BIOLOGICAL CONTROL OF THE TWO-SPOTTED SPIDER MITE, *Tetranychus urticae* Koch (ACARI: TETRANYCHIDAE)

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BIOLOGICAL CONTROL OF THE TWO-SPOTTED SPIDER MITE, *Tetranychus urticae* Koch (ACARI: TETRANYCHIDAE)

by

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Dedicated to my wife Jane, my sons Arsene and Bruno, and my daughters Cindy and Dora

Abstract

The two-spotted spider mite (TSM), *Tetranychus urticae* Koch, is an important pest of many greenhouse and field crops worldwide. The development of resistance in TSM populations to chemical acaricides, allied with public health concerns about pesticide residues, has motivated the search for alternative control measures to suppress the pest. Hyphomycetous fungi are promising agents for mite control and the fungus *Beauveria bassiana* (*Bb*) (Balsamo) Vuillemin was investigated in this study as a biocontrol agent. The principal objectives of this study comprised: a) screening *Bb* strains for their pathogenicity against *T. urticae;* b) testing the effect of adjuvants on the efficacy of *Bb;* c) studying the effect of plant type on persistence of *Bb* and the efficacy of control of *Bb* against *T. urticae;* d) evaluating the field efficacy of *Bb* applications against *T. urticae;* e) testing the compatibility of *Bb* with selected fungicides; and f) assessing the synergy between *Bb* and soluble silicon for *T. urticae* control.

Screening bioassays of sixty-two strains of *Bb* identified the two most effective strains, PPRI 7315 (R289) and PPRI 7861 (R444), that caused mortality levels of more than 80% of adult mites at 9 d post-inoculation with 2×10^8 conidia ml^{-1}. These strains performed significantly better than the *Bb* commercial strain PPRI 5339, in laboratory bioassays. The two strains also attacked mite eggs, causing 53.4% and 55.5% reduction in egg hatchability at 2×10^8 conidia ml^{-1} respectively. However, PPRI 7861 showed relatively higher production of conidia in culture and was, therefore, selected for further trials under greenhouse and field conditions.

Greenhouse evaluations of the effects of two adjuvants (Break-thru® and a paraffin oil-based emulsion) on efficacy of *Bb* demonstrated a higher efficacy of the biocontrol agent (BCA) when it was applied with Break-thru® or the oil solution than with water alone. Moreover, *Bb* conidia applied in Break-thru® solution resulted in greater control of TSM than conidia applied in the mineral oil. There was also a dose-response effect and the control of TSM by *Bb* increased when the concentration of conidia was increased.

The control of TSM by *Bb* in beans (*Phaseolus vulgaris* L), cucumber (*Cucumis sativus* L.), eggplant (*Solanum melongena* L.), maize (*Zea mays* L.) and tomato (*Solanum lycopersicum* L.) was tested in greenhouse trials. On these crops, the persistence of conidia declined over time. The rate of decline was significantly higher on maize. However, TSM mortality was positively correlated with the amount of conidia deposited on leaves immediately after spraying, rather than their persistence over time. Higher levels of mortality of TSM due to *Bb* application were observed on beans, cucumber and eggplants, suggesting that the type of crop must be taken into consideration when Bb is applied as a BCA.

Field efficacy of *Bb* against mites was evaluated in two trials on eggplants. Based on assessment of population densities of mites and leaf damage assessments; both trials showed that the strain PPRI 7861 controlled TSM in the field. Two commonly used fungicides, azoxystrobin and flutriafol, were investigated *in vitro* tests on culture medium and laboratory bioassays on detached bean leaves (*Phaseolus vulgaris* L.) for their effects on *Bb*. Azoxystrobin (a strobilurin) was less harmful to *Bb* while flutriafol was found to be inhibitory.

Another important finding of this study was the substantial enhancement of *Bb* efficacy by soluble silicon. When *Bb* was combined with soluble Si, the control of TSM was better than when either of the two products was applied alone. Moreover, application of soluble Si as a plant fertilizer in hydroponic water nutrient increased accumulation of peroxidase, polyphenoloxidase and phenylalanine ammonia-lyase enzymes in leaves of plants infested with TSM. Increased activity of these defense enzymes in leaves deters feeding behaviour of mites. We suggested that feeding stress renders them susceptible to *Bb* infection, which would explain the synergy observed between the two agents.

Declaration

I, **Gatarayiha** declare that:

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Table of contents

List of tables

Chapter 1

Chapter 2

Chapter 5

Chapter 6

Chapter 7

Chapter 8

List of figures

Chapter 2

Chapter 3

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Introduction

The two-spotted spider mite (TSM), *Tetranychus urticae* (Koch), belongs to the family Tetranychidae. It is perhaps this species, which is the most economically important pest of the family affecting many field and greenhouse crops (Meyer, 1996). The increase in the pest status of this mite can be linked to to the rise in large-scale use of chemical insecticides against insect pests (Gerson & Cohen, 1989) which reduced natural enemies, therby causing a decline in predation pressure, which allowed the TSM numbers to increase.

Chemical pesticides have mostly been relied upon to control TSM populations; however, their continuous use has also resulted in pesticide resistance among TSM populations. The factors contributing to this are its great egg-laying potential, very short life cycle allowing numerous generations in a growing season, a high mutation rate (Ramasubramanian *et al.,* 2005). Besides the problem of pest resistance, there is also concern about the high residues and toxicity of common miticides to humans.

Concerns about the use of agrochemical control measures have led to search for alternative control measures to suppress TSM populations, including the use of biological control, particularly by applying predatory phytoseiid mites (McMurtry & Croft, 1997). Nonetheless, the control provided by predatory mites is often insufficient and supplementary sprays with selective chemical acaricides have been required (Jacobson *et al*., 1999). The use of microbial control agents as part of an integrated pest management (IPM) strategy is likely to reduce the reliance on these chemical acaricides. Fungi of the genera *Neozygites* (Zygomycetes: Entomophthorales) are specific pathogens of TSM under natural conditions (Chandler *et al.*, 2000). However, artificial production of this fungus is reported to be difficult and therefore its application as an inundative biological pesticide is so far impracticable (de Moraes & Tamai, 1999).

Members of the mitosporic (Hyphomycetes) entomopathogens are also promising microbial control agents against acari (Chandler *et al*., 2000). These fungi invade the host by growing through the external cuticle, which is important for pests with sucking moutnparts such as TSM, which are less likely to acquire pathogens *per os*; they can be mass produced using low input technology (Chandler *et al.*, 2000); they can be formulated as myco-pesticides suitable for spraying using conventional chemical spraying equipment (Bateman, 1996); and are less harmful to non-target arthropods and mammals and are therefore ideal for integrated pest management (IPM) program strategies.

Unfortunately, there are very few research reports on successful utilization of these fungi against TSM, most having been developed and registered for use against insect pests. The most difficult problem facing TSM control with entomopathogenic fungi is that this mite generally occurs in dry and hot environments, which are unfavourable for the development of the entomopathogenic fungi. A second problem is that entomopathogenic fungi take some time to kill their hosts. Because of the short generation time of TSM and its high fecundity rate, this time delay in mycosis development may impede the short term efficacy of the entomopathogenic fungi. Hence for their successful utilisation as myco-pesticides, other strategies such as high virulence and speed in killing, survival under a challenging environment, and compatibility with other control agents (Lacey *et al*., 2001), are required.

Taking these strategies into account, the present thesis reports the work devoted to investigating the use of the Hyphomycetes *Beauveria bassiana* (*Bb*) (Balsamo) Vuillemin as a biological control agent (BCA) against TSM. Several strains were screened in laboratory and greenhouse experiments for their virulence against the pest. Results of these experiments are presented in Chapter 2. Oil enhancers are known to protect fungal biocontrol agents from harmful environmental conditions and to enhance their activity at the target site; this was addressed in Chapter 3 by comparing two adjuvants (Break-thru® and an oil emulsion) for their effects on *Bb* efficacy. Chapter 4 presents results of greenhouse experiments investigating the influence of crop type on persistence and control efficacy of *Bb*. Chapter 5 reports on field efficacy of *Bb* against TSM. Chapter 6 deals with the compatibility of *Bb* with two chemical fungicides (flutriafol and azoxystrobin)

commonly used in South Africa on various crops against fungal diseases. Chapters 7 and 8 report on enhancement of control efficacy of *Bb* by the element silicon and the possible mechanism involved.

This thesis consists of eight chapters; Chapter 1 reviews available literature on biocontrol of TSM with special reference to *Bb*. Finally there is an Overview of the thesis to review the research undertaken and to suggest future research activities in this field. The research chapters, Chapters 2 to 8, are discrete and self contained chapters, each written in the format of a scientific paper for publication. This is the format of PhD and MSc theses adopted at the University of KwaZulu-Natal as standard thesis format, as opposed to a monograph.

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Chapter 1

Review of literature

1.1 Two-spotted spider mite: pest status and general control

1.1.1 Taxonomical aspect of the pest

Two-spotted spider mite (TSM), *Tetranychus urticae* Koch, 1836, belongs to the group of acarines known as Acariformes, in the suborder Prostigmata, and the family Tetranychidae (Meyer & Ryke, 1959). This pest has been beset by problems of nomenclature and considerable confusion has been caused by changes in its specific name. It was first known as *Tetranychus telarius* Linneaus, 1758 (Meyer & Ryke, 1959). The same authors noted that the name *T. bimaculatus* Linneaus was commonly used in South Africa. Some of the scientific names were based on the colour of the different forms in which the pest may appear (Boudreaux, 1956). The two-spotted spider mite occurs in two forms: the carmine and the green form, formerly known under the names *T. cinnabarinus* Boisduval (=*T. bimaculatus* L.) and *T. urticae* (=*T. telarius*) respectively (Boudreaux, 1956; Meyer, 1996). Both carmine form and green form occur in South Africa (Meyer, 1996). The morphological distinction between these forms has been the subject of many studies and discussions. However, after many hybridization experiments by various acarologists, *T. cinnabarinus* has been used synonymously with *T. urticae* (Dupont, 1979; Annecke & Moran, 1982; Meyer, 1996). In the present study, the carmine form was used and was referred to as *T. urticae*.

1.1.2 Biology and behaviour

The life cycles of the more economically important tetranychid mites have been studied by several authors. Some of those referring to *T. urticae* are Boudreaux (1963), Coates (1974),

Hebert (1981), Helle & Sabelis (1985) and Meyer (1981). Two-spotted spider mite passes through five developmental stages during its life cycle: egg, larva, protonymph, deutonymph, and adult. Each active immature stage is followed by a quiescent period as protochrysalis, deutochrysalis and teliochrysalis respectively. Eggs are round and translucent, turn orange and larvae hatch in about 5 d under optimum conditions of 25-30°C and 45-55% RH. One TSM generation is completed in 10-14 d when the temperature is between 21ºC and 23ºC (Meyer, 1981). However, when temperatures are higher (30ºC), development time from egg to adulthood can be reduced to 7 d (Hebert, 1981).

The TSM exhibits arrhenotokous parthenogenesis (Boudreaux, 1963). Fertilized eggs will result in female offspring, whereas unfertilized eggs will produce male offspring. Males typically complete the last quiescent stage before adulthood earlier than females (Mitchell, 1973). It was thought that the male underwent only one nymphal stage (Zattler, 1951 and Lochner, 1951 quoted by Coates, 1974). This was corrected by Coates (1974) who found that the male underwent the same number of developmental stages as the females but that the time spent in each stage was slightly shorter for males. Coates (1974) noted that this had also been found by Anderson (1948) and Grasser (1948). It was thought that a 1:1 sex-ratio existed in tetranychid mites (Davis, 1961), but many studies subsequently showed that in natural populations, females outnumbered the males and that the sex-ratio varied periodically because of arrhenotokous reproduction (Boudreaux, 1963; Coates, 1974). Seasonal variation may be due to the fact that females are able to "balloon" and migrate on wind currents when the environment becomes unfavorable, but the males are unable to do so (Coates, 1974).

Instead of feeding, the males actively search out female deutonymphs and await their emergence from the quiescent period into adulthood (Cone *et al.,* 1971). Just before the female emerges, the male stays in close contact with the female, often touching her (Cone *et al.,* 1971; Potter *et al.,* 1976; Collins *et al.,* 1993). When the exoskeleton splits open, the male will often assist the female in freeing herself from the exuvium. Mating will sometimes take place as soon as the anterior portion of the exoskeleton is released (Cone *et al.,* 1971). Copulation can last from a few seconds to several minutes (Potter *et al.,* 1976).

When a female mates with more than one male, sperm precedence is given to the first male (Potter *et al*., 1976). Females generally lay an average of 38 eggs in total, but it is possible for a single female to lay over one hundred eggs during the oviposition period (Meyer, 1981). For example, it was found on rose plants (cv. Sonia) that a young fertilized female TSM could lay an average of 112 eggs during her whole lifespan (Van de Vrie, 1985). Higher numbers of eggs generally occur when relative humidity (RH) is low (25-30%) (Crooker, 1985). The life span of the adult female is divided into the preovipositional period and the ovipositional period, the former being the time between emergence from the teliochrysalis to the deposition of the first egg.

The preovipositional period can last less than 0.5 d and as long as 3 d depending on temperature. The period during which eggs are deposited (ovipositional period) can last from 10 d at 35°C to 40 d at 15°C (Sabelis, 1981) and from 5 d of adulthood onwards, the female TSM lays up to 10 eggs a day (Wrensh, 1985).

While on uninjured plants, TSM individuals are uniformly distributed over the leaf surfaces. When the plant begins to decline, resulting in a reduced food supply, the mites will enter a dispersal phase and aggregate on the uppermost parts of the plants (Hussey & Parr, 1963a). Mites in the dispersal phase show a greater directional response to light than in the sedentary phase (Hussey & Parr, 1963a). The declining condition of the plant partially triggers the change from the sedentary phase to the dispersal phase (Kennedy & Smitley, 1985). Dispersal is the movement away from the colony in which TSM developed. It includes both intraplant and interplant movement.

Interplant dispersal occurs under at least three separate conditions (Coates, 1974). The first is the gradual expansion of a mite population to completely occupy the habitat available to it. The second type of dispersal occurs under conditions of overcrowding. This phenomenon, also known as “swarming”, results from a large number of mites and a limited supply of food. Coates (1974) observed that, in a field of peas in which the population of *T. cinnabarinus* had almost completely destroyed the crop, the mites were so numerous that they hung from the apices of the dry leaves and pods in bunches of several

individuals. Crawling is a common means of dispersal across the host plant; however, it can also be an effective means of interplant dispersal (Coates, 1974). The mites will often climb over the intertwined foliage of adjacent plants. Where plants do not overlap, it seems possible for tetranychids to crawling on the ground, at least for limited distances. There still exists much speculation about crawling. Coates (1974) reported that *T. cinnabarinus* could travel over soil from plant to plant at about 0.3 m per hour. In contrast Boudreaux (1963) is skeptical of this ability and has observed a *T. ludeni* Zacher female on a smooth sandy soil at least 1 m from the nearest plant and judged from her activity, that a journey of another 1 m was not feasible.

Aerial dispersal begins with the mites aggregating on the uppermost portions of the plants (Hussey & Parr, 1963a). The mites produce threads of silk, which they use to "balloon" into the wind, which sometimes carry them great distances (Hussey & Parr, 1963a; Kennedy & Smitley, 1985). Fleschner *et al.* (1956) were however defiant that although a number of infestations were observed, in no instance was *T. telarius* observed to spin down or drift away from leaves. Ewing (1914), quoted by Coates (1974), confirmed that tetranychid mites could be carried long distances by wind but did not know whether they were supported by silken threads in the wind. With his experiments, Coates (1974) confirmed that *T. cinnabarinus* was able to disperse by "ballooning".

The third common method of dispersal is when mites move by 'hitchhiking' on other organisms (Weeks *et al.,* 2000). This form of accidental dispersal can occur at any stage of population growth.

The pest maximizes fitness in several ways. It is reported that reproductive rates of females increase greatly when the host plant begins to decline (Huffaker *et al.*, 1969). Since mating of females usually occurs just after emergence of the quiescent deutonymph stage, most are mated before they disperse, which increases their probability of founding new colonies. When a dispersing female reaches a new plant, she immediately begins to feed close to a leaf vein and produce webbing (Thomas & Walker, 1989).

Eggs are deposited beneath the webbing and larvae and nymphs develop within it. The webbing basically defines the colony boundaries, and as the colony grows, the webbing also expands (Brandenburg & Kennedy, 1987). In addition to providing the boundaries of the colony, the webbing also serves as a means of protection from rain, wind, and predators (Gerson, 1985; Morimoto *et al*., 2006). If the webbing is dense enough, protection may also be provided from acaricide sprays (Meyer, 1996). It is thought that the webbing and deposition of feacal pellets within the webbing is a mechanism to regulate humidity (Gerson, 1985).

Diapausing females or eggs are the most common overwintering stage for tetranychids (Veerman, 1977). In warm areas during the winter, some mites may continue to reproduce and diapause is facultative (Veerman, 1977; 1985). Diapause is often initiated in response to short day lengths and cooling temperatures (Mitchell, 1973). During diapause, TSM do not feed or oviposit, and they generally seek shelter in crevices in the bark of trees and shrubs, clods of dirt, and in leaf litter (Huffaker *et al.*, 1969). Diapause may be terminated by longer day lengths and warming temperatures (Veerman, 1985).

Host plant species, cultivar, or phenological stage can affect TSM developmental rate, survival, reproduction and longevity (East *et al.,* 1992). There is evidence that the nitrogen-phosphorus-potassium ratio can influence TSM female weight, preoviposition period and oviposition rate (Brandenburg & Kennedy, 1987). These authors also reported that high levels of nitrogen improved host quality, which resulted in higher female weight, shorter preovipositional period, and a high oviposition rate.

1.1.3 Damage caused by *Tetranychus urticae*

Two-spotted spider mite is an important economic pest of many host plants including, but not limited to, tomatoes, cucumbers, beans, roses, cotton, maize, soybean, strawberries and many orchard crops and ornamental plants (Simmonds, 1972; Hamlen & Lindquist, 1981; Sances *et al.,* 1981; Annecke & Moran, 1982; Riba & Silvy, 1989; Meyer, 1996; Meyer & Honnibal, 1998; Park & Lee, 2002; Opit *et al.*, 2004). The two-spotted spider mite feeds on

the underside of leaves by piercing the epidermal tissue of the host plant and from cells of the palisade layer and the spongy mesophyll and sucking the contents (Park & Lee, 2002). This results in typical "stippling" damage, with white- or grayish-coloured spots due to the punctures made by feeding (Park & Lee, 2002). Mites insert their stylets into the plant cells and suck out the cell contents. Feeding can damage protective leaf surfaces, stomata, and the palisade layer (Sances *et al.,* 1979). They may also damage the lowest parenchymal layer (Park & Lee, 2002). The degree of leaf damage by *T. urticae* is a function of its stylet length and leaf thickness (Park & Lee, 2002). The stylet length of *T. urticae* is typically 132±27 µm (Sances *et al.,* 1979) and can vary from 103 µm (larvae) to 157 µm (adult females) depending on developmental stage (Avey & Briggs, 1968).

Defoliation, leaf bronzing, and even plant death occur due to direct feeding damage in severe infestation (Meyer, 1996; Meyer & Craemer, 1999). Indirect effects of feeding may include decreases in photosynthesis and transpiration (Park & Leee, 2002). This combination of direct and indirect effects often reduces the amount of harvestable material (Hussey & Parr, 1963b; Sances *et al.,* 1981). It has been reported that in natural, relatively undisturbed habitats, the pest causes minimal damage to plants as it is in a fluctuating equilibrium with its own natural enemies (Huffaker & Flaherty, 1966).

1.1.4 Control of *Tetranychus urticae*

1.1.4.1 Chemical control

Several pesticides (acaricides) are listed for the control of TSM on different crops (Meyer, 1996; Nel *et al.,* 1999). Some synthetic pyrethroids (SPs), although known as broad-spectrum insecticides, are also effective for spider mite control (Iftner & Hall, 1983; Mckee & Knowles, 1984; Gerson & Cohen, 1989). While these pesticides are effective against mobile forms of TSM, little is known about their ovicidal properties (Jacobson *et al.,* 1999).

Resistance development in TSM due to intensive use of chemical pesticides has been discussed by many researchers (Cranham & Helle, 1985; Young-Joon *et al.,* 1993). For example, the pest has developed resistance to fenbutatin oxide (Jacobson *et al.,* 1999) and to organophosphates (Stumpf *et al.*, 2001). The factors contributing to this are its great egg-laying potential, a very short life cycle allowing numerous generations in a growing season and a high mutation rate (Saito *et al.*, 1983; Meyer & Craemer, 1999; Stumpf *et al*., 2001). Resistance of TSM to acaricides was first observed by Compton and Kearns in 1937 when it developed resistance to ammonium potassium selenosulfide (Selecide®) (Saito *et al.,* 1983). To date the pest is also reported to be resistant to dicofol (Dagli & Tunc, 2001), fenbutatin oxide (Jacobson *et al.*, 1999) and abamectin **(**James & Price, 2000**).** For example, the pest developed up to 100-fold resistance to dicofol (Dagli & Tunc, 2001). Campos *et al*. (1996) examined TSM strains from nurseries in California, Florida, the Canary Islands and Holland and found abamectin resistance levels ranging from 0.5-175 fold. Since the pest could develop resistance to an acaricide after it had been used for several consecutive seasons, the use of combination and/or alternation of acaricides with different chemical compositions was recommended (Meyer, 1996).

1.1.4.2 Effect of pesticide application, with special reference to synthetic pyrethroids, on natural enemies

Huffaker & Flaherty (1966) presented three statements:

1. Plant-feeding mites in natural areas untreated with chemicals or influenced by man, dust, etc., seldom extensively damage their hosts and are often so scarce that they are difficult to collect;

2. Before introduction of DDT and other synthetic materials, these mites, though injurious at times, were not as perennially severe as they became after extensive use of these materials;

3. The overriding common denominator is that in natural situations, the natural enemies of these mites were effective as control agents.

