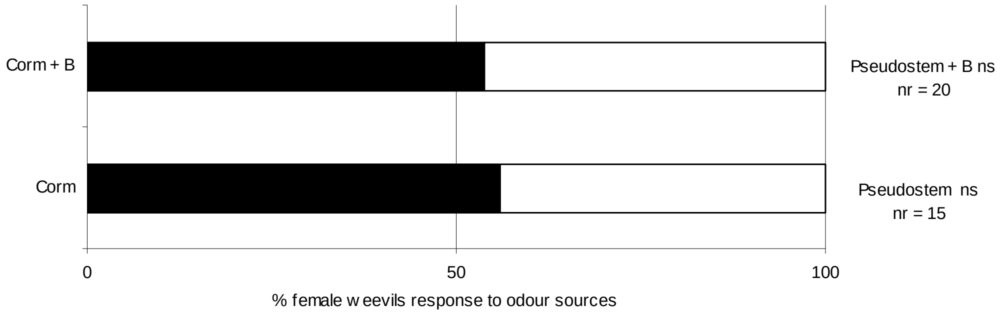

Figure 3. Response of female banana weevils (*C. sordidus)* to volatiles from banana corm or pseudostem tissue from plants with or without *B. bassiana* (+B) in an olfactometer test. The percentage of responding individuals of each treatment per comparison set, choosing one odour source or the other is given. Number of tested female, n = 100, Asterisks indicate significant differences between test and control within the same treatment, ns = not significant differences, $\chi 2$ – test and nr = number of no respond weevils.

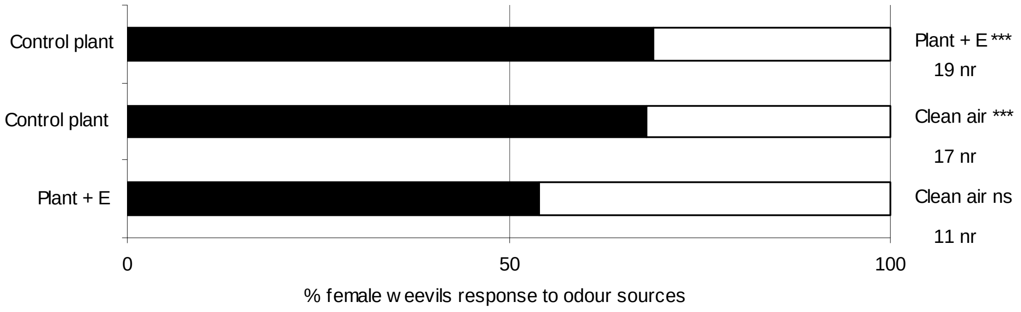

Figure 4. Response of female banana weevil *C. sordidus* to volatiles from banana intact plants treated with or without *F. oxysporum* (+E) in an olfactometer test. The percentage of responding individuals of each treatment per comparison set, choosing one odour source of the other is given. Number of tested female, n = 100. Asterisks indicate significant differences between test and control within the same treatment * $P \leq 0.05$, ** $P \leq 0.010$ *** $P \leq 0.001$, ns = not significant differences, $\chi 2$ – test, and nr = number of no respond weevils.

The inoculation of banana plants with *F. oxysporum* had no effect on weevils' choice in the olfactometer test over clean air ($P > 0.05$). On the other hand, more weevils moved to the side with control plants over clean air (*P* = 0.001). In addition, significantly more female weevils chose the side of the olfactometer with control plants than the side with plants inoculated with *F. oxysporum* when they were offered simultaneously (*P* = 0.001) (Fig. 4).