From these statements, various hypotheses have been raised to explain the general upsurge in mite outbreaks with agricultural intensification in recent years. The one strongly

supported theory is the detrimental effects of pesticides. Many records showed that for the most part, the outbreaks followed the use of new broad-spectrum pesticides, first DDT and then others (Huffaker & Flaherty, 1966; Bartlett, 1968). Some of the possible explanations for association between these outbreaks of previously minor pests and the use of synthetic materials are the destruction of natural enemies or stimulation of mite reproduction. Bartlett (1966) tested 59 compounds on TSM and *Aphis gossypii* in comparing pest stimulation and with natural enemy destruction. He found a few cases, which were probably due to pest stimulation, but most were however due to destruction of natural enemies.

Rield & Hoying (1980) reported population levels of 94 mites (TSM) per 10 pear leaves treated with fenvalerate 7 wk post-treatment compared with 0.2 mites per 10 leaves on untreated controls. Resurgence following pyrethroid applications is reported also for other phytophagous mites. Plaut and Mansour (1981) found that an apple-infesting *Panonychus ulmi* (Koch) population treated with cypermethrin, deltamethrin, and permethrin increased 30-50 times over control populatons within four weeks. Hoy *et al.* (1979) noted that populations of the pacific spider mite (*Tetranychus pacificus* McGregor) and Willamette mite, *Eotetranychus willamettei* (McGregor), on grapes increased within 17 d following permethrin applications.

The detrimental effect of synthetic pyrethroids (SP) on natural enemies is well documented and has frequently been regarded as the main cause of spider mite resurgence (Rock, 1979; Hull & Starner, 1983). Mair *et al.* (1984) (in Gerson & Cohen, 1989) studied the effects of various SPs on *P. ulmi* outbreaks and attributed them to the greater SP-associated mortality of predators. Predatory mites of the family Phytoseiidae, considered to be of prime importance in the natural control of spider mites including TSM, were shown in laboratory studies to be more susceptible to SPs than their prey (Rock, 1979). Other predators of spider mites that are adversely affected by SPs include coccinellid beetles of the genus *Stethorus* (Hull *et al.,* 1985) and the stigmaeid mite *Zetzellia mali* (Ewing) (Gerson & Cohen, 1989).

The effect of SPs is also reported on entomopathogenic fungi and this raises the possibility that SPs might adversely affect fungi, which attack spider mites, thereby freeing them from another natural enemy. For example, Anderson & Roberts (1983) found that SPs inhibited *Beauveria bassiana* (Balsamo) Vuillemin. On crops such as tomato and cucumber, where fungicides are applied throughout the growing season, serious outbreaks can occur. It was reported that some fungicides can increase mite populations (Smith & Monzingo, 1983). Predators are not capable of suppressing the high densities of TSM, which results in application of yet another pesticide. Obviously, there is also a concern about the high residues and toxicity of common acaricdes to humans (Butt *et al.*, 2001). Concerns about the use of these chemicals have led to emphasis on alternative control measures to suppress TSM populations.

1.1.4.3 Biological control

Diverse natural enemies have an important role in the ecology of TSM (Brandenburg & Kennedy, 1987). The orders of Arthropoda that prey on the pest include Thysanoptera, Coleoptera, Hemiptera, Neuroptera, Diptera, Acarina, and Araneida (Hussey & Huffaker, 1976). Among these predators, the phytoseiid, *Phytoseiulus persimilis,* has been shown to be most efficient in the biological control of TSM (Hussey & Scope, 1985). It was mass-produced by private companies for commercial use in the United Kingdom, Switzerland, Finland, and Holland, and in 1972 about 1/3 of the cucumber crop in those countries was treated with this predator (Bravenboer, 1972). Successful control of mites by use of *P. persimilis* was reported also on tomatoes (Hussey & Huffaker, 1976; Park & Lee, 2002), roses (Simmonds, 1972) and other ornamental plants (Hamlen & Lindquist, 1981; Opit *et al.,* 2004).

Recently, another species of phytoseiid mite *Phytoseuilus macropilis* was reported to efficiently control TSM population on strawberries (Oliveira *et al.*, 2007). Although predators are reported to dominate in most situations, fungi (Table 1.1) and viruses can also attack the mites under certain conditions (Hussey and Huffaker, 1976). No bacteria or parasitoid-type of natural enemies have been reported (Hussey & Huffaker, 1976). Fungi of

the genus *Neozygites* (Zygomycetes: Entomophthorales) are specific pathogens of tetranychids and play an important role as regulators of TSM under natural conditions (Chandler *et al.*, 2000).

Table 1.1 Some of the fungal species infecting *Tetranychus urticae*.

Species	Origin of the fungal isolate	References
Zygomycotina:		
Basidiobolus sp.	Former USSR	Jenina (1976) quoted by van der Geest (1985)
Conidiobolus thromboides	" "	" " " "
Neozygites sp.	Albama (USA)	Carner & Canerday (1968)
	N. Carolina (USA)	Carner (1976)
	S. Carolina (USA)	Brandenburg & Kennedy (1981)
	Iowa (USA)	Klubertanz *et al.* (1991)
Neozygites floridana	Poland	Mietkiewski *et al.* (1993)
Zoophthora radicans	Former USSR	van der Geest (1985)
Mitosporic fungi:		
Aspergillus parasiticus	-	Lipa (1971)
Beauveria bassiana	-	Lipa (1971)
Hirsutella thompsonii	Florida (USA)	Gerson *et al.* (1979)*
Paecilomyces terricola	Israel	Ben-Ze'ev (1993)*
Verticillium lecanii (=Lecanicillium longisporum)	Netherlands	van der Geest (1985)
Metarhizium anosopliae	Israel	Batta (2003)*
Acaromyces ingoldii	"	Paz *et al.* (2007)
Meira geulakonigi	"	Paz *et al.* (2007)
Meira argovae	"	Paz *et al.* (2007)

* *T. urticae* is referred to as *T. cinnabarinus*

A species of *Neozygites* has previousely been used to control the cassava green mite *Mononychelus tanajoa* (Bondar), one of the most important pests of cassava in Africa, causing up to 80% reduction in the crop yield (Yaninek *et al*., 1996; Hountondji *et al*., 2002; Delalibera & Hajek, 2004). However, artificial production of this fungus is reported to be difficult and therefore, its application as an inundative biological pesticide is so far impractical (Oduor *et al*., 1996; Leite *et al*., 2000; van der Geest *et al*., 2000; Delalibera & Hajek, 2004).

Isolates of *B. bassiana*, *Hirsutella thompsonii* and *Lecanicillium lecanii* have been reported as highly pathogenic to TSM (Alves *et al.,* 2002; Irigaray *et al.,* 2003). However, Chandler *et al.* (2005) were the first to study and compare a wide range of species and isolates of anamorphic entomopathogenic fungi against TSM. The use of *B. bassiana* in the control of TSM is discussed further in this Chapter.

1.2 Pest control by the entomopathogenic fungus *Beauveria bassiana*

1.2.1 Use of *Beauveria bassiana* as a microbial agent for pest control

The development of resistance to chemical pesticides and the side effects of these pesticides on natural enemies, human health and the environment have prompted considerable research into alternative methods for managing pests. The use of fungi as biological control agents (BCAs) is one of the methods that have been successful (Allen *et al.*, 1993). Among these BCAs, *B. bassiana* is a classical entomopathogen and has been used extensively for control of many important pests of various crops around world (Varela & Morales, 1996). For example, *B. bassiana* was reported to constitute 33.9% of all fungal species developed for the control of insects and acarines (Faria & Wraight, 2007).

In the USA, high mortalities in the test populations of the nut weevil *Curculio caryae* (Horn) were recorded after exposure of adults to inocula of *B. bassiana* and *Metarhizium anisopliae* Metcsh. (Gottwarld & Tedders, 1984). Similar results were observed on bark beetle *Dendroctonus ponderosae* (Hopk.) populations when they were inoculated with *B. bassiana* conidia (Hunt *et al.,* 1984). In China, application of conidia to different leafhopper populations resulted in high infection rate 15 d after incubation (Li, 1988). *Beauveria bassiana* has also been used in that country for the control of pine caterpillar *Dendrolimus punctata* (Walker) (Li, 1988), while in France, field-collected diapausing larvae of the European corn borer (*Ostrinia nubilalis* Hübner) showed high mortality after infection with *B. bassiana* (Marcandier & Riba, 1986). Other isolates have been under investigation for the control of additional pests, including fire ants *Solenopsis* spp., black

vine weevil *Otiorhynchus sulcatus* (Fabricius) and the citrus root weevil *Diaprepes* sp. (McCoy, 1990). The fungus is also a candidate to be tested in integrated pest management programs against TSM (Chandler *et al.,* 2000).

1.2.2 Mechanism of action of *Beauveria bassiana* for pest control

Different species of *Beauveria* including but not limited to *B. bassiana* (Balsamo) Vuillemin, *B. brongniortii* Sacc and *B. velata* Sans & Evans are recognized as pathogens (Ambethgar, 2001; Rehner & Buckley, 2005). Like most of the entomopathogenic fungi (Inglis *et al*., 2001), *B. bassiana* infects its host through the external cuticle, but there is also evidence that it may infect insects *per os*, especially the chewers, e.g. the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Fernandez *et al.,* 2001).

Conidia strongly adhere to host cuticles and the attachment is thought to involve non-specific adhesion mechanisms mediated by hydrophobicity of the conidial cell wall (Boucias *et al*., 1988; 1991). The eventual germination of a propagule follows its contact with host cuticle. Germination structures may be produced from which penetration hyphae are formed. Penetration of the fungus into cuticle is influenced by different factors such as moisture conditions and the presence of inhibitory factors (fatty acids or melanin) within the cuticle (Inglis *et al*., 2001). The same author also reported that the entomopathogenic Hyphomycetes utilize a combination of enzyme and mechanical mechanisms to penetrate the cuticle.

When the insect haemocoel is colonized by the hyphal bodies of the fungus, host death may result from a combination of actions, including depletion of nutrients, physical obstruction or invasion of organs, and release toxins (Vey *et al.*, 2001). Toxic compounds produced by *B. bassiana* are beauvercin, bassianin, bassianolide, beauverolides, oosporein and tenellin. The structure and function of these compounds are discussed by Vey *et al*. (2001).

Following host death, the oosporein metabolite produced by *B. bassiana* is reported to be involved in the competitive exclusion of competing microorganisms from the cadaver, which may enable the fungus to grow saprophytically within the host (Inglis *et al.,* 2001). Soon after the host's death and under favourable conditions, hyphae emerge from the cadaver and sporulation occurs on the host surface (Inglis *et al.,* 2001).

A threshold of inoculum is required to induce disease and this is not static. It is influenced by all aspects of the disease tetrad (e.g. host susceptibility and environment) (Inglis *et al.*, 2001). An understanding of conditions under which entomopathongenic fungus epizootics are initiated and developed is necessary if these pathogens are to be used successfully in integrated pest management (IPM) (Inglis *et al.,* 2001).

1.2.3 Environmental impact on fungal pathogenesis

The ability of *B. bassiana* propagules to persist in an environment is another important factor in its success as a BCA, and this is most influenced by abiotic factors such as temperature, humidity and sunlight (Fargues & Luz, 1998). For example, with the control of grasshoppers, Inglis *et al.* (1997) explained that despite the deposition of large numbers of virulent conidia in a sunny period, disease progression may be prevented or reversed because the insect is able to elevate its body temperature to 40ºC. The cardinal temperature of *B. bassiana* is 35ºC (Inglis *et al.,* 1996). In contrast, the same authors hypothesized that under cool conditions, when grasshoppers are incapable of elevating their body temperature, the pathogen's inoculum threshold may drop too low to result in disease initiation. The most suitable temperature for application of *B. bassiana* was found to be 20ºC at 80% RH (Fargues & Luz, 1998), although its infection on rice weevil *Oryzaephilus surinamensis* (L.) could occur at 70ºC (Searle & Doberski, 1984).

Relative humidity (RH) can influence the persistence of *B. bassiana* (Fargues & Luz, 1998). Humidity in combination with temperature influences evaporation of spray droplets, which can result in the loss of small particles and thereby adversely affect targeting. Moisture can also have significant effects on the persistence of fungal inocula. Maximum

germination of conidia has been reported to occur at 90-100% RH (Ferron, 1977). It was also observed that a RH of > 97% was required for conidial production of *B. bassiana* on mummified cadavers of cone-nosed bug *Rhodnius prolixus* (vector of *Trypanosoma cruzi*) (Fargues & Luz, 1998). However, low ambient humidity is reported to be less detrimental to *B. bassiana* and *M. anisopliae* var. *acridium,* provided that moisture within the microhabitats is sufficient (Ferron, 1977; Riba & Marcandier, 1984; Marcandier & Khachatourians, 1987; Fargues *et al*., 1997). For example, Ferron (1977) demonstrated that while > 92% RH was required for conidial germination of *B. bassiana*, this fungus would infect the bean weevil, *Acanthoscelides obtectus* (Say), regardless of the ambient RH.

Conidia of *B. bassiana* are susceptible to the detrimental effects of sunlight (Daoust & Pereira, 1986; Inglis *et al.*, 1995; Fargues *et al*., 1996). Ultra-violet (UV) radiation causes damage to exposed microorganisms, which may lead to cellular death (Tevini, 1993). Inglis *et al.* (1995) observed that *B. bassiana* was highly susceptible to artificial UV-B radiation and it is likely that the extreme sensitivity of this fungus to the UV-B portion of the solar spectrum (280-320 nm), limits its persistence in epigeal habitats. However, in contrast to other entomopathogens, fungi seem to be more resistant to UV. In their radiation experiments on the UV sensitivity of entomopathogens, Kreig *et al*. (1981), quoted by Kambona (1996) found that *B. bassiana* conidia were more resistant to UV than granulosis virus (GV), nuclear polyhedrosis virus (NPV) and *Bacillus thuringiensis*. Detrimental effects of UV were also reported on *M. anisopliae* by Zimmermann (1982). He observed that exposing conidia to between 6-12 h of artificial solar radiation from UV lamps produced a significant decline in the viability of conidia. Inglis *et al.* (1995) considered that in order to enhance the longevity of fungal propagules they should be protected against sunlight by a UV-protectant, especially if epigeal habitats are to be targeted for the management of pest populations.

1.2.4 Culture and mass production of *Beauveria bassiana*

Culture and mass production of *B. bassiana* are discussed in Thomas *et al.* (1987) and Feng *et al.* (1994). Two different propagules of *B. bassiana* are produced; these are conidia and

blastospores or hyphal bodies. *Beauveria bassiana* is one of the hyphomycetes which are easily grown on a wide range of conventional mycological media (Goettel & Roberts, 1992). One of the most commonly used media for isolation and culture of these fungi is Sabouraud dextrose agar supplemented with 0.2% yeast extract (SDAY) (Goettel & Inglis, 1997). Aerial conidia are produced on solid media. Feng *et al.* (1994) reported that no difference exists in the morphology and infectivity of conidia produced on solid media and those produced on the surface of insect cadavers. Blastospores are produced by yeast-like budding from single parent cell, or they may be formed in the insect haemocoel by schizolytic separation at septa, or by mechanical fragmentation of hyphae due to shearing forces in the liquid medium (Alves *et al*., 2002). The blastospores of *B. bassiana* are thin walled and unstable during drying after fermentation or when applied under field conditions (Wraight *et al.*, 2001). Thus conidia are much more resilient in production and application for pest control. On the other hand, mass production of aerial conidia (by diphasic fermentation) is reported to be labour-intensive and unsuitable for conventional processing of fungal material in fermenters (Feng *et al.,* 1994). The possibilities of producing submerged conidia that are similar to aerial ones in environmental stability and virulence as well as in morphology have been investigated (Thomas *et al.,* 1987; Feng *et al.*, 1994). Ultimately, the amenability of *B. bassiana* to mass production by both solid substrate and liquid fermentation renders it highly suitable for development as a mycoinsecticide.

1.2.5 Formulation and application of *Beauveria bassiana* in the biological control of insects

There have been many reviews on the formulation and application of *B. bassiana* (e.g. Feng *et al.,* 1994; Inglis *et al.,* 2001). This has been discussed even earlier by Ferron (1978) and Ignoffo *et al.* (1979). Most of these reviews are focus on the possibility of using the fungus for insect control.

Formulation is the most critical aspect for the development of microbial pesticides and has to fulfill several criteria including:

a) Allowing the microbial agent to retain and express its pesticidal properties (Jones *et al.*, 1997);
b) Providing a long shelf life (6-18 mo) at ambient conditions (Feng *et al*., 1994);
c) Allowing the active ingredient to be applied with existing application equipment (Bateman & Chapple, 2001).

Table 1.2 *Beauveria bassiana* products formulated for insect and acarine control (Butt *et al*., 2001; Wraight *et al*., 2001).

Product	Formu-lation	Active ingredient	Target pest	Producer and country
Conidia	WDG	Ae-conidia	Coffee-berry borer	Live System Technology, Colombia
Ostrinil	G	Ae-conidia	Corn-borer	Natural Plant Protection (NPP), France
CornGuard			European corn-borer	Mycotech, USA
Mycotrol & BotaniGard	WP, ES, OF	Ae-conidia	Whitefly, aphids, thrips and grasshoppers	Mycotech, USA
Naturalis L	ES	Ae-conidia	Cotton pests including boll-worms	Troy Biosciences, USA
Proecol	WP	Ae-conidia	Army worm	Probioagro, Venezuela
Boverin	WP	Ae-conidia Sub-conidia Blastospore	Colorado beetle	Former USSR
Boverol	WP	Ae-conidia	Colorado beetle	Czechoslovakia

Ae-conidia: Aerial conidia; **Sub-conidia**: Submerged conidia; **WDG:** water dispersal granular; **OF:** oil flowable; **ES**: oil-based emulsifiable suspension; C/**WP**: wettable or contact powder; **G**: granule.

Good formulation will also increase the efficacy of microbial biopesticides by improving their application efficiency and coverage, activity, and persistence on the leaf surface (Jones *et al.*, 1997). All three developmental stages of *B. bassiana* (conidia, blastopores and mycelia) have been successfully formulated for small-scale field trials or large-scale application (Feng *et al*., 1994). However, conidia are the propagule of choice for most commercial formulations due to their strong hydrophobic walls, which confer environmental stability, thus contributing significantly to their production efficiency and storage stability (Wraight *et al.*, 2001) (Table 1.2).

Conidia of *B. bassiana* are smooth-walled, globulose and only 2-3 µm in diameter; they can be suspended in aqueous liquids or mixed with a powder carrier and sprayed as a mist or a dust with conventional equipment used for application of chemical pesticides. Pure conidial powder can be stored in airtight containers at 4ºC and still retain a viability of 71% after 21 months if the water content is below 10% (Feng *et al.,* 1994). The water content is considered as a key factor influencing the shelf-life of the conidia of several fungi, including *B. bassiana* (Wraight & Carruthers, 1999). Conidial formulation of *B. bassiana* has been attempted in different countries (Table 1.2) (Faria & Wraight, 2007). For example, in the USA, a wettable formulation of *B. bassiana* conidia, based on Isolate ARSEF 252, was developed by Abbott Laboratories and used against Colorado potato beetle during the 1980s (Anderson *et al.,* 1988).

During the mid-1980s, a new *B. bassiana* preparation, Boverol®, was developed in Czechoslovakia (Feng *et al.,* 1994). The formulation, a whitish powder and insoluble in water, is applied as a water suspension alone or in combination with selected pesticides and wetting agents (Feng *et al.*, 1994). Boverol® contains > 10^{10} conidia g^{-1} with > 70% viability. In the former USSR, all *B. bassiana* preparations were referred to as Boverin regardless of their infective forms (Feng *et al.*, 1994). The annual application of Boverin in former USSR was said to be > 10000 ha (Deacon, 1983), mainly against potato beetle and codling moth. It was reported that in a field experiment in the USA, spraying a 5% Boverin preparation (7.4×10^{14} conidia ha^{-1}) onto field plots of kale resulted in about 50% reduction of larval populations of the cabbage looper and 87% reduction of leaf damage (Ignoffo *et al.,* 1979).

Finally, considerable effort has been devoted to the evaluation of various oils for formulation of aerial conidia (i.e. Inglis *et al.*, 1993; Wraight & Carruthers, 1999). The reported effects of vegetable- and petroleum-based oils on the stability of conidia stored over a broad range of temperatures are highly variable and even contradictory (Wraight & Carruthers, 1999). Nevertheless, selected oils are highly compatible with the hydrophobic conidia of hyphomycetes (Wraight *et al.*, 2001). For example, paraffinic oil formulations of

B. bassiana are claimed to have a shelf-life of approximately 1 year at 25ºC (Wraight *et al.,* 2001).

1.2.6 *Beauveria bassiana* strains as BCAs for control of acarines

Beauveria and other entomopathogenic mitosporic fungi (Hyphomycetes), e.g. *Metarhizium* and *Paecilomyces,* show adaptations to the soil environment, exhibit wide host ranges within Insecta, occupy broadly similar trophic niches and possibly form guilds (Chandler *et al.,* 2000). All these fungi have world-wide distributions and they can be cultured readily in bulk (Chandler *et al.,* 2000). To date they have been the most popular choices for development as mycoinsecticides. This group of fungi is also known to kill Acari including *T. urticae* (Kaaya *et al.,* 1996; Chandler *et al.,* 2005).

Table 1.3 Acari species reported to be infected by *Beauveria bassiana* (adapted from Chandler *et al.,* 2000).

Order	Family	Species
Ixodida	Ixodidae	*Amblyomma variegatum*
		Boophilus microplus
		Dermacentor sp
		Ixodides ricinus
		Rhipicephalus appendiculatus
Mesostigmata	Ascididae	*Proctolaelaps* sp.
	Varroidae	*Varroa jacobsoni*
Prostigmata	Eupodidae	*Halotydeus destructor*
	Tarsonemidae	*Tarsonemus spirifex*
		Polyphagotarsonemus latus
	Tetranychidae	*Byobia rubrioculus*
		Mononychelus sp.
		Tetranychus urticae

However, there are few reports on the use of *B. bassiana* as a BCA against acarines. Moreover, it is not known whether the pathotypes involved are opportunists or have evolved a degree of specialization for Acari (Chandler *et al.,* 2000). Most reports concern

mycoses in species that spend all or part of their lives near to the soil or in detritus, where entomopathogenic mitosporic fungi are common.

For example, Verissimo (1995) (in Chandler *et al.*, 2000) observed *B. bassiana* infecting soil-dwelling stages of the cattle tick, *Boophilus microplus* Canestrini in Brazil. *Beauveria bassiana* and *M. anisopliae* are reported as potential microbial control agents of cattle ticks, which are a major constraint to livestock production in many areas of the world. Kaaya *et al.* (1996) reported that these fungi caused 100% mortality in free-living ticks in the field. In Kenya the cattle tick *Rhipicephalus appendiculatus* Neumann was susceptible in laboratory bioassays to an isolate of *B. bassiana* originating from the banana weevil *Cosmopolites sordidus* Germar (Mwangi *et al*., 1991). Different Acari infected by *B. bassiana* are listed in Table 1.3.

1.2.7 Compatibility of *Beauveria bassiana* with other control methods

Beauveria bassiana may be applied alone or in combination with other control methods, although it is reported that control of insects with *B. bassiana* alone is often slower than with chemical insecticides (Feng *et al.*, 1994). Studies have showed *B. bassiana* preparations to be compatible with several chemical and microbial pesticides. Some of these are listed in Table 1.4; however, some insecticides, especially emulsifiable-concentrate insecticide formulations, herbicides and fungicides have been found to inhibit the germination and growth of the fungus (Anderson & Roberts; 1983; Loria *et al*., 1983; Todorova *et al.,* 1998; Jaros-Su *et al*., 1999).

The compatibility of *B. bassiana* with the predatory mite *Neoseiulus cucumeris* (Oudemans) was also studied in laboratory and glasshouse on cucumbers with no adverse effects on the predatory mite population (Jacobson *et al.,* 2001). Entomopathogenic nematodes (EPNs) are increasingly being considered for control of a range of insects (e.g. Barbercheck & Kaya, 1991; Medeiros *et al.,* 2000; Rosa & Simões, 2004). The work of Barberecheck & Kaaya (1991) indicated that co-infection of *B. bassiana* could speed up the

lethal infection rate on larvae of the lepidopteran, lesser armyworm *Spodoptera exigua*, and cause higher mortality in a pest population treated with EPNs.

Furthermore, in the control of grasshoppers, Inglis *et al.* (1997) observed that the combination of *B. bassiana* and *M. anisopliae* var. *acridium* increased mortality of the pest more than *M. anisopliae* var. *acridium* alone; this suggested that the co-application of *B. bassiana* and *M. anisopliae* var. *acridium* could be used to increase the temperature range over which the individual fungi alone would induce mortality. As indicated previously in Section 1.2.3, the detrimental effects of high temperatures are recognized as an important constraint on the use of *B. bassiana* against grasshoppers.

Table 1.4 Some compatibility cases of *Beauveria bassiana* preparations with chemical and microbial pesticides.

Class	Common name	Target	References
Insecticides	Carbaryl 50	Colorado potato beetle (CPB)	Anderson & Roberts (1983); Anderson *et al.*(1989)
	Azinphos methyl 50	CPB	Anderson & Roberts (1983)
	Diflubenzuron 25	CPB	Anderson & Roberts (1983)
	Abamectin 0.1EC	CPB	Anderson *et al.* (1989)
	Carbofuran	European corn borer, *Ostrinia nubilalis* in maize	Lewis *et al.* (1996)
	Imidacloprid	Sugar cane rootstock borer	Quintela & McCoy (1998)
		Leafhopper	Feng *et al.* (2004)
	Bacillus thuringiensis	CPB	Anderson *et al.* (1989)
		European corn borer	Lewis *et al.* (1996)
Acaricides	Pyridaben 15EC	Eggs of *Tetranychus cinnabarinus*	Shi *et al.*(2005)
	Propargite 73EC	Eggs of *Tetranychus cinnabarinus*	Shi *et al.*(2005)
	Hexythiazox 5EC	Eggs of *Tetranychus cinnabarinus*	Shi *et al.* (2005)
	Triflumuron 4F	*T. urticae*	Irigaray *et al.* (2003)
Fungicides	Metalaxyl	-	Loria *et al*. (1983)
	Copper hydroxide	-	Jaros-Su *et al.* (1999)
Herbicide	Diquat	-	Todorova *et al.* (1998)

1.2.8 Safety of *Beauveria bassiana*

A review of the present knowledge of potential fungal biocontrol agents indicates that these organisms pose only a minimal risk to man, domestic animals, wildlife and non-target invertebrates (Zimmermann, 2007). Several isolates of *B. bassiana* have undergone detailed vertebrate laboratory safety tests as required for their registration. Overviews of some of the vertebrate safety test results are provided for Isolate GHA (Mycotrol and Botanigard) by Goettel & Jaronski (1997) and for Naturalis-L by Copping (2001). In general these tests indicated that the fungus is essentially non-toxic and non-infectious to vertebrates but that caution must be exercised to avoid inhalation of a large quantity of conidia. Field studies conducted by Peveling & Weyrich (1992), Goettel *et al*. (1996), Pingel & Lewis (1996) and Brinkman & Fuller (1999) also found no evidence of detrimental effects of the fungus to non-target organisms.

However, there have been some reports on natural epizootics of *B. bassiana* on non-target organisms. Natural prevalence of the fungus was monitored in Zimbabwe in *Neochetina bruchi* (Hustache), a curculionid biocontrol agent of water hyacinth (Chikwenhere & Vestergaard, 2001). These authors noted a correlation between the prevalence and use of herbicide treatments, suggesting that herbicide application predisposed the insect to infection. Cases of implication of *B. bassiana* in human and vertebrate animal infections have been discussed by Henke *et al.* (2002) and Vestergaard *et al*. (2003). It was reported that the pathogen associated with infections in those cases was not *B. bassiana* (Vestergaard *et al*., 2003).

Ultimately, despite a wide host range, evidence to date is that *B. bassiana* can be used with minimal impact on non-target organisms, especially when isolate selection and spacio-temporal factors are taken into consideration (Vestergaard *et al*., 2003).

1.3 Problems and perspectives in developing fungal biological control agents

Biological control agents (BCAs) may offer more environmentally friendly alternatives to chemical pesticides and could also be used where pests have developed resistance to conventional pesticides. Unfortunately, investment in the research and development of these organisms is reported to be minimal compared with that spent on the discovery of chemical pesticides (Butt *et al*., 1999; 2001). Reasons for this are that BCAs usually have narrow host ranges, and that they often give inconsistent and poor control in field trials (Butt *et al.,* 1999). Many studies have concluded that UV irradiation is one of the major abiotic factors limiting the viability of fungi on foliage (Daoust & Pereira, 1986). Temperature and low relative humidity can also negatively affect entomopathogenic fungi (Ferron *et al.,* 1991). However, some exceptions exist. For example, Kouassi *et al.* (2003) found that the Strain MK 2001 of *B. bassiana* was not affected by these factors and could provide an acceptable level of control of tarnished plant bug on celery. Other factors such as plant species (Kouassi *et al*., 2003) and plant physiology (Hajek & St Leger, 1994) may influence the efficacy of foliar application of entomopathogenic fungi, thus resulting in poor control under field conditions.

Consequently, more attention is being given to the selection of broad-spectrum biopesticides (Butt *et al*., 1999) and improvements in their efficacy through formulation (Goettel & Roberts, 1992) and application technologies (Chapple & Bateman, 1997; Bateman, 1997; Bateman & Chapple, 2001). Efforts are also being made to optimize the impact of these agents by integrating them with other novel crop protection strategies (Irigaray *et al.,* 2003; Wraight & Ramos, 2005).

Another problem with BCAs is that their market potential has not been realized (Butt *et al.,* 2001). The reasons given by these authors are the absence of strong incentives to develop these agents and/or discourage chemical pesticides; availability of new biodegradable chemical pesticides; absence or breakdown of the infrastructure, which facilitates transfer

of new technology to the end-user; absence of universally acceptable registration procedures; restriction in the use of exotic BCAs; lack of robust and reliable field effects; and relatively few growers or extension workers who know how to use BCAs.

Improvements in microbial products, grower awareness of the benefits that microbial control offers and the need to develop alternatives to conventional chemical insecticides should overcome many of the obstacles that microbial control is now facing (Butt *et al.,* 2001). Microbial pesticides are presently experiencing a renaissance as regulatory constraints are resolved and mass production technology is improved (Lacey *et al.,* 2001). Their success, however, will ultimately depend on the willingness of consumers to employ new methods of pest control and integrate microbial agents into IPM.

1.4 References

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Chapter 2

Screening of *Beauveria bassiana* strains against the two-spotted spider mite in laboratory and greenhouse trials[1]

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Abstract

South African strains (62) of *Beauveria bassiana* (Balsamo) Vuillemin were screened for their pathogenicity against two-spotted spider mite, *Tetranychus urticae* Koch, in laboratory bioassays on detached bean leaves, *Phaseolus vulgaris* L., and under greenhouse conditions. In the first bioassay, strains of *B. bassiana* were applied at a single concentration of 10^7 conidia ml^{-1}. A mortality of mites of 4-92.5 % was observed, with 40% of the strains causing mortality levels higher than 50%. The median lethal times (LT_{50}s) ranged between 5.5 and 8.6 days. Six strains were the most virulent, with an LT_{50} of less than 6 days. These were compared in a second screening, together with the commercial strain PPRI 5339 contained in *Bb* Plus, using five concentrations (2×10^4, 2×10^5, 2×10^6, 2×10^7 and 2×10^8 conidia ml^{-1}) on female mites and using three concentrations (2×10^6; 2×10^7 and 2×10^8 conidia ml^{-1}) on eggs. Mortality of mites assessed in this bioassay showed that Strain PPRI 7315 and Strain PPRI 7861 performed similarly and were the most efficient, causing a mortality > 80%, 9 days after inoculation, at the highest concentration, with a median lethal concentration (LC_{50}) of 1.13×10^6 and 1.22×10^6 conidia ml^{-1} respectively. Both strains were far better than the commercial strain PPRI 5339 and showed good control of *T. urticae* in greenhouse trials.

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2.1 Introduction

Efforts have been made to use *Beauveria bassiana* (Balsamo) Vuillemin (*Bb*) and other entomopathogenic fungi against tetranychid mites in laboratory and greenhouse experiments (Alves *et al*., 2002; Irigaray *et al*., 2003; Shi & Feng, 2004; Chandler *et al*., 2005; Shi *et al*., 2008). The rationale behind these research efforts has been the development of a myco-acaricide for use in an integrated control programme and thereby to reduce reliance on chemical acaracides in the management of *Tetranychus urticae* (two-spotted spider mite, TSM) populations.

Although most isolates tested have been infectious to *T. urticae,* they have provided varying levels of control. Some strains resulted in efficient control of the mite (Irigaray *et al*., 2003; Wekesa *et al*., 2006). In other works, control by *Bb* was poor (Andreeva & Shternshis, 1995; Tamai *et al*., 1999) or effective control could only be achieved with high concentrations of conidia, resulting in an unacceptable cost of application (Shi & Feng, 2004). Evidence to date is that there are few microbial pesticides developed for the control of TSM (Faria & Wraight, 2007).

In South Africa both indigenous and exotic strains of *Bb* have been tested by Agricultural Research Council (ARC) but most of these strains were initially screened against Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) and other secondary cereal-aphid species at the Small Grain Institute[2] of ARC (Hatting, 2002). In our study 62 South African strains of *B. bassiana* were tested against TSM. The objectives were to identify the most pathogenic strains against TSM in laboratory bioassays and to compare their efficacy with the South African commercial strain PPRI 5339, under laboratory and greenhouse conditions.

[2] Small Grain Institute-ARC, Bethlehem, South Africa

2.2 Materials and methods

2.2.1 General procedure for bioassays

2.2.1.1 Rearing of *Tetranychus urticae*

Individual TSM colonies used in experiments were reared on bean plants (*Phaseolus vulgaris* L. var. Tongati) in the greenhouse (23-26°C; 12 h light). These mites were originally collected from a cassava plant, *Manihot esculenta* L., at Ukulinga[3] and maintained on tomato plants in a greenhouse (Controlled Environment Facility[4]- CEF). When plants started to die because of heavy infestations, new colonies were initiated on newly grown tomato plants (*Solanum lycopersicum* L.). For experiments, mites were transferred to bean plants by placing infested tomato leaves on bean plants. Plants were grown in pots (200 mm diameter and 200 mm deep) placed in a gravel bed with circulating sub-gravel irrigation, sustained by a pump with a single-phase induction motor (Type: MY 8012)[5]. Nutrients were constantly provided in the irrigation system.

To get single-age populations for bioassays, mites were prepared according to Chandler *et al*. (2005), with slight modifications. Tomato leaflets infested with TSM were collected from the stock cultures in the greenhouse and placed on whole bean plants for 1 h to allow mites to move onto the new plants. Leaflets were then removed. After 24h, leaves of the newly infested bean plants were collected (Fig. 2.1) and observed under a dissecting microscope. Two-spotted spider mite individuals were removed using a dissecting needle, leaving only eggs, which were used to rear adult mites or used directly in bioassays where necessary. To obtain adult mites, leaves with eggs were placed on wet cotton wool in Petri dishes (90 mm-diameter) and incubated at 21ºC (16:8 h L: D regime) for 6 d. New bean plants were infested with the TSM nymphs; after 10 d, leaves with adult TSM were collected for bioassays.

[3] Research Farm of the University of KwaZulu-Natal, Pietermaritzburg, South Africa
[4] University of KwaZulu-Natal
[5] Rapid Allweiler (Industry) Pump & Eng. Co. (Pty) Ltd, South Africa

2.2.1.2 Fungal strains and conidia preparation

The fungal strains were provided by the Natural Collection of Fungi – Plant Protection Research Institute - Agricultural Research Council[6] (ARC-PPRI). In total 62 strains were screened against TSM (Table 2.1). All the strains were stored on 1.5% malt extract agar (MEA) in sealed 10-ml bottles kept at -20°C.

To produce conidia, strains were recovered and cultured on Sabouraud dextrose agar[7] (40 g dextrose, 10 g mycological peptone and 15 g agar in 1 l of distilled water as directed by the manufacturer) supplemented by 1% yeast extract (SDAY) (Goettel & Inglis, 1997). A plug of MEA with *Bb* growth (≈ 25 mm^2) was inoculated onto SDAY Petri dishes; these were enclosed in polystyrene plastic bags to reduce dehydration of the medium and to maintain adequate moisture levels for fungal growth. Petri dishes were incubated at 26°C in the dark for 21 d. After this time, they were removed from the bags and allowed to dry on a laminar flow bench for 2 d. Conidia were then harvested by gently scraping them off the surface of the dried medium using a sterile scalpel blade. Fungal material so collected was passed twice through a 100 mm diameter sieve (110 µm pore size) to separate conidia from mycelial mats. The conidial powder was stored in sealed containers at 4°C until used in bioassays.

Conidial suspensions were prepared by suspending a given weight of conidial powder in 10 ml sterile distilled water containing 0.01% Tween 80 in a sterile 20 ml bottle. The concentration of conidia in the sample suspensions was determined using a Neubauer haemocytometer and adjusted to the final concentration required in the experiment by diluting the conidial suspension with 0.01% Tween 80 in water or adding more conidia. The final conidial suspension prepared was immediately used for bioassays.

[6] ARC-PPRI, Gauteng, South Africa
[7] Biolab Diagnostics (Pty) Ltd, Midrand, South Africa

2.2.1.3 Assessment of conidial viability

The viability of conidia for suspensions was assessed on SADY medium following a modified protocol of that described by Goettel & Inglis (1997). A volume of 0.1 ml of the sample suspension was pipetted and spread on SDAY Petri dishes using an L-shaped glass rod and incubated at 26ºC for 24 h. Germinated conidia were examined under a compound light microscope at 400 × magnification. All conidia with an apparent germ tube were considered viable. Counts were made at four different random locations per Petri dish and there were three Petri dishes per sample suspension. At each field of view all conidia were counted and percentage germination calculated. All the strains used in experiments had more than 97% viable conidia.

2.2.1.4 Assessment of strains for conidial production

Production of conidia was assessed from a concentration of 10^6 conidia ml,$^{-1}$ in a sterile Tween 80 solution (0.01% v/v), prepared for each strain. An aliquot of 100 ml of the suspension was inoculated onto SDAY petri dishes (90 mm diameter) and incubated at 26 ± 1°C for 14 d. Discs (*ca*. 180 mm^2) were cut from the SDAY using a sterilized 15.1 mm diameter-punch.

Table 2.1 *Beauveria bassiana* strains used in laboratory experiments for *Tetranychus urticae* control: collection site and conidial production.

PPRI No	Code	Province of origin [(a)]	Town of origin	Conidial production (×10^3 conidia mm^{-2}) [(b)]
7269	Vopi 47	Gauteng	Roodeplaat	11.33 ± 3.10
7270	Sasex 67	KwaZulu-Natal	Pongola	36.02 ± 3.20
7271	Sasex 69	KwaZulu-Natal	Stanger	17.22 ± 4.20
7272	Univ 18	KwaZulu-Natal	Pietermaritzburg	34.17 ± 7.10
7276	NISSV 35	Mpumalanga	Nelspruit	42.36 ± 9.00
7277	NISSV 46	Mpumalanga	Nelspruit	30.11 ± 8.20
7278	NISSV 58	Mpumalanga	Nelspruit	25.12 ± 6.40
7279	NISSV 59	Mpumalanga	Nelspruit	20.64 ± 3.10
7280	SGI 132	Western Cape	Clanwilliam	18.20 ± 4.70
7281	SGI136	Western Cape	Clanwilliam	26.31 ± 9.70
7282	SGI 137	Western Cape	Clanwilliam	17.40 ± 4.20
7283	SGI 138 (2 van 2)	Western Cape	Clanwilliam	8.90 ± 2.30
7285	SGI 139A	Western Cape	Clanwilliam	11.01 ± 5.30
7286	SGI 141	Western Cape	Clanwilliam	12.12 ± 4.00
7287	SGI 146	Western Cape	Clanwilliam	42.14 ± 12.0
7287	SGI 146	Western Cape	Clanwilliam	42.14 ± 12.0

Table 2.1 continued.

PPRI No	Code	Province of origin[a]	Town of origin	Conidial production ($\times 10^3$ conidia mm^{-2}) [b]
7290	SGI 153	Western Cape	Vanrhynsdorp	22.06 ± 4.50
7295	Rooi 65	Free State	Bloemfontein	5.37 ± 1.10
7298	Rooi 146	Western Cape	Clanwilliam	27.39 ±10.0
7300	Rooi 148	Western Cape	Clanwilliam	26.10 ± 4.00
7301	Rooi 150	Western Cape	Clanwilliam	17.09 ± 4.70
7302	Rooi 167	Western Cape	Clanwilliam	16.25 ± 5.00
7303	Rooi 191	Western Cape	Clanwilliam	11.03 ± 2.30
7305	Rooi 199	Western Cape	Clanwilliam	22.40 ± 5.37
7306	Rooi 200	Western Cape	Clanwilliam	7.00 ± 2.800
7307	Rooi 213	Western Cape	Clanwilliam	12.17 ± 4.50
7308	Rooi 217	Western Cape	Clanwilliam	28.02 ± 3.10
7310	Rooi 223	Western Cape	Clanwilliam	29.40 ± 8.00
7312	Rooi 203	Western Cape	Piketberg	17.30 ± 6.10
7314	Rooi 274	Western Cape	Piketberg	16.05 ± 2.00
7315	Rooi 289	Western Cape	Piketberg	27.31 ± 1.91
7316	Rooi 301	Western Cape	Piketberg	11.90 ± 4.00
7318	Rooi 325	Western Cape	Clanwilliam	3.31± 0.10
7319	Rooi 337	Western Cape	Clanwilliam	38.12 ± 9.10
7321	SGI 138 (1 van 2)	Western Cape	ClanWilliam	11.01 ± 3.70
7539	Sasex 314	KwaZulu - Natal	Pongola	40.02 ± 9.40
7568	Vopi99	Gauteng	Roodeplaat	24.13 ± 6.30
7583	Sasex 153	KwaZulu - Natal	Mount Edgecombe	21.36 ± 9.03
7589	Sasex 273	KwaZulu - Natal	Mount Edgecombe	8.10 ± 4.12
7606	Rooi 351	Western Cape	Clanwilliam	17.25 ± 9.40
7617	Rooi 299	Western Cape	Piketberg	15.03 ± 3.10
7620	Rooi 377	Western Cape	Vanrhynsdorp	14.12 ± 3.58
7621	Rooi 328	Western Cape	Clanwilliam	40.01 ± 9.81
7629	Rooi 396	Western Cape	Vanrhynsdorp	20.07 ± 8.70
7644	Sasex 370	KwaZulu - Natal	Mount Edgecombe	17.41 ± 3.13
7769	SGI 168	Free State	Bloemfontein	25.78 ± 7.80
7773	Sasex 240	KwaZulu - Natal	Ottawa	21.31 ± 7.18
7774	Sasex 373	KwaZulu - Natal	Mount Edgecombe	27.14 ± 4.35
7776	Rooi 320	Northern Cape	Calvinia	38.09 ±13.0
7787	INF 40	Western Cape	Paarl	10.67 ± 3.03
7793	Vopi 96	Gauteng	Roodeplaat	7.11 ± 2.21
7794	Rooi 503	Western Cape	Clanwilliam	14.32 ± 3.90
7809	INF 67	Western Cape	Worcester	21.30 ± 3.80
7812	ITSC 61	Mpumalanga	Nelspruit	18.20 ± 3.10
7846	INF 66	Western Cape	Stellenbosch	19.31 ± 3.01
7847	INF 32	Western Cape	Montagu	9.97 ± 4.19
7854	ITSC 93	Mpumalanga	Hazyview	32.19 ±12.4
7861	Rooi 444	Western Cape	Graafwater	38.70 ± 11.9
7867	Rooi 330	Western Cape	Clanwilliam	4.39 ± 0.32
7869	Sasex 221	KwaZulu - Natal	Gingindlovu	5.13 ± 0.37
7870	Sasex 184	KwaZulu - Natal	Richardsbay	21.01 ± 5.05
7873	ITSC 108	Mpumalanga	Hazyview	7.61 ± 1.40
7939	ITSC 113	Mpumalanga	Hazyview	17.73 ± 6.13

[a] All the strains are South African strains and were provided by ARC–PPRI, South Africa
[b] Mean ± S.E of conidia produced by strains cultured on SDAY petri dishes after 14 d of incubation

They were placed in glass tubes and washed in 10 ml aqueous Tween 80 (0.01%) to extract conidia from the surface of the medium. Conidia collected from 5 Petri dishes per strain (1 disc per Petri dish) were counted in a Neubauer haemocytometer. The data were analysed using ANOVA (GenStat, 2007). The means (conidia mm^{-2}) for the strains were compared by Tukey-Honestly Significant Difference (Tukey-HSD) test at a 5% level of significance (Table 2.1).