Discussion

This study showed that female weevils are attracted to volatiles emitted by the host banana plant. A previous study mentioned that weevils are regularly more attracted to pseudostem than to corm material (Sumani, 1997). On the other hand, it has been stated that weevils are more attracted to corm than pseudostems (Masanza, 2003). However, in this study female weevils did not show a significant preference between corm or pseudostem selection (Fig. 3). Conversely, when *C. sordidus* were confronted with plant material (corm and pseudostem) treated with *B. bassiana* they preferred the clean air. Nevertheless, for leave tissue the preference was not significant. However, the highest number of weevils without response was in this set with 40 insects out of 100 individuals and for the set of pseudostem + *B. bassiana* vs clean air 32 insects did not respond. The results of these sets of tests were significantly different from the respective controls (24 and 21 no responded weevils out of 100 respectively; Fig. 1). This give a perception of the disorientation this insects may have when are faced by the volatiles emitted by plants treated with *B. bassiana*.

Our results suggest that *B. bassiana* induce changes in the banana plants at the volatile emission level. These emissions could reduce weevils' acceptance as we can see in Fig. 1, where weevils showed a high preference for control plant material and clean air when infected material was offered. Many foliar endophytic fungi have a deterrent effect on herbivores feeding (Vicari *et al*., 2002). Some weevils have shown sensitivity to endophytes, like Argentine stem weevil (*Listronotus bonariensis* Kuschel) the major pest of *Lolium perenne* L. pastures in New Zealand is sensitive to *Neotyphodium lolii* an endophyte found in *L. perenne* (Christensen *et al*., 1993). A study carried out by Gerard

(2000) showed that weevils spend less time and feeding on endophytic pants. This can guide our results to the fact that weevils can detect an abnormal smell in those plants infected with *B. bassiana* and base on that information they make the decision to avoid them.

Plant infection with endophytes may alter herbivore populations and could interact at higher trophic levels (Omacini *et al.*, 2001). Omacini *et al*. (2004), studied the effect of *Lolium multiflorum* (Italian rye-grass) grown from *Neotyphodium* infected or endophyte-free seeds on aphids. They found that aphid herbivores density was three times higher on endophyte-free grass monoculture than on endophyte infected plots.

Once weevils were allowed to choose between intact control banana plants and plants inoculated with the endophyte *F. oxysporum,* they showed a preference for the control. Although, when it was offered a plant with endophyte against clean air, weevils choose the plant (Fig. 4). Weevils may be less sensible to *F. oxysporum* than to *B. bassiana*. They were capable to differentiate between control vs infected plants, but when infected plant was offered against clean air they went for the plant. This is a different interaction between plants and endophytes; different induction of volatiles can be activated according to the endophyte.

Recently research has described the potential benefit of the mycofumigation of certain endophytic fungus. Some endophytes are related with induction of a blend of volatiles. For instance, Strobel (2006) found a novel fungal genus *Muscodor albus* that emits a mixture of volatile compounds that vary in quality and quantity and can be lethal to a wide range of plant pathogenic fungi and bacteria and also has been effective against nematodes and insects. This sort of analysis can be done in banana plants carrying *F.*

oxysporum and *B. bassiana* in order to have a better picture about what is going on in the system and its potential as a strategy to decrease or reject the attraction of the banana weevil.

Use of the pathogens *B. bassiana* and *F. oxysporum* in banana plantation in East Africa to control *C. sordidus* seem to be a biological novelty in agriculture. These biological control agents may affect the volatiles released by the banana plant, which seem to decrease weevils host attraction.

Conclusions

In conclusion our results suggest that the response of the banana weevils differs from the interaction of the banana plant with endophytes. Weevils are capable of discriminate between infected and control banana tissue. Nevertheless, more research should be conducted to study the effect of integrating *B. bassiana* and *F. oxysporum* in banana plants and the efficiency of this technique under field conditions on weevil population and damage. Theoretically, if the strategy is found promising the data will be used in exploiting opportunities for the integration of *B. bassiana* and *F. oxysporum* into a broader IPM programme to control banana weevils.

Deeper information and further studies are needed to understand the exact mechanism and the chemistry of the emission of volatiles in banana plants and how the change by the inoculation with endophytes. In addition, more studies are needed to evaluate fungus colonization in the different organs of the plant such as leaves, pseudostem and corm. Investigations have to focus in long term rigorous studies, with multiple and accurate diagnostic methods to provide credible conclusions.

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