2.2.1.5 Preparation and inoculation of *Tetranychus urticae*

Infested bean leaves described above were collected and kept in a refrigerator (4°C) overnight to immobilize the individual TSMs. These were then counted under a dissecting microscope and transferred to a single bean leaf using a dissecting needle (on the abaxial surface), placed on two layers of $N_{0.}$ 1 Whatman® filter paper[8], inside a Petri dish (90 mm diameter). Except when otherwise stated, 20 TSM females were counted per leaf. In total, 60 mites were treated per strain and per trial event.

Conidial suspensions of 0.01% Tween 80 prepared for each of the strains was sprayed onto the bean leaves in Petri dishes using a Burgerjon spray tower (Fig. 2.2), built after Burgerjon (1956) by the Mechanical Instruments Workshop (Faculty of Science and Agriculture, University of KwaZulu-Natal, Pietermaritzburg, South Africa). The spray was fitted with an air-atomizing nozzle (Fluid Cap 2850 + Air Cap 73160 – ENP) mounted in a ¼ J nozzle body[9]. The nozzle was connected to a regulator valve providing a constant air flow of 5 l min^{-1}. The spray targets (bean leaf with spider mite placed in the lid of a Petri dish containing wet filter papers) were positioned on a revolving 215 mm diameter turntable (33 revolutions per minute) during application. To calibrate the sprayer, 5 consecutive sprays with 0.01% Tween 80 solution were performed always on 3 Petri dishes.

Spray deposition collected and quantified revealed that, at the level of the target surface, there was no significant difference between the three Petri dish lids sprayed and the periods of spray; and the quantity deposited was approximately 0.01 µl mm^{-2} (resulting

[8] Merck NT Laboratory Supplies (Pty) Ltd, Bloemfontein, South Africa
[9] Spraying Systems Co, Wheaton, IL, USA

from spraying of a 5 ml aliquot)**.** Three Petri dishes with leaves and mites were sprayed per strain at the same time (Fig. 2.2). The control Petri dishes consisted of leaves with mites sprayed with a Tween solution alone. The number of conidia deposited by the spray (conidia mm^{-2}) was estimated following the enumeration protocol described by Wraight *et al*. (1998). After each strain was applied, the spray tower was cleaned with 70% ethanol and rinsed with distilled water to eliminate cross–contamination between treatments. Spray deposits on leaves were allowed to dry before covering the Petri dishes. Petri dishes were kept in the laboratory at room temperature (22 ± 1°C) with a 12 h light regime. The filter paper was regularly wetted to prevent leaves from fading; with this care, treated leaves remained green ± 8 d before turning chlorotic. All bioassays were performed over a period of 5 months using successive generations of TSM.

2.2.2 First screening bioassays

All 62 strains of *Bb* were used in the first screening against TSM in single dose bioassays under laboratory conditions. Suspensions of 2×10^7 conidia ml^{-1} of Tween 80 solution were sprayed on the leaves with TSM (20 mites per leaf) in the Petri dishes. There were in total 189 Petri dishes treated (63 treatments replicated three times), including the control. Using CycDesigN (2002), treatments were arranged in a resolvable incomplete block design; there were, in total, 27 blocks split into three replicate groups, each of 9 blocks (7 treatments per block) with each treatment occurring once in each replicate. This experiment was repeated on four occasions, following the same procedures.

On the first occasion, the bioassay was performed with a conidial concentration of 10^4 conidia ml^{-1} and all the strains caused a mortality of TSM less than 50%, with a mortality of 12% in the control treatment 9 d after treatment application. These data were discarded and the bioassay was repeated three more times with a conidial suspension of 10^7 conidia ml^{-1}. Data were combined for analysis. The mortality of the mites was first observed at Day 5 post-spray. It was then recorded every day for another 5 d.

Figure 2.1 Bean leaf with two female *Tetranychus urticae* and eggs, 24 h after infestation. Eggs were used to produce mite individuals for bioassays. The bar corresponds to 1mm.

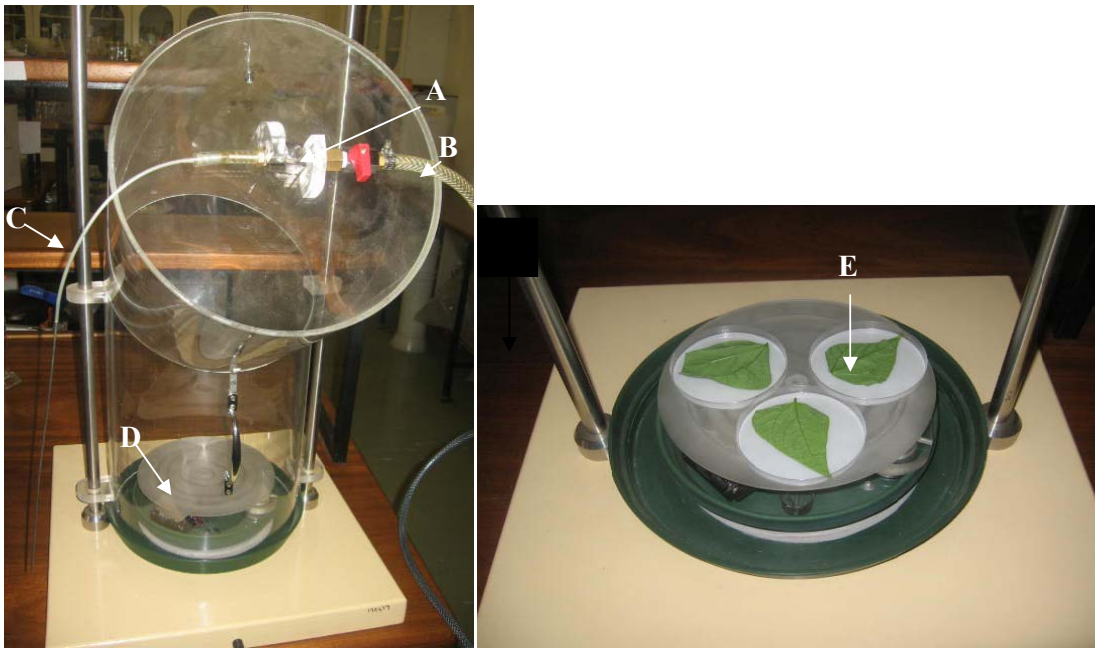

Figure 2.2 The Burgerjon spray tower (left) with the nozzle (A), the air input (B), inoculum input (C) and the turntable, 215 mm diameter (D). Bean leaves (right) with mites in Petri dishes placed on turntable for spraying (E). The air flow was provided by a compressor.

Dead individuals counted every day in each treatment were removed, placed on water agar and incubated at 25 ± 1°C and 12 h light. The growth of the fungus from the mite body was then checked to confirm the mortality was due to the fungus.

Percentage mortalities of TSM were corrected relative to the control, using Abbott's formula (Abbott, 1925). However, where mortality of TSM in the control exceeded 5%, the bioassay was discarded and repeated (Butt & Goettel, 2000). Data were transformed (angular transformation) prior to analysis and submitted to analysis of variance (ANOVA) (GenStat, 2007); means were compared using Tukey-HSD test at a 5% level of significance (Jones, 1984). Probit analysis was also performed to determine the median lethal time (LT_{50}) for the strains.

2.2.3 Second screening: multiple dose bioassays

Six strains selected from the first screening were used in the second screening against TSM; *Bb* Plus[10], a commercial biopesticide, based on *Bb* Strain PPRI 5339, was included in this bioassay. This strain was provided as a technical powder with approximately 2×10^{10} conidia g^{-1}. The product is registered in South Africa for the control of aphids and TSM. Conidia were recovered by inoculating 1 mg of the technical powder into 20 ml of liquid culture (2% sucrose and 5% peptone) in 50-ml flasks and incubating for 48h in a water bath shaker (120 rpm) at 26 ± 1°C. Conidia were produced by spreading 0.01 ml of the culture onto SDAY medium, then following the protocol described above. The viability of these conidia was also assessed before application.

Five different doses (2×10^4, 2×10^5, 2×10^6, 2×10^7 and 2×10^8 conidia ml^{-1}) of conidial suspension in 0.01% Tween 80 aqueous solution were prepared for each strain and sprayed onto infested leaves using the Burgerjon sprayer as described above. The dose of 2×10^8 conidia ml^{-1} was first prepared and the subsequent doses were prepared from this, using the dilution procedure as suggested by Butt & Goettel (2000). There was one Petri dish, with leaf and mites (25 per leaf), per replicate for each dose and one Petri dish sprayed with the Tween 80 solution as a control treatment for each strain in

[10] Biological Control Products, SA (Pty) Ltd, Durban, South Africa

each replicate. Petri dishes were arranged in a laboratory in a resolvable 6 × 7 row-column design with 3 replications. This bioassay was performed twice and data were pooled for analysis. Mortalities of TSM recorded were corrected on a basis of natural mortality observed in the control treatment (Abbott, 1925) and were submitted to probit analysis (Finney, 1971) to generate a concentration-mortality relationship for estimating the median lethal concentration (LC_{50}) at 95% confidence intervals (CI) for each of the strains. The first two strains with lowest LC_{50} values were selected and used in further studies. The relative potencies of the strains were calculated by dividing the LC_{50} of *Bb* Plus by the LC_{50} of the tested strains. These analyses were performed using GenStat (2007).

2.2.4 Effect of *Beauveria bassiana* strains on egg hatchability

The *Bb* strains, including *Bb* Plus, used in the second screening (Section 2.2.3), were also tested for their effect on mite egg hatchability. Eggs were collected on bean leaves 24 h after infesting plants with *T. urticae* females (Section 2.2.1.1). The number of eggs varied per leaf collected (20 to 45 eggs) and were all used in bioassay. Leaves with mite eggs were placed on wet filter paper in a petri dish and sprayed with the fungal suspensions. Three doses (2×10^6; 2×10^7 and 2×10^8 conidia ml^{-1}) for each strain were applied. A control blank was also included for each strain. The bioassay protocol was the same as on adult TSM (Sections 2.2.2 and 2.2.3), except that the leaves with eggs were maintained in an incubator at 26°C and 12h light.

The number of eggs that hatched in each treatment was counted every day until 10 d post–inoculation but no more eggs hatched after 7 d. Eggs that did not hatch were checked for outgrowths of fungal mycelia under a dissecting microscope. Eggs with fungal outgrowths in the sprayed treatments were recorded as unhatched eggs due to fungal infection.

2.2.5 Germination and development of *Beauveria bassiana* on adult *Tetranychus urticae* cuticle and eggs

Bean leaves with TSM females and eggs were sprayed with a conidial suspension (2×10^8 conidia ml^{-1}) of *Bb* Strains PPRI 7315 and PPRI 7861 and incubated at 21 ± 1 ºC (16:8 h L:D regime). Adult mites and eggs were assessed for germination and development of Bb every day for 10 days after fungal conidia application. Plant materials containing TSM were kept in a deep freezer at -80ºC overnight and placed in a freeze drier for another 24 h. Freeze dried samples were then coated with 10 nm gold/palladium (ratio 3:2) and viewed under a Philips XL 30 environmental scanning electron microscope[11] (ESEM) operating at high vacuum and a voltage of 15kV.

2.2.6 Greenhouse experiments: comparison of three *Beauveria bassiana* strains for their virulence against *Tetranychus urticae*

These experiments were performed on bean plants (var. Tongati). Plants were grown in pots (75 mm diameter × 75 mm deep) placed in a plastic and water trough with a constantly circulating hydroponic solution. Water troughs were placed on tables in a hydroponic tunnel[12]. The base of each table support was set in container filled with water to prevent ants from reaching the plants. Silicone glue was applied on the support at 300 mm from the floor as a second ant trap. Ants were controlled because they can feed on living and dead mites, making it difficult to assess TSM mortality due to fungal application. Bean seeds were directly sown into pots and plants were inoculated with TSM by placing 10 females on a new emerging leaf of 2-wk old plants.

Beauveria bassiana Strain PPRI 7315 (R289) and Strain PPRI 7861 (R444) were used in the greenhouse experiments (Temperature: 16-27°C and RH: 55-70%) were compared to PPRI 5339. For each of the two first strains, conidia were formulated and provided by Plant Health Products (PHP)[13]. *Bb* Plus (PPRI 5339) is bought as a wettable

[11] Philips Electron Optics, Eindhoven, Holland
[12] Controlled Environment Facility,University of KwaZulu-Natal Pietermaritzburg, South Africa
[13] Plant Health Products (Pty) Ltd, Nottingham Road, South Africa

powder (see Section 2.2.3). Conidial concentrations were 1.9×10^9, 2.1×10^9 and 2×10^{10} conidia g^{-1} for PPRI 7315, PPRI 7861 and PPRI 5339, respectively.

The formulated products were suspended in a 0.01% (v/v) Break-thru® (polyether-polymethyl siloxane-copolymer)[14] solution acting as a surfactant. Conidial concentrations were adjusted to 2×10^7 conidia ml^{-1} for each strain. This concentration corresponded to the recommended rate of *Bb* Plus, equivalent to 1 g per litre of water. After checking the viability of conidia, suspensions were sprayed onto plants 2 wk after mite inoculation, using a 500 ml hand-held sprayer. Two sprays were applied with an interval of 7 d. The experiment involved four treatments (three *Bb* strains and a control sprayed with a Break–thru® without fungal inoculum) arranged in randomized complete block design (RCBD) with four replications. Each experimental unit (plot) consisted of two plants. Leaves were systematically removed from plants 14 d after the first spray. Mites (juveniles, adults and eggs) were counted under a dissecting microscope. Percentage mortalities of adult and juvenile mites were calculated based on total number (dead + alive), counted per plot. The number of eggs was evaluated per mm^2 of leaf area. The surface areas of leaves were measured using a portable area meter, L-3000[15]. Percentage mortalities of adults and juveniles were calculated based on total numbers counted per treatment. Data were transformed using $\log_{10}(x+1)$ or an angular transformation for percentages prior to analysis to stabilize the variance. However, means presented in the results are untransformed. Data were analyzed using ANOVA and means compared by contrasts (GenStat, 2007). The experiment was repeated once to confirm results.

2.3 Results

2.3.1 First screening bioassay

Data of total mite mortality analyzed showed that there was not much variation between the series of assays ($\chi^2 = 0.78$; $p = 0.15$; cv = 11.1%) and within replicates in each assay (cv = 11.9%). The pooled corrected mortality of adult TSM varied among strains and

[14] Evonik Industries (previously Degussa) Africa (Pty) Ltd. Halfway House, South Africa
[15] Lambda Instrument Corporation, USA

ranged from 0.5-92.8%; 23 isolates resulted in confirmed mortality levels > 50%; 31 strains caused TSM mortalities of 30-49% and the remaining 8 cause mortality of < 30% 10 d after application.

Conidial production by the strains on SDAY ranged from 3.31×10^3 to 42.36×10^3 conidia mm^{-2} (Table 2.1). In general, strains that resulted in higher mortality of mites also had a higher level of sporulation ($r = 0.59$; $p = 0.037$); among the 23 strains that caused > 50% mortality, the LT_{50} varied from 5.5 to 8.6 d (Table 2.2). Based on the LT_{50} and production of conidia, the strains PPRI 7270, PPRI 7300, PPRI 7308, PPRI 7310, PPRI 7315 and PPRI 7861 were selected for a second screening. These strains had significantly lower LT_{50} values compared to the rest of the strains ($p < 0.001$) and had higher levels of conidial production (26.1×10^3 to 42.14×10^3 conidia mm^{-2}).

Table 2.2 Mean percentages of total mortality and corrected mortality of *Tetranychus urticae* due to the application of *Beauveria bassiana* strains and their median lethal times (LT_{50s}) in the 1st laboratory screening with 2×10^7 conidia ml^{-1}.

Strains[a]	Total mortality [b]	Corrected mortality [d]	LT_{50} in days	95% confidence intervals (CI)	
				Lower limit	Upper limit
PPRI 7315	92.8 ± 4.7	92.1 ± 4.7	5.5	4.6	6.1
PPRI 7861	88.1 ± 5.7	85.9 ± 5.7	5.7	5.4	6.5
PPRI 7270	83.3 ± 4.3	80.6 ± 4.7	5.7	3.3	6.6
PPRI 7308	85.7 ± 6.8	84.3 ± 5.2	5.8	4.9	6.4
PPRI 7300	90.1 ± 4.2	89.0 ± 3.1	5.9	5.1	6.5
PPRI 7310	89.0 ± 6.7	88.1 ± 4.1	5.9	5.2	6.4
PPRI 7319	83.7 ± 6.7	82.1 ± 4.1	6.5	5.7	7.1
PPRI 7287	74.1 ± 6.3	71.5 ± 6.3	6.3	5.6	7.0
PPRI 7568	84.7 ± 6.4	83.2 ± 8.1	6.7	6.0	7.3
PPRI 7301	81.3 ± 3.7	79.5 ± 5.6	7.0	6.4	7.5
PPRI 7606	89.3 ± 3.8	88.1 ± 7.4	7.1	6.7	7.5
PPRI 7318	86.3 ± 4.2	85.0 ± 6.8	7.2	6.8	7.7
PPRI 7769	86.4 ± 4.3	85.1 ± 7.6	7.3	6.9	7.6
PPRI 7280	85.7 ± 7.0	84.3 ± 6.7	7.4	6.9	7.9
PPRI 7321	69.9 ± 2.8	67.0 ± 4.3	7.5	6.9	8.3
PPRI 7302	70.0 ± 7.6	67.0 ± 8.3	7.8	7.1	8.7
PPRI 7939	67.4 ± 4.6	64.2 ± 3.4	7.9	7.5	8.5
PPRI 7312	57.5 ± 5.6	53.3 ± 7.7	8.0	7.1	8.7
PPRI 7776	63.2 ± 6.1	59.7 ± 9.2	8.1	7.4	8.8
PPRI 7277	57.1 ± 5.1	52.8 ± 8.4	8.1	7.1	8.9
PPRI 7539	58.3 ± 3.4	54.2 ± 7.0	8.3	7.6	8.8
PPRI 7278	58.8 ± 3.1	54.7 ± 4.2	8.4	7.7	8.8
PPRI 7774	54.9 ± 6.2	50.4 ± 4.7	8.6	7.8	8.9

[a] Results for strains with less than 50% mite mortality at the end of experiment (9 d after treatment application) are not included in the table

[b] Mean ± SE of total mite mortality at the end of experiment

[c] Mean ± SE of Abbott-corrected mortality. .

Table 2.3 Mean percentage mortality and corrected mortality of mites 10 d after fungal application using five different conidial concentrations.

Strains	Concentrations (conidia ml^{-1})							
	Control	2×10^4	2×10^5	2×10^6	2×10^7	2×10^8	F [3]	P value
a)Total percentage mortality [1] [2]								
PPRI 5339	3.9 (11.4)	36.5 (38.0)	44.4 (41.8)	50.5 (45.2) ab	63.0 (52.1) c	65.5 (54.6) d	(53.1)	(<0.001)
PPRI 7270	4.3 (12.3)	41.0 (39.8)	44.7 (41.9)	50.1 (45.0) ab	79.3 (63.4) b	81.3 (66.3) c	(74.3)	(<0.001)
PPRI 7300	4.1 (11.5)	38.8 (38.9)	40.3 (39.6)	48.0 (43.8) bc	87.0 (69.2) a	89.1 (70.7) b	(83.4)	(<0.001)
PPRI 7308	3.7 (11.7)	35.1 (36.3)	42.5 (40.6)	48.1 (43.0) bc	80.3 (65.8) b	82.3 (66.8) c	(67.9)	(<0.001)
PPRI 7310	4.0 (11.1)	40.5 (39.7)	42.2 (40.1)	43.3 (40.7) c	87.6 (69.4) a	85.1 (67.7) b	(60.1)	(<0.001)
PPRI 7315	3.6 (11.4)	42.6 (40.8)	45.3 (42.3)	55.2 (48.0) a	89.1 (70.7) a	94.1 (72.1) a	(86.7)	(<0.001)
PPRI 7861	3.9 (11.3)	37.8 (38.2)	41.2 (39.9)	55.4 (48.1) a	91.5 (72.1) a	95.6 (73.4) a	(84.0)	(<0.001)
$F_{6;33}$	(1.1)ns	(3.75)ns	(2.32)ns	(4.31)	(8.87)	(4.57)		
P value	(0.51)	(0.09)	(0.10)	(0.034)	(<0.001)	(0.012)		
b)Corrected percentage mortality [1] [2]								
PPRI 5339	–	32.6 (34.8)	40.4 (39.4)	46.4 (42.9) ab	59.1 (50.3) c	61.4 (51.6) d	(43.9)	(<0.001)
PPRI 7270	–	36.3 (38.0)	39.2 (38.8)	45.1 (42.1) ab	72.6 (59.6) b	76.9 (60.5) c	(54.1)	(<0.001)
PPRI 7300	–	33.5 (35.0)	35.3 (36.1)	42.9 (40.9) bc	84.6 (67.3) a	83.6 (66.7) ab	(59.3)	(<0.001)
PPRI 7308	–	31.2 (33.2)	37.7 (38.2)	44.3 (41.8) bc	75.0 (60.4) b	78.1 (63.0) bc	(49.7)	(<0.001)
PPRI 7310	–	36.0 (37.8)	38.0 (38.1)	38.6 (38.9) c	81.2 (66.3) a	79.2 (63.4) bc	(53.6)	(<0.001)
PPRI 7315	–	38.2 (38.4)	40.1 (39.3)	50.7 (45.2) a	84.7 (67.3) a	87.9 (69.5) a	(101.3)	(<0.001)
PPRI 7861	–	33.3 (35.0)	36.3 (38.0)	51.5 (45.8) a	86.8 (69.0) a	89.3 (70.8) a	(123.1)	(<0.001)
$F_{6;33}$	–	(3.62)ns	(2.33)ns	(4.30)	(8.88)	(4.51)		
P value	–	(0.11)	(0.12)	(0.031)	(<0.001)	(0.012)		

[1] Figures in brackets are angular transformed data

[2] Lower case letters: In each column, means followed by the same letter are not significantly different by Tukey-HSD at 5% level of significance

[3] F value, 5 and 30 degrees of freedom for total mortality; 4 and 25 degrees of freedom for confirmed mortality

ns: means were not significantly different according to F test (α=5%)

2.3.2 Screening in multiple dose bioassays

Cumulative percentage mortality of TSM at 10 d after inoculation varied among the strains ($p < 0.001$; $F_{6;\ 210} = 14.14$) and between the different concentrations within the strains ($p < 0.001$; $F_{35;\ 210} = 57.39$) (Table 2.3). Different mortalities resulting from different strains of *Bb* were observed with the concentration of 2×10^8 conidia ml^{-1} and varied from 65.5-95.6%. Strains PPRI 7861 caused the highest mortality of TSM, followed by PPRI 7315. The effectiveness of these strains was not significantly different at the concentration of 2×10^8 conidia ml^{-1} (Table 2.3)

Table 2.4 Regression analysis of probit mortality and median lethal concentration (LC_{50}) estimates with 95% confidence intervals (CI) for different strains of *Beauveria bassiana* against *Tetranychus urticae* females.

Strains	Intercept	Slope ± SE	[(1)] χ^2	P	LC_{50} with 95% CI ($\times 10^5$ conidia ml^{-1})	Relative potencies [(2)]
PPRI 5339	-1.94	0.28 ± 0.04	8.46	0.813	2.36 (0.87 – 4.82)	1.00
PPRI 7270	-1.52	0.27 ± 0.01	5.32	0.688	2.27 (0.71 – 4.65)	1.04
PPRI 7300	-1.87	0.34 ± 0.03	4.25	0.988	2.13 (0.73 – 4.18)	1.11
PPRI 7308	-1.38	0.24 ± 0.02	5.79	0.950	2.33 (0.30 – 3.99)	1.01
PPRI 7310	-1.63	0.27 ± 0.03	9.26	0.753	2.29 (0.04 – 4.38)	1.03
PPRI 7315	-1.89	0.35 ± 0.04	9.14	0.763	1.22 (0.66 – 2.66)	1.93
PPRI 7861	-1.96	0.36 ± 0.02	3.31	0.993	1.13 (1.04 – 2.28)	2.08

[(1)]Chi-square (df =28), critical region set at 5%
[(2)]The LC_{50}s were compared to a commercial standard strain, PPRI 5339, to calculate the relative potencies.

Mortalities of TSM (total and confirmed mortality) resulting from the two highest concentrations (2×10^7 and 2×10^8 conidia ml^{-1}) for each of the strains, were not statistically different (Table 2.3).The standard strain PPRI 5339 used in this bioassay provided less control of TSM compared to the other strains, but it also killed more than 50% of TSM at higher concentrations (2×10^7, 2×10^8 conidia ml^{-1}).

For all conidial concentrations, the tested strains resulted in significant TSM mortalities; these mortalities tended to increase as conidial concentrations of the strains were increased. But there was no parallelism between the slopes of the strains ($\chi^2 = 2.1$; df. = 6; $p = 0.023$). Indeed, at low concentrations (2×10^4 and 2×10^5 conidia ml^{-1}), the strains had similar effects, which varied at higher concentrations. For the reason of

non-parallelism, probit analyses were performed separately for each strain and results are presented in Table 2.4. The test of goodness of fit (low χ^2 values and $p > 0.05$) indicated that there was no significant heterogeneity in concentration–mortality relationship for each of *B. bassiana* strains (Table 2.4). The lowest LC_{50}s were observed for Strain PPRI 7315 and PPRI 7861 and were far different from the commercial strain PPRI 5339, as indicated by the potency ratios in Table 2.4.

2.3.3 Effect on *Beauveria bassiana* strains on egg hatchability

The percentages of unhatched eggs were calculated based on total number and the cumulative number of hatched eggs counted in the bioassays. Numbers of unhatched eggs differed as a function of different conidial concentrations within each strain (Table 2.5). All strains reduced the level of hatchability, and there was a substantial reduction in eggs hatchability with higher conidial concentrations. Differences in egg hatchability were also observed between the strains at the same conidial concentrations ($F_{21;\ 140} = 10.61$; $p < 0.001$). Almost all of the eggs that did not hatch showed fungal outgrowths after observation under a microscope. As with adult mites, the strains PPRI 7861 and PPRI 7315 also caused more infection of eggs compared to other strains, with > 50% of reduction in egg hatchability at a concentration of 2×10^8 conidia ml^{-1}, and lowest LC_{50} values (Table 2.5).

2.3.4 Conidial germination on adult *Tetranychus urticae* cuticle and eggs

There were no apparent differences in conidial germination between the Strain PPRI 7315 and Strain PPRI 7861. Scanning electron micrographs taken on different days after inoculation showed that the fungi grew better on adult mites than eggs (Fig. 2.3). For both strains, germination of conidia occurred earlier on adult mites than eggs. Germination occurred on mites between 24 h and 48 h after *Bb* inoculation while the first germination on eggs was observed on Day 4 after *Bb* inoculation. On Day 8 after fungal application, the first cadavers with fungal outgrowths and sporulation from mite body were observed for both strains; in contrast, on eggs, fungal outgrowths were limited and no apparent sporulation was observed.

Table 2.5 Mean percentages (mean ± SE) of unhatched eggs of *Tetranychus urticae* and the LC_{50} estimates, 10 d after treatment with conidia of seven *Beauveria bassiana* strains applied at three concentrations.

Strains	Concentration (conidia ml^{-1})	% unhatched eggs [b]	% eggs with mycosis [b]	LC_{50} ($\times 10^8$ conidia ml^{-1}) and 95 % C I [a,c]
PPRI 5339	2×10^8	47.3 ± 7.3a	45.9 ± 2.7a	–
	2×10^7	43.4 ± 5.1ab	41.8 ± 3.1ab	
	2×10^6	34.1 ± 8.9b	32.3 ± 2.9b	
	Control	2.4 ± 0.3c	–	
$F_{3;21}$ (α= 5%)		18.1; p < 0.001	1.1; P = 0.04	
PPRI 7270	2×10^8	38.2 ± 6.4a	36.8 ± 3.9a	–
	2×10^7	34.7 ± 4.7a	33.0 ± 4.1a	
	2×10^6	20.1 ± 3.3b	18.2 ± 1.4b	
	Control	1.7 ± 0.5c	–	
$F_{3;21}$ (α= 5%)		17.2; p < 0.001	3.1; P = 0.01	
PPRI 7300	2×10^8	36.0 ± 5.5a	34.4 ± 3.3a	–
	2×10^7	32.2 ± 4.6a	30.5 ± 3.1ab	
	2×10^6	24.0 ± 4.3b	22.1 ± 2.4b	
	Control	2.1 ± 0.7c	–	
$F_{3;21}$ (α= 5%)		9.7; p < 0.001	1.9; P = 0.04	
PPRI 7308	2×10^8	37.2 ± 7.1a	35.6 ± 2.1a	–
	2×10^7	30.9 ± 6.4ab	29.2 ± 2.8ab	
	2×10^6	21.3 ± 6.6b	19.2 ± 3.1b	
	Control	0.9 ± 0.07c	–	
$F_{3;21}$ (α= 5%)		11.1; p < 0.001	1.9; P = 0.02	
PPRI 7310	2×10^8	43.4 ± 6.8a	41.9 ± 4.2a	–
	2×10^7	45.3 ± 4.3a	43.9 ± 3.7a	
	2×10^6	25.2 ± 5.2b	23.6 ± 3.0b	
	Control	2.6 ± 0.4c	–	
$F_{3;21}$ (α= 5%)		21.3; p < 0.001	5.1; P = 0.009	
PPRI 7315	2×10^8	53.4 ± 5.3a	51.9 ± 3.5a	0.967 (0.732– 6.887)
	2×10^7	47.2 ± 3.8a	43.7 ± 4.8a	
	2×10^6	34.4 ± 3.2b	32.6 ± 2.7b	
	Control	2.7 ± 0.8c	–	
$F_{3;21}$ (α= 5%)		19.7; p < 0.001	2.2; P = 0.03	
PPRI 7861	2×10^8	55.5 ± 3.1a	53.5 ± 3.9a	0.705 (0.162 – 4.273)
	2×10^7	51.6 ± 4.8a	48.6 ± 4.1a	
	2×10^6	34.2 ± 3.4b	31.2 ± 2.8b	
	Control	1.9 ± 0.6c	–	
$F_{3;21}$ at 5% level		26.7; p < 0.001	1.9; P = 0.01	

[a] Median lethal concentrations with 95% confidence intervals; [b] Mean values for each strain bearing the same letter within the same column were not significantly different by Tukey's HSD test at α = 5%. [c] The LC_{50} values higher than 2×10^8 conidia ml^{-1} are not included in the Table,

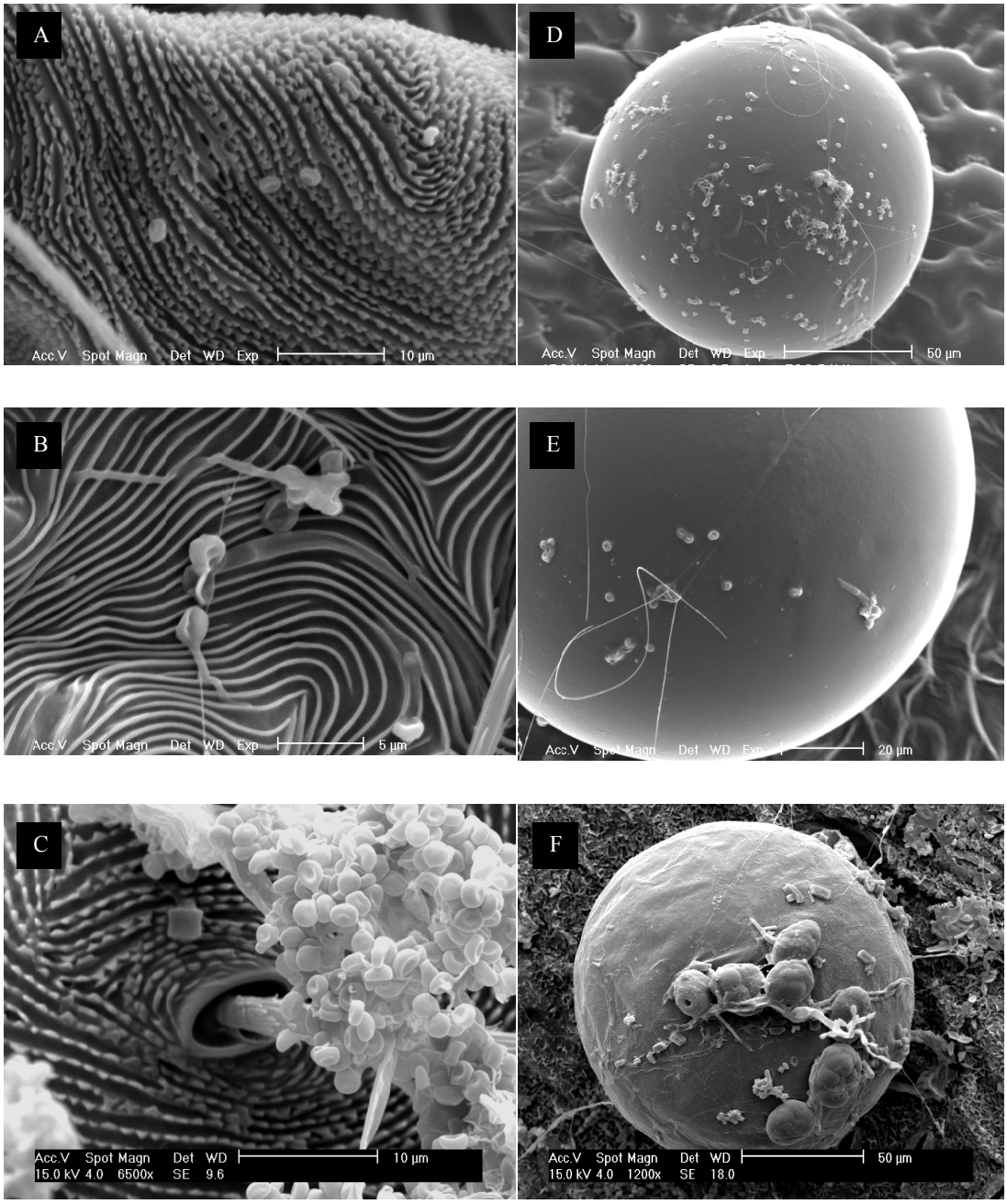

Figure 2.3 Scanning electron micrographs of *Beauveria bassiana* growth (Strain PPRI 7861) on adult females of *Tretranychus urticae* (A – C) or eggs (D-F); On Day 1 after inoculation, no germination of conidia found on either adults (A) and eggs (D); germination of conidia and penetration by germ tubes viewed at Day 2 on adults (B); conidial germination and elongation of germ viewed at Day 4 on egg (E); (C) shows intense sporulation of *Bb* from a mite body on Day 8; growth of the fungus on an egg on Day 10 less prolific (F).

2.3.5 Comparison of three *Beauveria bassiana* strains for their virulence to *Tetranychus urticae* in greenhouse trials

Application of *Bb* preparations in the greenhouse trials resulted in efficient control of TSM as measured particularly by the high percentage mortalities of adult mites in the trials (Table 2.6). Results presented in Table 2.7 showed that all the strains performed significantly better than the control ($p < 0.001$ for both juveniles and adults; and in both the trials). However, there was no significant difference between the commercial strain *Bb* Plus and the rest of the strains ($p > 0.05$), neither was PPRI 7861 different from PPRI 7315 ($p > 0.05$).

Table 2.6 Mean percentage mortalities (means ± S.E) of *Tetranychus urticae* 14 d after application of *Beauveria bassiana* strains at the concentration of 2×10^7 conidia ml^{-1} (untransformed data).

Strains	Trial 1		Trial 2		Means (*)	
	Adults	Juveniles	Adults	Juveniles	Adults	Juveniles
Control	4.8 ± 0.67	1.3 ± 0.1	3.7 ± 1.9	2.1 ± 0.9	4.25 ± 1.3	1.4 ± 0.5
PPRI 5339	77.5 ± 3.4	10.3 ± 4.6	75.9 ± 5.3	15.1 ± 2.3	76.7 ± 4.4	12.7 ± 3.5
PPRI 7315	81.3 ± 4.7	15.7 ± 1.7	79.9 ± 6.1	11.9 ± 5.1	80.6 ± 5.4	13.8 ± 3.4
PPRI 7861	85.7 ± 2.7	13.2 ± 1.1	78.1 ± 8.3	13.2 ± 4.8	81.9 ± 5.5	13.2 ± 3.0

(*) Means of pooled data of greenhouse trials (Trial 1 and Trial 2)

Table 2.7 Results of orthogonal contrasts of strains for mortality of mites 14 d after treatment.

	% adult mortality (*)		% juvenile mortality (*)	
Contrasts	F value	P value	F value	P value
a) 1st trial				
Control vs all strains	744.14	< 0.001	64.29	< 0.001
PPRI 5339 vs (PPRI 7861+ PPRI 7315)	0.03	0.984	0.41	0.536
PPRI 7861 vs PPRI 7315	1.73	0.227	0.04	0.849
a) 2nd trial				
Control vs all strains	933	< 0.001	63.69	<0.001
PPRI 5339 vs (PPRI 7861+ PPRI 7315)	0.01	0.983	0.41	0.538
PPRI 7861 vs PPRI 7315	1.89	0.213	0.04	0.847

(*) The degrees of freedom for numerator and denominator are respectively 1 and 9 with $\alpha = 5\%$; data were angular transformed prior to ANOVA.

Despite the overall significant effect of fungal treatment on adult and juvenile mites, there were no differences in the number of eggs per mm^2 of leaf area attributable to the effect of fungal conidia application because means were similar in all treatments ($p =$

0.41; $F_{3;9} = 0.4$ in the 1st trial and p = 0.087; $F_{3;9} = 1.37$ in the 2nd trial). The means of the number of eggs estimated per mm^2 of leaf area were 1.1, 0.93, 1.12 and 1.01 in Trial 1 for the control, PPRI 5339, PPRI 7315 and PPRI 7861, respectively. In Trial 2 the mean number of eggs was 0.8, 1.0, 0.7, and 0.93 per mm^2 for the control, PPRI 5339, PPRI 7315 and PPRI 7861, respectively.

2.4 Discussion

Among the 62 *B. bassiana* strains tested, few of them were highly pathogenic to adult TSM, but the mortalities of mites caused by most of them were higher than that found in the control treatment. The mortality level of the control in this study was low (less than 5%) which indicated that the conditions of the bioassay protocols were reliable (Butt & Goettel, 2000). Where it was more than 5%, the data were discarded and the bioassay was repeated to avoid misinterpretation of TSM mortality due to non-fungal effects. Higher control mortalities have been a concern in previous tetranychid mite bioassays with entomopathogenic fungi (Chandler *et al*., 2005; Shi *et al*., 2008). These are generally associated with the short life spans of the mites, and stress factors that occur during bioassays (Chandler *et al*., 2005). The control mortalities in this study were comparable to those observed by Wekesa *et al*. (2006) in *T. evansi* bioassays with *B. bassiana* and *Metarhizium anisopliae.* Almost all mite cadavers in the *Bb* treatments showed outgrowths of the fungus after incubation, confirming that the fungus was the main cause of mite death, because this was not observed on cadavers in the control treatments.

The six strains selected for the first screening bioassay had low LT_{50} values, indicating rapid infection of TSM, which is an important feature for selecting fungal isolates as potential biological control agents (Tanada & Kaya, 1993). Among other criteria there was also a high level of conidial production on SDAY, and a high final mortality of TSM attributable to the strains. The LT_{50} values of the strains measured in the first screening (5.5 to 8.9 days with a concentration of 10^7 conidia ml^{-1}) were lower than those observed by Alves *et al*. (2002) on *T. urticae* (3.9 days) with the *B. bassiana* Isolate 447, but this was applied at a concentration of 10^8 conidia ml^{-1}, equivalent to ten times the conidial concentration of the strains tested here. However, these LT_{50} values of the strains obtained in the first screening were close to those observed by Barreto *et*

al. (2004) when *Bb* isolates were tested against female green mite *Mononychellus tanajoa* at a concentration of 10^8 conidia ml^{-1}. Recently, Shi *et al*. (2008) observed an LT_{50} as low as 3.6 d on carmine mite *T. cinnabarinus* with *B. bassiana* Strain *Bb* 2860 but this could not be compared with our results because the concentration they appliedwas evaluated in conidia mm^{-2}.

Another important factor in this study was the low coefficient of variation observed among replicates in most of the bioassays and the consistency in virulence of the strains against TSM throughout the trials. For example, the strains selected in the first screening were conspicuously effective on TSM in the second bioassay. Although the strains did not have the same regression lines when probit mortalities were plotted against log-concentrations, it was observed that there was a positive linear relationship between the concentrations and the mortality of mites, and all the strains performed best at the higher concentrations. All the strains performed better than the commercial strain PPRI 5333 in the bioassay. The best strains, PPRI 7861 and PPRI 7315, had similar effects, causing more than 80% TSM mortality. A similar level of control by *Bb* isolates against tetranychid mites in laboratory trials was observed by Barreto *et al.* (2004) and Wekesa *et al*. (2006). Control of TSM with the fungal strains in this study was far better than that observed by Tamai *et al.* (1999) with the *B. bassiana* Isolate 447 and Chandler *et al.* (2005) with *B. bassiana* Isolate 432.99 when these fungal isolates were applied to mites in similar conditions.

With regard to egg bioassays, the same *Bb* strains tested were less effective than they were on juvenile or adult mites. At high concentrations of conidia, only PPRI 7861 and PPRI 7315 reduced the rate of hatching by > 50%, with an LC_{50} which was higher than that observed in the adult mite bioassays. As in the adult mite bioassays, the effect of different strains on egg hatchability was concentration-dependant. This finding corroborated with other reports on bioassays of *Bb* applied to TSM eggs (Irigaray *et al.,* 2003; Shi & Feng, 2004; Shi *et al.* 2005; Wekesa *et al.,* 2006). The poor performance of the fungal strains on eggs compared to adults has been hypothesized to be due to the topography of eggs, which is not suitable for the establishment of conidia (St. Leger *et al.,* 1991). In contrast, observations made with scanning electron microscopy in this study showed a higher number of *Bb* conidia on eggs than on adult TSM 2 d post-

inoculation. However, conidia deposited on eggs showed a poorer germination rate compared to conidia on adult mites (Fig. 2.3). Bidochka & Khachatourians (1992) concluded that there is a lack of nutrients (lipids) on mite egg shell surfaces and that these are important for the germination of conidia and subsequent growth of the fungus.

In greenhouse experiments, two strains, PPRI 7861 and 7315, were effective against TSM but were not significantly more effective than the commercial strain PPRI 5339. The control provided by Strain PPRI 5339 was worse in the laboratory than in the greenhouse. It is hypothesized that the formulation of the product may have increased the efficacy of the fungus. *Bb* Plus is a powder with *B. bassiana* conidia formulated in a carrier which enables an easy suspension of powder in water for spraying against mites. The formulation of PPRI 7861 and PPRI 7315 conidia for greenhouse application did not increase the efficacy of the strains compared to prior laboratory results.

Ultimately, the distinctive performance of *Bb* Strain PPRI 7861 (R444) and Strain PPRI 7315 (R289) compared to other strains tested in this study showed that they were the best two strains. They were highly effective on adult mites and relatively effective on mite juveniles and eggs. Also, these two strains reduced TSM under greenhouse conditions, although they caused less mortality to juveniles.

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Chapter 3

Effects of adjuvant and conidial concentration on the efficacy of *Beauveria bassiana* for the control of the two-spotted spider mite, *Tetranychus urticae*[1]

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Abstract

Greenhouse experiments were conducted on various crops to ascertain the effects of Break-thru® and a mineral oil emulsion, on *Beauveria bassiana (Bb)* applications for the control of two-spotted spider mite. The objectives were to compare a) the efficiency of *Bb* control when applied in aqueous Break-thru® or an oil emulsion; b) the effects of various concentrations of *Bb* conidia, as affected by each surfactant; and c) the effects of Break-thru® on the activity of the fungus. Conidia were suspended either in an aqueous Break-thru® or an oil-based emulsion at different conidial concentrations (1.05×10^6; 2.1×10^6 and 4.2×10^6 conidia ml^{-1}) and sprayed onto leaves 2 wk after pest inoculation. Two sprays with an interval of one week from one spray to another were performed and *T. urticae* population counts (both motile and egg stages) were made on plant leaves 7 d after each spray. *Beauveria bassiana* conidia applied in Break-thru® suspension were more efficacious than conidia suspended in a mineral oil emulsion. With the highest rate of conidia (4.2×10^6 spore ml^{-1}), mortality of adult mites ranged from 60 ± 4.2% to 85.7 ±4.3% in the Break-thru® suspension and 39.4 ± 7% to 61.3 ± 6% in the oil emulsion. Results are discussed in the present chapter.

[1] Gatarayiha, M.C., Laing, M.D. & Miller, R.M., 2009. Effects of adjuvant and conidial concentrations on the efficacy of *Beauveria bassiana* for the control of the two spotted spider mite, *Tetranychus urticae*. *Experimental and Applied Acarology.* DOI 10.1007/s10493-009-9307-6

3.1 Introduction

The most difficult problem facing two-spotted spider mite (TSM) control with entomopathogenic fungi is that this mite generally occurs in dry and hot environments (Huffaker *et al*., 1969), which are unfavourable for the development of the fungi. It has been reported that the ability of *Beauveria bassiana* (Balsamo) Vuillemin (*Bb*) propagule to persist in an environment is an important factor in its success as a biological control agent (BCA) and this is most influenced by abiotic factors such as temperature, humidity and sunlight (Fargues & Luz, 1998). The use of oil as adjuvant in BCA applications is known to protect agents from harmful environment factors and enhance their activity at the target site (Hong *et al.*, 2005). In foliar sprays for the control of plant pests, *Bb* is usually applied as conidia. These are hydrophobic and are more easily suspended in oils rather than in water. Moreover, TSMs feed primarily on the ventral leaf surfaces and produces webs; they are therefore difficult to reach by control agents during spray applications (Morimoto *et al*., 2006; Burges, 2007). It was hypothesised in this study that the use of adjuvants would increase the spread of conidia on the leaf undersides and therefore increases the chance of the target pest acquiring a lethal dose of infectious propagules.

Therefore, this study evaluated the efficiency of the *Bb* Isolate PPRI 7861, for the control of TSM, when applied with Break-thru® (a silicon wetter) or in an oil emulsion. Results from prior laboratory experiments indicated that this strain has the potential to be effective for TSM control (Chapter 2). Concentrations of conidia were compared in order to obtain the optimum application rate for each formulation. Moreover, it was investigated whether soaking conidia in Break-thru® over time would increase or decrease the efficiency of the fungal isolate.

3.2 Materials and methods

3.2.1 Plant materials and mite inoculation

Cucumber, *Cucumis sativus* L. (variety: Ashley), tomato, *Solanum lycopersicum* L. (variety Heinz), brinjal, *Solanum melongena* L. (variety: Black Beauty) and bean, *Phaseolus vulgaris* L. (variety: Tongati) were used in separate trials in this study. All these vegetable crops are important in greenhouse production in South Africa (Coertze, 1995) and susceptible to TSM attacks (Visser, 2009). Seeds were sown in trays, and seedlings were fertilized daily with nutrient solution under greenhouse conditions until they were transplanted. Seedlings were transferred into plastic pots (150 mm diameter by 150 mm deep; one seedling per pot) containing composted pine bark as growing medium and placed on gravel in a hydroponic tunnel (Controlled Environment Facility[2] - CEF) with constant circulation of nutrient solution. The daily temperature during the experiments ranged between 16ºC (night) and 31ºC (day) for the first series of trials and between 12ºC (night) and 28ºC (day) for the second series.

A TSM population was reared and maintained on tomato plants (variety: Heinz) in the greenhouse. The same variety was also used in the experiments. During the experiments, adult females were artificially inoculated onto plants at transplanting by placing a tomato leaf disc, containing 20 mites, onto the youngest fully expanded leaf of each plant. The number of mites on each leaf disc was counted under a dissecting microscope prior to inoculation.

[2] CEF, University of KwaZulu-Natal, Pietermaritzburg, South Africa

3.2.2 Fungal control agent

A formulated conidial powder containing 2.1×10^9 conidia g^{-1} of the strain PPRI 7861 was provided by Plant Health Products[3] (Pty) Ltd. (PHP). The strain had shown potential for TSM control during screening in laboratory and greenhouse trials (Chapter 2).

3.2.3 Experimental designs

The formulated conidial powder was suspended in a mineral oil (paraffin) based emulsion (0.3%), in water or in a 0.01% aqueous suspension of Break-thru® (polyether-polymethyl siloxane-copolymer[4]) at concentrations of 0, 0.5, 1 and 2 g l^{-1} (*ca. 0,* 1.05×10^6, 2.1×10^6 and 4.2×10^6 conidia ml^{-1} respectively) in a 3 × 4 factorial trial design. The mineral oil contained an emulsifier, a technical preparation by PHP (5%). The emulsifier in oil is compatible with *Bb* conidia (Dr Mike Morris, personal communication). The emulsifier and the carrier in the *Bb* formulation constitute proprietary technical information of PHP company (www.plant-health.co.za), and were not disclosed in this thesis.

Before application, the viability of conidia was assessed on Sabouraud dextrose agar medium supplemented by 0.2% yeast extract (SDAY) (Goettel & Inglis, 1997). One milligram of technical powder was suspended in 10 ml sterilized water and 0.1 ml of the suspension was spread on SDAY medium in Petri dishes using an L–shaped glass rod and incubated at 26ºC for 24 h. Germinated conidia were examined under a light compound microscope at 400 × magnification. In all experiments, more than 95% of conidia were viable. The final conidial suspension prepared was immediately used for experiments.

In each trial *Bb* was applied twice at an interval of 1 wk from one application to another, using a 500 ml hand-held sprayer. Suspensions of conidia were sprayed to runoff on the target plants. In most cases, spraying was done in the late afternoon, as suggested by Chandler *et al*. (2005), and was started 2 wk after TSM inoculation. Treatments were

[3] Plant Health Products (Pty) Ltd, Nottingham Road, South Africa
[4] Evonik Industries (previously Degussa) Africa (Pty) Ltd, Halfway House, South Africa

replicated 3 times in a randomized complete block design (RCBD). Plots consisted of five pots each and were separated at 1 m for beans and 2 m for eggplants, cucumbers and tomatoes. This separation of plots combined with pruning and trimming of the plants, prevented intermingling of foliage and contaminations of treatments with spray drift to adjacent plots. Trials on each crop were repeated twice, following the same protocol or with minor modifications in some trials. However, the bean trials consisted of checking the effect of soaking conidia in Break-thru® over time on the activity of the fungal isolate. For this, conidia (2 g l^{-1}) were suspended in aqueous Break-thru® (0.01%) and kept for different times (0.5, 1, 6, 12, 24 and 48 h) at room temperature prior to spraying.

All the trials were carried out in the greenhouse and the trial on each crop was repeated once. There were eight trials in total (Cucumber trial 1 and 2, Bean trial 1and 2, Tomato trial 1 and 2, and Eggplant trial 1 and 2). Abamectin (EC, a.i. 18 mg l^{-1}), a common acaracide used for mite control, was applied as a positive control in the trials. Except for Alto (cyproconazole), which was sprayed early in cucumber trials for the control of powdery mildew, there was no additional chemical pesticide applied in the trials.

Table 3.1 Percentage germination (mean ± S.E) of *Beauveria bassiana* conidia on PDA medium mixed with Break-thru®, 24 h after incubation at different temperatures.

Break-thru® concentration	% germination		
	20°C	25°C	30°C
Control (PDA alone)	80.2 ± 3.3a	93.1 ± 2.3a	50.1 ± 2.3bc
PDA + 0.01%	82.3 ± 1.9a	91.2 ± 3.3a	60.1 ± 3.3a
PDA + 0.02%	80.3 ± 2.4a	90.1 ± 4.0a	55.1 ± 4.3ab
PDA + 0.04%	79.2 ± 1.3a	91.2 ± 4.7a	61.1 ± .3.0a
PDA + 0.08%	60.1 ± 3.3b	59.1 ± 2.3b	47.1 ± 5.3c
PDA + 0.16%	-	-	-
F value (d.f: 5;20)	10.9	14.8	6.1
P value	p <0.001	p <0.001	p <0.001
C.V. (%)	25.3	15.6	21.1

Means followed by the same letter within the same column are not significantly different by Tukey's HSD (5% of significance level).

The effect of Break-thru® concentrations on the germination of *Bb* conidia was initially tested in the laboratory using potato dextrose agar (PDA) medium with different

concentrations of the surfactant (Table 3.1). There were no significant differences in the effect of various concentrations of Break-thru®, from 0.01 to 0.04%, on the germination of *Bb* conidia at any of the temperatures. However, at the concentration of 0.08%, there was less germination of conidia. At 0.16% Break-thru® affected the PDA medium which would not solidify. Thus, the concentration of 0.01% was adopted for making Break-thru® solutions in this study.

3.2.4 Assessment of *Tetranychus urticae* population and leaf damage

One day before the first spray and seven days after each of the two sprays, five leaves per plant from each treatment were collected and the number of mites (adults, juveniles and eggs) was counted under a stereo microscope in the laboratory. Since the laboratory was close to the greenhouse, samples were progressively collected per replication and immediately processed for mite counting to minimize within-block variation due to the sampling time. It was easy to distinguish living from dead individuals by observing their movement. Generally, living mites are mobile, whereas immobile (dead) mites fail to respond with leg movements after being lightly nudged with a fine dissecting needle. Mortality for treatments was estimated by dividing counts of cadavers over the total counts of live and dead mites.

Leaf damage index (LDI) was also assessed per plot *in situ* by scoring mite damage on plant leaves from 0 to 5 according to Meyer (1996) with few modifications: 0 = no damage; 1 = 1-20% of leaf area damaged; 2 = 21-40% of leaf area damaged; 3 = 41-60% of leaf area damaged; 4 = 61-80% of leaf area damaged; and 5 > 80% of leaf area damaged. The following formulae were used to estimate the LDIs in the trials: $LDI = \sum LDI_p / N_p$; and $LDI_p = \sum LDI_f / N_f$., where LDI is the mean leaf damage index per plot; LDI_p: the leaf damage index scored per plant; N_p: total number of scored plants per plot; LDI_f: the leaf damage index scored per leaf and N_f: the total number of leaves scored per plant.

Mite population densities on plants assessed before the first spray in the first series of trials averaged 4, 5, 6 and 8 mites per leaf respectively in cucumber, eggplant, green beans and

tomato; and in the second series of trials, on average 7, 9, 10, and 13 mites per leaf were counted respectively in eggplant, tomato, green beans and cucumber. The population density counted prior to spray was the same in the different plots for almost all trials. Where it was not the same, leaf damage index was not assessed. Differences in TSM mortality and the LDI among treatments were compared on separate sample days after analysis of variance (ANOVA) by Tukey's Honestly Significance Difference (Tukey's HSD) using the GenStat computer package (GenStat, 2007).

3.3 Results

3.3.1 Effects of two adjuvants and different *Beauveria bassiana* inoculum levels

3.3.1.1 Mite mortality

Mortality of mites assessed before the application of *Bb* did not show significant differences between plots and was less than 1% for all trials. Moreover, Break-thru® and oil solutions without the fungal conidia were sprayed as blanks. Neither adjuvant significantly controlled mites compared to unsprayed plots (Fig. 3.1).

In Cucumber trial 1, *Bb* at the rate of 2 g l^{-1} in a Break-thru® suspension efficiently controlled adult populations of mites, but not juveniles at 14 d after initial spraying, and did not significantly differ from abamectin, but was significantly different from the rest of the treatments (Fig. 3.1d & e). In Tomato trial 1 and Cucumber Trial 2, there were no mites on plants treated with abamectin at 14 d after initial spray (Fig. 3.1a & c).

In general, conspicuous mortality of mites due to *Bb* was observed at 14 d after the first spray, which was supplemented by a second spray. At 7 d after the first spray, mortality data did not show any significant difference among *Bb* conidial concentrations applied but these were highly different from the controls (unsprayed, oil alone and Break-thru® alone) and from abamectin application in which more than 70% of mite mortality was observed (Fig. 3.1e).

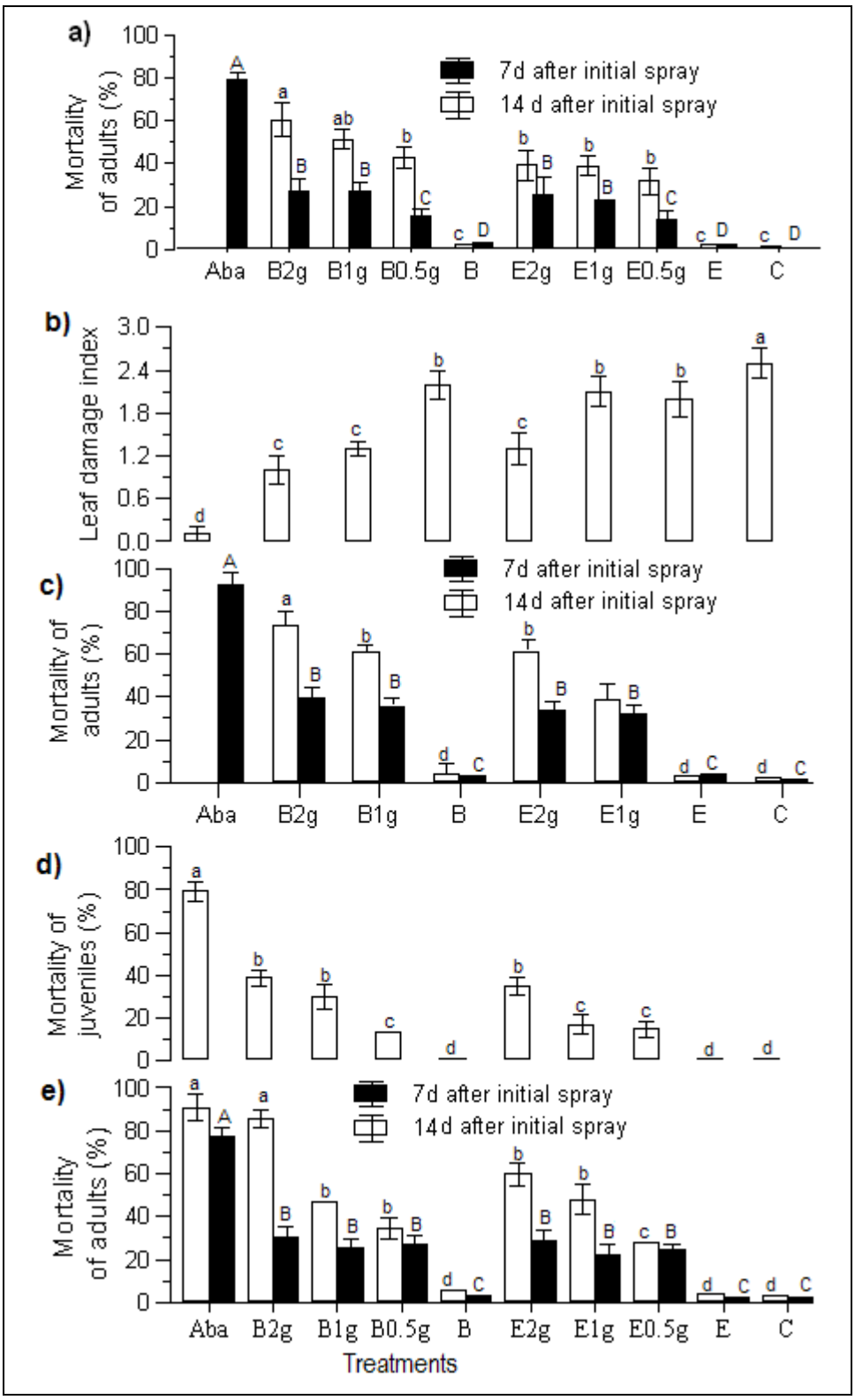
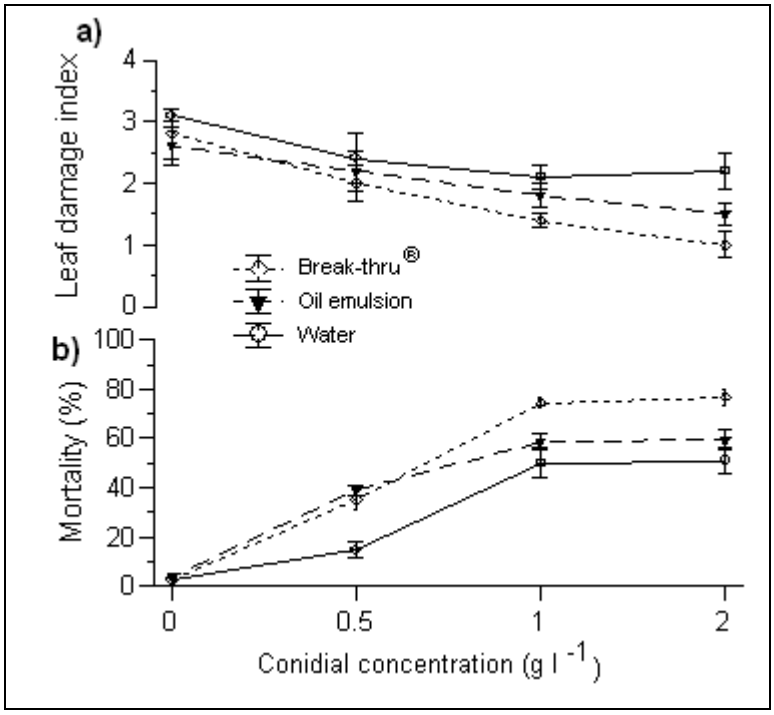


Figure 3.1 Mean (±SE) mortality of mites and leaf damage indexes in Tomato trial 1 (a), Cucumber trial 2 (b, c) and Cucumber trial 1 (d, e). Treatments: abamectin (Aba), *Bb* applied with Break-thru® at 2g l^{-1} (B2g), at 1gl^{-1} (B1g), at 0.5gl^{-1} (B0.5g); *Bb* applied with oil emulsion at 2g l^{-1} (E2g), at 1g l^{-1} (E1g), at 0.5gl^{-1} (E0.5g); Break-thru® alone (B), oil emulsion alone (E) and untreated control (C). Within each series, means followed by the same letter are not significantly different (Tukey's HSD, α=5%).

In most of the experiments, mortality levels of mites increased with the increase of conidial inoculum regardless of the adjuvant used. However, in Eggplant trial 1, mortality of adult mites assessed 14 d after spray application showed an interaction between the *Bb* concentrations and the type of adjuvant used (Table 3.2). In Eggplant trial 2 (Fig. 3.3) mite mortalities varied among *Bb* application treatments. In this trial, mite cadavers incubated at 25°C for 4 d showed growth of fungus from the body only for cadavers from *Bb* treatments. The mean percentage of mites with fungal growth was neither correlated to conidia concentrations ($r = 0.31$; $p > 0.1$) nor affected by the type of adjuvant used ($r = 0.42$; $p = 0.06$).

The effect of Break-thru® was significantly higher than that of oil in most of the experiments. Although *Bb* performed better in Break-thru® than in the oil emulsion, the addition of oil also improved the efficacy of the fungus for mite control because in Tomato trial 2 and Eggplant trial 1 significantly lower mite mortalities were observed when the fungus was applied in water (Fig. 3.2 & Table 3.2).



igure 3.2 Trends of leaf damage index (a) and mite mortality (adults + juveniles) (b) for treatments in Tomato trial 2, 14 d after initial spray. The bars are standard errors.

For juvenile populations, high mortality levels were also observed with increased concentrations of *Bb* conidia. Dead juveniles were only observed and recorded for Cucumber trial 1 (Fig. 3.1d) and Eggplant trial 1 (Table 3.2) and were remarkably low compared to adult mortalities. In neither trial were Break-thru® and oil emulsion treatments significantly different. *Beauveria bassiana* application was not very effective on the egg stage of TSM. There were no infected eggs observed during the counting of mites whether at 7 d or 14 d after initial spray and the number of eggs counted on leaves remained the same among the different treatments (Table 3.2).

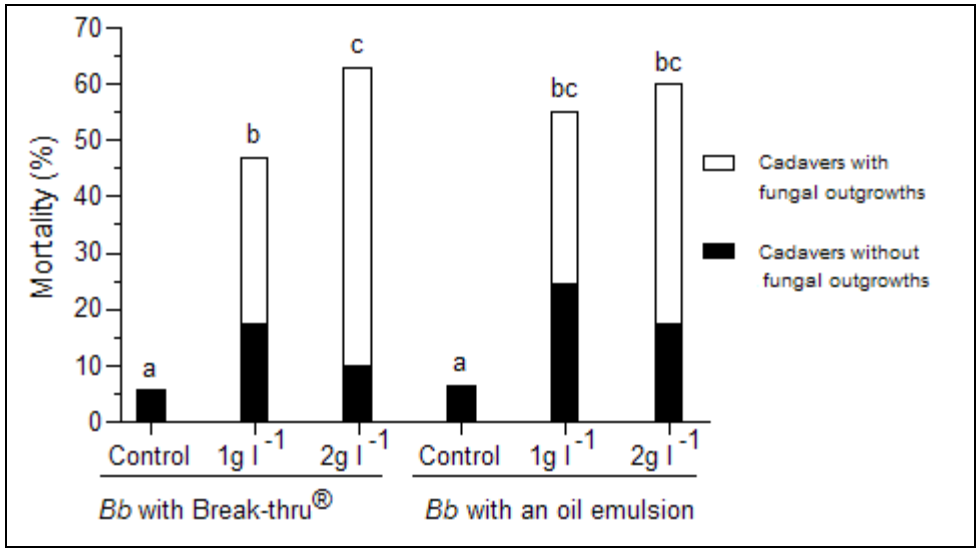


Figure 3.3 Mean percentages of dead mites and cadavers showing external development of the fungus (Eggplant Trial 2). In each of the adjuvants, *Bb* was applied at 0 (control), 1 g and 2 g of conidia per litre of solution.

3.3.1.2 Leaf damage index

Although there was no remarkable damage on Cucumber in Trial 1 or on Tomato in Trial 1, the LDI could not be assessed since the initial population density of mites before application of *B. bassiana* treatments varied among plots. In Cucumber trial 2, the means of LDI were significantly different among treatments ($F_{7, 15} = 7.2$; $p < 0.01$; Fig. 3.1b). In this trial *Beauveria bassiana* in Break-thru® solution reduced the LDI to 53.3% (2 g l^{-1}) and 49.1% (1 g l^{-1}) relative to the unsprayed control.

Table 3.2 Mite mortality and number of eggs (mean ± S.E) in Eggplant trial 1. Data were collected on 18 November 2006 (1 wk after the first spray) and 25 November 2006 (1 wk after initial spray).

Treatments		Leaf damage index		% dead adult mites		% dead juvenile mites		Number of egg.mm^{-2}	
Adjuvant	*Bb* (g l^{-1})	18th Nov.	25th Nov.	18th Nov.	25nd Nov.	18th Nov.	25th Nov.	18th Nov.	25th Nov.
Break-thru®	0	2.6 ± 0.3	3.0 ± 0.9	1.4 ± 1.3	4.1 ± 0.50	1.5 ± 0.9	0.04 ± 0.01	0.7 ± 0.2	1.2 ± 0.04
	0.5	1.7 ± 0.3	3.2 ± 0.3	21.8 ± 2.4	22.7 ± 1.2	4.8 ± 0.7	0.04 ± 0.03	0.8 ± 0.6	0.8 ± 0.03
	1	2.2 ± 0.1	2.7 ± 0.3	27.1 ± 2.1	63.6 ± 6.8	6.4 ± 1.1	0.04 ± 0.01	0.8 ± 0.3	0.7 ± 0.06
	2	2.1 ± 0.5	2.6 ± 0.3	24.5 ± 4.1	69.6 ± 4.2	10.1 ± 3.4	5.89 ± 0.8	1.2 ± 0.5	0.5 ± 0.01
Oil	0	1.9 ± 0.3	3.1 ± 0.7	2.5 ± 0.5	2.2 ± 0.90	0. 3± 0.01	1 ± 0.3	0.3 ± 0.1	1.0 ± 0.1
	0.5	2.0 ± 0.6	3.2 ± 0.9	17.4 ± 2.0	55.6 ± 7.5	0.2 ± 0.01	2 ± 0.2	1.3 ± 0.4	0.7 ± 0.4
	1	2.0 ± 0.5	2.4 ± 1.1	21.2 ± 3.1	57.8 ± 7.2	0.8 ± 0.07	1.84 ± 0.3	0.6 ± 0.3	0.9 ± 0.3
	2	1.5 ± 0.3	2.2 ± 1.0	22.6 ± 1.4	59.1 ± 2.2	1.7 ± 0.80	5.15 ± 1.1	0.7 ± 0.4	0.4 ± 0.1
Water	0	2.2 ± 0.4	2.9 ± 0.9	0.3 ± 0.02	4.9 ± 2.10	1.3 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	1.4 ± 0.5
	0.5	1.6 ± 0.9	3.2 ± 0.3	9.3 ± 2.1	35.6 ± 9.9	0.6 ± 0.4	0.7 ± 0.1	1.1 ± 0.5	1.9 ± 0.3
	1	1.7 ± 0.3	3.4 ± 0.3	20.8 ± 3.4	46.3 ± 6.7	2.9 ± 1.9	1.12 ± 0.1	0.9 ± 0.4	1.0 ± 0.4
	2	2.3 ± 0.1	3.0 ± 0.7	24.9 ± 5.0	48.4 ± 7.1	3.6 ± 0.6	5.37 ± 1.3	0.5 ± 0.1	1.3 ± 0.3
Adjuvant	$F_{2, 22}$	0.96 ns	2.55 ns	1.26 ns	18.1*	2.89 ns	0.31 ns	0.50 ns	1.93 ns
Bb	$F_{3, 22}$	0.71 ns	1.61 ns	5.30*	43.3***	0.41 ns	2.53 ns	0.85 ns	0.56 ns
Bb × adjuvant	$F_{6, 22}$	0.79 ns	0.91 ns	1.60 ns	21.1*	0.40 ns	1.86 ns	2.43 ns	1.45 ns

* Means significantly different at $p < 0.05$
*** Means highly significantly different at $p < 0.001$
ns: not significantly different

The greater reduction of mite damage using *Bb* in Break-thru® compared to water and oil was also observed in Tomato trial 2 (Fig. 3.2) and in both Cucumber trial 2 and Tomato trial 2, the LDI values were correlated to the mortality of mites ($p = 0.042$ for Cucumber trial 2; $p < 0.022$ for Tomato trial 2). However, in Eggplant trial 1 the LDI values were not significantly different among treatments (Table 3.2); the control of mite was indeed less on this crop compared to other crops.

3.3.2 Effect of Break-thru® on *Beauveria bassiana* activity

In the bean trials, the fungus performed better in Break-thru® compared to the oil emulsion treatment. There were two consecutive sprays with a 7 d interval in each trial and Fig. 3.4 represents results of data assessed at 14 d after the first spray.

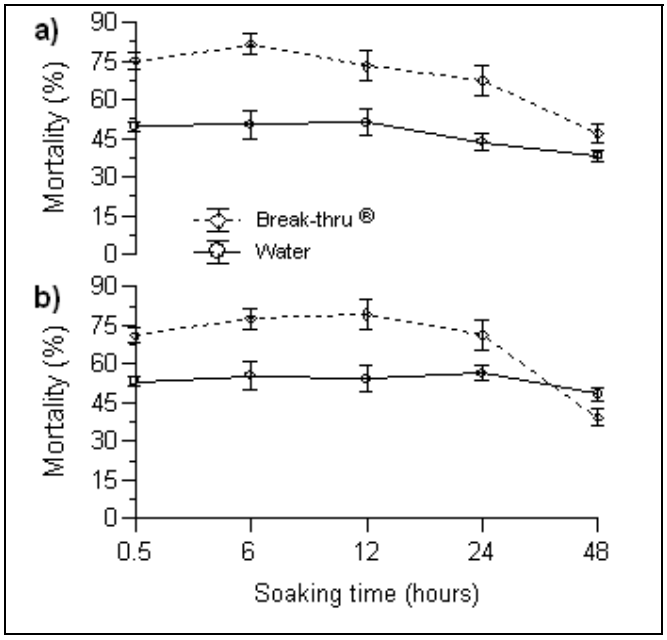


Figure 3.4 Mean (± SE) mortality of mites in different treatments 14 d after the first spray in Bean trial 1 (a) and trial 2 (b).

For the Break-thru® treatments, control efficacy did not change significantly until after 24 h of soaking time. But when the conidial suspension was kept for 48 h in Break-thru®, a decline in *Bb* control efficacy was observed ($F_{4, 18} = 5.1$; $p = 0.009$ for Trial 1 and $p = 0.03$; $F_{4, 18} = 4.1$ for Trial 2). In contrast, there was no significant difference

among treatments in water ($F_{4, 18} = 1.2$; $p = 0.2$ for Trial 1 and $F_{4, 18} = 2.3$; $p = 0.11$ for Trial 2), although mite mortality tended to decrease when *Bb* suspensions stayed in water 48 h before spraying, as in Trial 1 and Trial 2. Observations of the suspensions made under the microscope prior to spraying revealed a significant germination rate of conidia after 48 h of soaking in Break-thru® suspension ($F_{4, 11} = 4.7$; $p < 0.001$) and water ($F_{4,11} = 3$; $p = 0.02$).

3.4 Discussion

Beauveria bassiana is normally applied as conidia; these are hydrophobic and difficult to suspend in water during the foliar application. The two surfactants tested allowed conidia to be suspended uniformly and enhanced the activity of the fungus for mite control compared to water-based suspensions. *Bb* conidia applied in Break-thru® solution were more efficacious than those applied in mineral oil, as measured by a greater mortality of mites observed. Break-thru® has been proven to be an efficient surfactant registered for the use of chemical pesticides (Stevens, 1993).

However, there is evidence of the value of mineral oils in the enhancement of fungal BCA efficacy (Prior *et al*., 1988; Jones *et al.*, 1997; Kaaya & Hassan, 2000). The oil emulsion used in this study was mineral paraffin and it has been reported to be biologically compatible with fungal conidia (Smart & Wright, 1992). When comparing mineral oil and Silwet L-77 (an organosilicone) for *Bb* efficacy to control red flour beetle, Akbar *et al.* (2005) found lower median lethal concentration (LC_{50}) of conidia in the mineral oil and concluded that this was more efficient than Silwet L-77. According to these authors, the spreading quality of Silwet L-77 resulted in fewer conidia per unit area due to run off, which may explain the poor performance of the fungal conidia with this surfactant. On the other hand, when spider mites infest plants and start feeding on leaves, they produce webs which can protect them from sprays (Gerson, 1985; Morimoto *et al*., 2006). Break-thru® is a spreader and penetrant, and therefore it was presumed that its application could have resulted in a higher incidence of mite individuals acquiring infectious conidia through webs than oil emulsion during spray. This may alternatively best explain the differences in efficacy control of TSM obtained between the two surfactants.

However, there is less information available for the use of Break-thru® as an adjuvant to microbial pesticides; this study also investigated any toxicity effect on the fungal BCA. It was found that soaking conidia overnight (24 h) in Break-thru® neither increased nor decreased the control efficiency of the fungal conidia, provided that they had not germinated. After 48 h, when the fungal conidia had germinated, less control of mites was observed. When *Bb* conidia were soaked in water, conidia also germinated at 48 h but the mortality of mites was not significantly different as a result of the different periods of soaking; it was low compared to Break-thru® treatments. It was also clear that Break-thru® did not stimulate the germination of conidia.

With regard to increased mite mortality due to increased conidial concentrations, similar results were found in a previous study under laboratory conditions (see Chapter 2). Tamai *et al*. (1999) used a range of between 5×10^6 and 1×10^9 conidia ml^{-1} of *Bb* Isolate 447 on TSM females. They observed a total mortality higher than 50% only with the highest concentration. The efficiency of higher concentrations of *Bb* was also reported on cassava green mite *Mononychellus tanajoa* (Barreto *et al.*, 2004) and tomato spider mite *Tetranychus evansi* (Wekesa *et al.,* 2006).

Furthermore, the *Bb* concentrations were only effective at 14 d after initial spray. It was hypothesized that the second spray made at 7 d immediately after mite sampling might have improved the control observed at 14 d by increasing *Bb* inoculum in the treated plots. However, there has also been evidence of a delay in the mortality of mites (Feng *et al*., 1994; Alves *et al.,* 2005). It was demonstrated in previous laboratory experiments that infected mites died between 6 and 11 d following *Bb* application (see Chapter 2). This explains why explains why there were substantial number of eggs present after the different treatments with *Bb*. The fungus killed adult mites after they had laid eggs. For example, with *T. evansi*, Wekesa *et al*. (2006) reported that the female adult could still lay eggs six days after *Bb* treatment. TSM is known to lay up to 10 eggs a day (Wrensh, 1985) and has a life span that varies between 13 d and 15 d depending on the host plant and the environmental conditions (Chandler *et al.,* 2005). Application of *Bb* had an insignificant effect on the control of eggs in the greenhouse; these findings are consistent with prior results in the laboratory (see Chapter 2) where the fungal application affected eggs less than adult mites.

Another unexpected result in this study was the relatively poor control of mite juveniles by the fungus. The mortality of TSM juveniles was less than 50% in all trials performed, even with the highest concentration of conidia. Similarly, Irigaray *et al*. (2003) reported TSM juveniles to be less susceptible to *B. bassiana* infection than adults. Recently, Wekesa *et al*. (2006) found similar results on *T. evansi*. This finding contrasts with that of Susilo *et al.* (1994) who reported TSM juveniles to be more susceptible to *Neozygites floridana* than adults. According to these authors, the probable cause is the thinner cuticle of juvenile mites, which should make them more vulnerable to fungus infection.

An alternative possibility is that the series of moults made in the life cycle of juvenile mites could be responsible for the differential susceptibility between the two stages. To achieve infection, *B. bassiana,* like other entomopathogenic fungi, adheres to its host, germinates and has to penetrate through the cuticle. During moulting, any attached *B. bassiana* conidia may be shed without effecting penetration. This would be especially true for arthropods with short ecdysis intervals (Vey & Fargues, 1977). TSM juvenile stage covers three developmental stages and each of them lasts 2d at 25°C or 1d at 30°C (Wrensh, 1985).

Although *B. bassiana* Isolate PPRI 7861 was relatively ineffective against eggs and juveniles of TSM, it delivered good control of adult mites when it was applied with Break-thru® at the concentration of 2×10^7 conidia ml^{-1} as adjuvant. This treatment reduced the leaf damage index from 70% (unsprayed) to 40% in the cucumber trial.

Break-thru® was revealed to be an efficient adjuvant for the application of *Bb*, and the tests showed conclusively that it was compatible with the fungus. Thus it should be recommended for the application of the fungus in the management of TSM. Because it has to be sprayed on leaves, its toxicity to some plants will need to be investigated.

Under the greenhouse conditions of these trials, foliar application of *B. bassiana* PPRI 7861 showed promising control of against TSM, however, it still needs to be improved for an adequate level of control.

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Chapter 4

Effects of crop type on persistence and control efficacy of *Beauveria bassiana* against the two-spotted spider mite

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Abstract

The influence of five different crops (beans, *Phaseolus vulgaris* L.; cucumber, *Cucumis sativus* L.; eggplant, *Solanum melongena* L.; maize, *Zea mays* L. and tomato, *Solanum lycopersicum* L.) on persistence and the efficient control by *Beauveria bassiana* (*Bb*) of *Tetranychus urticae* Koch was investigated in the greenhouse in two trials. Plants were artificially infested with *T. urticae*. *Bb* conidia were suspended in Break-thru® solution (0.01%) at three different concentrations (0.5, 1, 2 g l^{-1}) and sprayed once onto plants. Plants in the control treatment were sprayed with Break-thru® solution alone. The population of conidia and mite mortality were evaluated. Colony-forming unit (CFU) counts of conidia recovered from plant leaves reduced over time regardless of the concentrations of *Bb* applied. The rate of decline was different among crops and significantly high on maize. However, more than 50% of the initial populations of conidia were still viable on crops three weeks after application. A longer persistence of *Bb* on a crop did not result in better control of mites. Rather mortality was positively correlated to the amount of inoculum deposited on leaves immediately after spraying. Mortality of mites was concentration-dependent and influenced by the host crop, with less control observed on maize and tomato than eggplant, beans and cucumber. Results were consistent for the two trials and confirmed the hypothesis that the type of crop can influence the efficacy of *Bb* against *T. urticae*.

4.1 Introduction

Foliar application of *Beauveria bassiana* (*Bb*) in previous experiments (Chapter 3 of the present thesis) provided varying levels of control of two-spotted spider mite (TSM) *Tetranychus urticae* Koch, on various crops. Since the crops were individually used in separate experiments, it was not possible to make a conclusion whether the crop type had an effect on the efficacy of the biocontrol agent.

However, there is evidence that the host plant of phytophagous arthropods can influence their susceptibility to entomopathogens (Tanada & Kaya, 1993). For example, the susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), to *Bb* was observed to vary with host plant, and where the insects were not suited to the host they were more susceptible to infections (Hare & Andreadis, 1983). Naturally, TSM is a highly polyphagous arthropod (Rodriguez & Rodriguez, 1987) and has a high capacity to adapt to a range of hosts (Fry, 2004), but it behaves differently on diverse host plant species (Greco *et al*., 2006), which may influence its susceptibility to *Bb* infections.

Alternatively, plants can also contain chemical compounds inhibitory to entomo-pathogens, thereby reducing their infectivity to herbivores (Costa & Gaugler, 1989; Inyang *et al*., 1999). Another factor influencing the success of foliar application of microbial control agents is their persistence on plant leaf surfaces (Ignoffo, 1992) and this can also be affected by the crop (Kouassi *et al*., 2003). Hence, in the present chapter five different crops were investigated to determine on a) whether the persistence of *Bb* conidia on leaf surfaces differed among these crops; b) whether mortality of the mite due to *Bb* application varied among the crop type; and c) whether there was a relationship between the persistence of conidia and the mortality of mites. *Bb* conidia were applied at a range of concentrations and its interaction with the crop type was investigated.

4.2 Materials and methods

4.2.1 Host plant, mite inoculation and *Beauveria bassiana* application

Beans, *Phaseolus vulgaris* L. (var. Tongati), cucumber, *Cucumis sativus* L. (var. Ashley), eggplant, *Solanum melongena* L. (var. Black Beauty), maize, *Zea mays* L. (var. Colorado) and tomato, *Solanum lycopersicum* L. (var. Heintz) were used in the experiments. These plants are commonly attacked by TSM and belong to four different families. Maize belongs to the family Poaceae, beans belong to the family Fabaceae, and cucumber belongs to the family Cucrbitaceaet, while eggplant and tomato belong to the Solanaceae family. Except for maize, these plants were also selected because they were used in previous experiments (Chapter 3) in which varying levels of *Bb* control to TSM were observed.

Bean seeds were provided by Pro-Seed[1], while cucumber, eggplant, maize and tomato seeds were provided by McDonald Seeds[2]. Seedlings were prepared in trays and transplanted into 2.5 l pots (one plant per pot) containing composted pine bark as the sole growing medium. Maize seeds were directly sown into pots. Plants were grown in the greenhouse[3] under natural light conditions. Plants were supplied with water and fertilizer through drip irrigation three times a day for five minutes. Mites were artificially inoculated onto plants at transplanting by placing them onto the youngest fully expanded leaf of each plant (Chapter 3, Section 3.2.1).

Suspensions of *Bb* conidia in Break-thru® solutions (0.01% v/v) were sprayed once onto plant leaves, to run-off, two weeks after mite inoculation, using a 2.5 l Solo 402 hand-held sprayer[4]. A formulated conidial powder of *Bb* conidia Strain PPRI 7861, containing 2.1×10^9 conidia g^{-1} was provided by Plant Health Products[5]. In the control treatment, Break-thru® solution without *Bb* was sprayed onto plants.

[1] Pro-Seed cc (Pty) Ltd, Pietermaritzburg, South Africa
[2] McDonald Seeds cc (Pty) Ltd, Pietermaritzburg, South Africa
[3] Controlled Environment Facility, University of KwaZulu-Natal, Pietermaritzburg, South Africa
[4] Solo Kleinmotoren GmbH, Germany
[5] Plant Health Products (Pty) Ltd, Nottingham Road, South Africa

4.2.2 Treatment and design structures

Conidia of *Bb* were suspended in a Break-thru® solution (0.01% v/v) at four different concentrations (0, 0.5, 1 and 2 g l^{-1}). Each concentration was applied onto each of the five crops in a split plot design with three replications, *Bb* being the whole plot (the *Bb* plots were arranged in a randomized complete block design) and crop type being the sub-plot. Each experimental unit consisted of five plants and the middle three plants were used for evaluation. The experiment was repeated once in the same greenhouse with the same protocol, and the data set of each of the trials was analysed separately.

4.2.3 Leaf sampling and recovery of *Beauveria bassiana* conidia from plants

Collection of leaf samples - To estimate the persistence of *Bb* conidia on plants, five leaf discs (*ca.* 40-60 mm of diameter each) were randomly collected from each of the three plants in each plot. Leaf discs were cut using a pair of scissors, placed into plastic bags and transported to the laboratory for processing. Between collections from one plot to another, scissors were rinsed in 70% ethanol and water. Samples were placed in a refrigerator at 4 ºC prior to being processed. Samples were processed the same day of collection. Leaf discs were collected immediately after *Bb* spray (0 wk); and at the following times after spray: 1, 2 and 3 weeks (respectively noted, 1 wk; 2 wk and 3 wk). During leaf disc collection at wk 2 and wk 3, care was taken to sample the older leaves present at the time of *Bb* application, because new leaves had developed on plants after spraying.

Recovery of conidia from leaves – Conidia were recovered from the leaf discs with the washing method described by Inglis *et al*. (1993) but with slight modifications. Leaf discs of each sample were washed in 30 ml of 0.01 M phosphate buffer solution (pH 7.0) containing 0.01% of Tween 80 in a 50 ml Erlenmeyer flask. Flasks with samples were placed on a shaker and agitated at ambient temperature for 2 h. The wash solutions were diluted 3 times at 10-fold serial dilutions. A 100-µl aliquot of each solution was inoculated onto Petri dishes with Sabouraud dextrose agar[6] medium (40 g dextrose, 10 g mycological peptone and 15 g agar in 1 l of distilled water as directed by the

[6] Biolab Diagnostics (Pty) Ltd, Midrand, South Africa

manufacturer) supplemented by 1% yeast extract (SDAY) (Goettel & Inglis, 1997). Chloramphenicol (0.04%) and cycloheximide (0.05%) were added to the medium to inhibit non-pathogenic fungi or bacteria. Petri plates were incubated at 26 ± 1ºC in darkness and the number of colony-forming units (CFUs) was recorded after four days. Conidial density (CFUs mm^{-2}) was determined per leaf disc area. Area of the leaf discs was measured using a portable area meter, L-3000[7]. To confirm the identity of *Bb*, well differentiated colonies were isolated and inoculated onto SDA medium grown on slide culture (Goettel & Inglis, 1997) and the morphological characteristics of the fungus were observed under a light microscope (400 × magnification).

4.2.4 Assessment of mite mortality

Mortality of mites (adults + juveniles) was assessed every week for three weeks from the first week after *Bb* spray application. Three leaves per plant (sections of leaves for maize) from the three plants in each plot were collected and the number of mites (adults + juveniles) was counted under a stereo microscope (see also Chapter 3, Section 3.2.3 of the present thesis). Mortality of mites in treatments was estimated by dividing counts of cadavers over the total counts of alive and dead mites. Because there was no interaction between treatments and time of assessment, mortality data for the three assessment-times were combined for analysis.

4.2.5 Statistical analysis

Data on conidial density (CFUs mm^{-2}) at each time of assessment were analyzed with factorial analysis of variance (ANOVA) (*Bb* x crop type) taking into account the split plot design for the random model (*Bb*-concentration as the whole plot). Data counts of CFUs were normalized by log transforming them (log_{10} CFUs mm^{-2}) prior to the analysis. There were very few or no conidia recovered from the control treatment (plot sprayed with Break-thru® solution alone), therefore, the analysis was performed without this treatment. Linear regression analysis was performed to determine whether conidial density declined over time with the fitted equation log CFUs = A+ BX, where A, B and X are respectively the intercept, the slope and the time of assessment. The percentage

[7] Lambda Instrument Corporation, USA

reduction of conidia three weeks after *Bb* spray was calculated relative to initial population (conidial density assessed immediately after spray) as follow:

$$Conidial\ \ density\ \ reduction\ (\%) = \frac{CFU_s t_0 - CFU_s t_i}{CFU_s t_0} x100\,;$$

where $CFU_s t_0$ are colony-forming units of conidia recovered immediately after *Bb* application and $CFU_s t_i$ are colony-forming units of conidia recovered at each of the subsequent times of recovery (1 wk, 2 wk and 3 wk after spraying).

Factorial ANOVA was also performed on mite mortality and percentage reduction of conidial population data. Percentage mortality data and reductions of conidia were angular transformed (arcsine square-root) prior to analysis; however, the means and the standard errors presented in the tables are untransformed. Where the separation of means was applicable, Tukey's Honestly Significant Difference (HSD) at 5% experiment-wise error rate was used (Jones, 1984). All analyses were done using GenStat (2007).

4.3 Results

4.3.1 Conidial population and persistence on plant leaves

Conidial density (log CFUs mm^{-2}) assessed on plant leaves varied among treatments (Table 4.1) and the results of each assessment time were consistent in both Trial 1 and Trial 2. At 0 wk (assessment done immediately after *Bb* sprays), the *Bb* concentrations applied were significantly different in their main effect ($p < 0.05$). Conidial density was also influenced by the crop type ($p < 0.001$). However there was no interaction between *Bb* concentrations and the crop ($p > 0.05$). A high number of *Bb* conidia were consistently recovered where *Bb* was sprayed at high concentrations; this was a good indication for the efficiency of the recovery. For all the *Bb* concentrations applied, conidial density was low on maize while not much difference existed between the rest of the crops (Table 4.1). As expected, the numember of conidia varied among the assessment times. For all the trials, the interaction effects *Bb* x time of assessment and *Bb* x time of assessment x crop were significant, however no significant interaction was found between time of assessment and *Bb* concentrations (Table 4.1).

Table 4.1 Population of conidia (log CFUs mm^{-2}) recovered at different times from crop leaves sprayed with different concentrations of *Beauveria bassiana.*

		Time of assessment							
		Trial 1[(a)]				Trial 2[(a)]			
***Bb* rate**	**Crop**	**0 wk**	**1 wk**	**2 wk**	**3 wk**	**0 wk**	**1 wk**	**2 wk**	**3 wk**
0.5g l^{-1}	Beans	1.12 a	1.01 a	0.79 ab	0.82 a	0.97 a	0.62 a	0.71 a	0.56 a
	Cucumber	1.22 a	1.19 a	0.95 a	0.89 a	1.01 a	0.73 a	0.68 ab	0.49 a
	Eggplant	0.91 a	0.95 a	0.68 b	0.55 b	0.87 a	0.70 a	0.60 ab	0.20 b
	Maize	0.51 b	0.42 b	0.38 c	0.28 c	0.60 b	0.31 b	0.16 c	0.10 b
	Tomato	1.37 a	1.23 a	0.95 b	0.72 b	1.10 a	0.60 a	0.51 b	0.47 a
1g l^{-1}	Beans	1.56 a	1.45 a	1.35 a	1.31 ab	1.23 b	1.01 a	0.70 b	0.72 a
	Cucumber	1.62 a	1.47 a	1.41 a	1.40 a	1.67 a	1.10 a	0.93 a	0.67 ab
	Eggplant	1.48 ab	1.41 a	1.22 a	1.05 b	1.31 b	0.98 a	0.76 ab	0.53 b
	Maize	0.83 b	0.72 b	0.62 b	0.37 c	0.78 c	0.55 b	0.32 c	0.19 c
	Tomato	1.50 a	1.46 a	1.38 a	1.20 b	1.30 b	1.12 a	0.91a	0.70 a
2g l^{-1}	Beans	1.77 a	1.63 b	1.54 a	1.28 b	1.99 a	1.50 a	1.32 a	1.11 a
	Cucumber	2.01 a	1.90 a	1.77 a	1.47 b	1.89 ab	1.38 a	1.21 a	0.99 a
	Eggplant	1.97 a	1.89 a	1.71 a	1.59 ab	1.76 b	1.31 a	1.12 a	0.86 a
	Maize	1.16 b	1.11 c	0.86 b	0.61 c	0.96 c	0.72 b	0.49 b	0.33 b
	Tomato	1.93 a	1.86 a	1.76 a	1.65 a	2.01 a	1.55 a	1.29 a	0.91 a
Bb	$F_{2;\ 4}$	18.1*	13.4*	35.3**	22.7**	13.4*	21.3*	16.1*	11.7*
Crop	$F_{4;\ 24}$	48.8***	30.2***	42.3***	31.7***	33.3***	32.6***	41.4***	27.8**
Bb x Crop	$F_{8;\ 24}$	1.7 ns	0.8 ns	2.2 ns	2.1 ns	2.4 ns	0.9 ns	2.1 ns	0.9 ns
Time	$F_{3;\ 90}$	216***				Time	$F_{3;\ 90}$	49.6***	
Time x Bb	$F_{6;\ 90}$	2.18 ns				Time x Bb	$F_{6;\ 90}$	1.7 ns	
Time x crop	$F_{12;\ 90}$	6.07***				Time x crop	$F_{12;\ 90}$	4.9***	
Time x Bb x crop	$F_{24;\ 90}$	2.7***				Time x Bb x crop	$F_{24;\ 90}$	3.7**	

[(a)] For each *Bb* concentration, means within a column followed by the same letter are not significantly different from one another according to Tukey's HSD test (at 5% of significance level); *** significant difference at $p < 0.001$; ** significant difference at $p < 0.01$; * significant difference at $p < 0.05$; ns: no significant difference.

Trends in conidial density recovered from leaves at 1 wk, 2 wk and 3 wk after *Bb* applications were consistent with those of conidia recovered immediately after *Bb* sprays (Table 4.1). Conidial density was different among *Bb* concentrations and it varied among crops. In neither trial was there any interaction effect between *Bb* and the crop at any of the three assessment times. Furthermore, the conidial density observed for each crop (regardless of the *Bb* application rates) declined significantly over time in both Trial 1 and Trial 2 as shown by the regression equations (Table 4.2). The comparison of the slopes of the regression equations showed that these were significantly different ($p = 0.029$ for Trial 1 and $p = 0.031$ for Trial 2), suggesting that

the rate of decline was different among crops. But the difference was only established between maize and the other crops, which showed similar trends in conidial decline. Additionally, changes in percentage reduction of conidial density were consistent in both the trials. At 1 wk after *Bb* sprays, there was no significant difference between the *Bb* concentrations ($p > 0.05$), the percentage reduction was unaffected by the type of the crop ($p > 0.05$), and the interaction effect *Bb* x crops was non-significant ($p > 0.05$) (Table 4.3).

Table 4.2 Regression analyses of conidial density (Log CFUs mm^{-2}) (*) assessed from plant leaves over time in the two trials.

	Intercept ± SE	t probability	Slope ± SE	t probability	R^2
Trial 1					
Beans	1.598 ± 0.071	<0.001	-0.089 ± 0.031	0.004	98.7
Cucumber	1.754 ± 0.017	<0.001	-0.124 ± 0.016	0.003	99.2
Eggplant	1.615 ± 0.071	0.002	-0.134 ± 0.026	0.035	89.6
Maize	1.144 ± 0.076	0.004	-0.151± 0.028	0.023	93.1
Tomato	1.931 ± 0.027	<0.001	-0.149 ± 0.031	0.002	99.5
Trial 2					
Beans	1.520 ± 0.111	0.005	-0.193 ± 0.032	0.041	87.9
Cucumber	1.672 ± 0.137	0.007	-0.201 ± 0.041	0.033	90.2
Eggplant	1.228 ± 0.060	0.002	-0.196 ± 0.029	0.012	96.4
Maize	0.763 ± 0.056	0.005	-0.212 ± 0.035	0.018	94.7
Tomato	1.772 ± 0.137	0.006	-0.188 ± 0.045	0.021	93.8

(*)Because there was no interaction between *Bb* levels and the time of assessment, it was possible to analyze data per crop regardless the levels of *Bb* applied.

Table 4.3 Percentage reduction (crop main effect means) of conidial population of *Beauveria bassiana* in the trials.

	Time after spraying					
	Trial 1			Trial 2		
Crops	**1 wk**	**2 wk**	**3 wk**	**1 wk**	**2 wk**	**3 wk**
Beans	24.7	46.1 b	58.5 b	54.1	64.7 b	72.0 b
Cucumber	20.1	46.6 b	59.3 b	63.6	71.6 b	76.3 b
Eggplant	10.2	52.2 b	65.8 b	50.0	64.7 b	79.1 b
Maize	18.3	66.0 a	79.8 a	54.6	75.3 a	89.3 a
Tomato	23.4	55.1 b	69.4 b	55.8	69.4 b	75.2 b
F (df = 4; 24)	ns	4.57*	5.12*	ns	5.19*	2.63*

Means within column followed by the same letter are not significantly different from one another; *significant difference at $p < 0.05$; ns: not significantly different

After 2 wk however, a significant effect for the crop main effect ($p < 0.001$ for Trial 1 and $p < 0.01$ for Trial 2); similarly, the crop main effect was significant after three weeks, ($p < 0.001$ for Trial 1 and $p < 0.01$ for Trial 2). The crop main effect means for the percentage reduction of conidial density three weeks after spraying varied between 22.47 – 53.85 and 42.96 – 72.88 respectively in Trial 1 and Trial 2 (Table 4.3). In both the trials, conidia were found to persist longer on bean, cucumber, eggplant and tomato than on maize plants.

4.3.2 Mortality of mites

Mortality data were analyzed with 4×5 (*Bb* concentration × crop) split plot ANOVA. In Trial 1, the *Bb* main effect was tested with a mean square error (MSE) (error a) of 49.89. The crop main effect and the *Bb* × crop interaction effect were tested with a MSE (error b) of 23.49. As expected, the *Bb* main effect was highly significant ($p < 0.001$, Table 4.4). A significant effect was found also for the crop main effect ($p < 0.01$), and there was a weak interaction effect between *Bb* levels and crop type ($p = 0.046$).

In Trial 2, the *Bb* main effect was tested with a MSE (error a) of 51.27 while the crop main effect and the *Bb* × crop interaction effect were each tested with a MSE (error b) of 29.21. As in Trial 1, there was a highly significant difference between the *Bb* concentrations applied ($p < 0.001$), the effect of crop was significant ($p < 0.01$), but the interaction effect *Bb* × crop fell short of statistical significance ($p > 0.05$), suggesting that the different *Bb* concentrations sprayed performed consistently among the crops. However, where *Bb* was not sprayed (control blank) there was no remarkable difference among crops, as expected.

In both these trials and as expected, a higher mortality of mites was found where *Bb* was sprayed at higher concentrations (2 g l^{-1}) (Table 4.4). Furthermore, the mortality of mites was consistently higher on beans, cucumber and eggplants. On tomato and maize, the mortality of mites was lower and much lower, respectively. Although *Bb* conidia persisted better on beans and cucumber as well, there was no linear correlation between the trends in mite mortality and the persistence of conidia observed in the crops ($p > 0.05$), but the percentage mortality of mites was positively correlated to the conidial

density recovered on crops immediately after spray ($p < 0.01$ for Trial 1; $p < 0.05$ for Trial 2).

Table 4.4 Mortality of mites (% means ± SE) assessed in Trial 1 and Trial 2.

***Bb* concentration**	**Crop**	**Trial 1**[(a) (b)]	**Trial 2**[(a) (b)]
Control	Beans	1.9 ± 0.01 a	2.5 ± 0.72 a
	Cucumber	2.8 ± 0.09 a	3.5 ± 0.81 a
	Eggplant	2.7 ± 0.11 a	2.5 ± 0.61 a
	Maize	1.7 ± 0.07 a	1.7 ± 0.79 a
	Tomato	3.6 ± 0.12 a	3.9 ± 0.50 a
0.5g l^{-1}	Beans	22.1 ± 3.9 a	24.4 ± 4.6 a
	Cucumber	23.8 ± 4.1 a	25.0 ± 2.7 a
	Eggplant	19.7 ± 3.7 ab	21.8 ± 3.9 a
	Maize	11.7 ± 2.4 b	12.4 ± 3.4 b
	Tomato	16.8 ± 4.1 ab	17.8 ± 3.8 ab
1g l^{-1}	Beans	40.7 ± 6.0 a	41.9 ± 5.7 ab
	Cucumber	39.8 ± 6.2 a	44.6 ± 7.8 a
	Eggplant	38.0 ± 4.0 a	33.6 ± 3.9 b
	Maize	13.7 ± 3.1 c	12.9 ± 4.2 c
	Tomato	23.8 ± 3.2 b	16.1 ± 4.0 c
2g l^{-1}	Beans	56.7 ± 5.3 a	50.9 ± 7.1 a
	Cucumber	50.3 ± 6.1 a	51.5 ± 9.2 a
	Eggplant	51.7 ± 3.6 a	47.6 ± 4.5 a
	Maize	25.0 ± 3.3 b	18.8 ± 3.1 c
	Tomato	34.6 ± 4.4 b	30.8 ± 5.0 b
Bb concentration	F (df:3; 6)	110.42 ***	46.03 ***
Crop	F (df:4; 32)	35.00 **	30.27 **
Bb conc. x crop	F (df:12; 32)	4.99 *	1.41 ns

[(a)] For each *Bb* concentration and in each column, means followed by the same letter are not significantly different from one another; [(b)]Except the F values which are from untransformed data, the means presented in the table are initial data ; *** significant difference at $p < 0.001$; ** significant difference at $p < 0.01$; * significant difference at $p < 0.05$; ns: not significantly different

4.4 Discussion

Conidial density of *Bb* declined over time. This was indicated by the substantial reduction of number of conidia recovered on leaves three weeks after application, relative to initial recovery (immediately after spraying). Moreover, the rate of decline was significantly different among the crops, but the difference was only established between maize and the rest of the crops because beans, cucumber, eggplant and tomato showed minimal differences in their effects. Despite a greater decline in conidial density

observed in Trial 2 than in Trial 1, the effects of treatments on the persistence of *Bb* conidia were consistent in both these trials.

Different factors can affect the persistence of *Bb* conidia on plant foliage (Ferron *et al.*, 1991; Ignoffo, 1992; Inglis *et al*., 1993; Thompson *et al*., 2006). Among them, ultraviolet (UV) light has been reported to be the most detrimental (Daoust & Pereira, 1986; Inglis *et al*., 1993). The UV light can affect differently the conidia on plant foliage depending on the nature of foliage and the light shielding provided by the plant (Ignoffo, 1992; Inglis *et al.,* 1993). In the present study, plants were grown in a greenhouse under natural light conditions, with the daily temperature varying between 15ºC (night) and 27ºC (day) in the first experiment; and between 18ºC (night) and 33ºC (day) throughout the second experiment. It is possible that the higher decline of conidial density observed on maize may be attributable to its poor coverage in plant canopy compared to other plants, resulting in less protection of conidia from the UV light. However, in greenhouse, the level of UV irradiation is normally reduced (Lipa & Smits, 1999) which may explain the longer persistence of *Bb* conidia in this study compared to field experiments in previous studies (e.g. Daoust & Pereira, 1986; Inglis *et al.,* 1993).

Under field conditions, Inglis *et al*. (1993) observed a reduction of more than 75% of the *Bb* conidial population in only four days after application on foliage of crested wheatgrass *Agropyron cristatum* (L.) Gaertn and alfalfa *Medicago sativa* L. Similarly, Daoust & Pereira (1986) reported a significant reduction in germination of *Bb* conidia recovery from cowpea foliage in a field setting, and no conidia were viable after one week. But *Bb* conidia could also persist up to 26 days on lettuce and celery foliage (Kouassi *et al*., 2003) and 28 days on alfalfa (James *et al*., 1995). For greenhouse experiments in the present study, the percentage reduction of conidial density calculated indicated that more than 50% of the initial population of conidia (recovery immediately after spray) was still viable on leaves of crops three weeks after spraying. In some crops, conidial density recovered after 3 wk where *Bb* was sprayed at the concentration of 2 g l^{-1} was noticed to be equivalent to the initial population when 1 g l^{-1} was sprayed (Table 4.1).

Persistence of entomopathogens on plant foliage has been associated with their efficacy as biocontrol agents (Ignoffo, 1992).When comparing application of *Bb* on celery and lettuce foliage for the control of tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), Kouassi *et al.* (2003) found better control on lettuce than on celery. They attributed this to the longer persistence of *Bb* propagules on leaves of the lettuce than on celery leaves. Similarly, in a field experiment with alfalfa and wheatgrass, Inglis *et al.* (1993) observed that the mortality of grasshopper nymphs due to *Bb* was correlated to the persistence of the fungal conidia on plant foliage. Given this evidence and the findings of the present study, a higher mortality of mites was expected in crops where *Bb* conidia persisted longer. Unfortunately, there was no obvious relationship between the persistence of conidia and the mortality of mites observed on plants three weeks after treatment. Rather, the control efficiency of TSM was correlated significantly to the amount of conidia recovered on plant leaves immediately after application. This suggests that *Bb* conidial residues had little effect probably because of the low mobility of mites on leaf surfaces (Kennedy & Smitley, 1985) which restrict them to a probable contact with conidia on the treated leaf surfaces.

When the mortality data of TSM for the trials were analysed, a significant crop effect was observed, suggesting that the efficiency of *Bb* varied among crops. Expectedly, the control efficacy of *Bb* was concentration-dependent and was better at 2g l^{-1}, with mean mortalities ranging from 25-56.7% in Trial 1 and from 18.8-51.5% in Trial 2. On the other hand, the inconsistency of results for the interaction *Bb* x crop type did not resolve whether there was a particular concentration of *Bb* which performed better on one crop than another. The level of control observed herein was, however, less than that found in previous experiments (Chapter 3 of this thesis), obviously because *Bb* was sprayed once, while it was sprayed twice in the previous experiments.

The comparison of mean mortalities of TSM among crops shows that control was better on beans, cucumber and eggplants than on tomato and maize plants. The lower control efficacy found on maize was probably due to low conidial inoculum retained on leaves as observed with conidial density assessed immediately after spray (Table 4.1). Low inoculum levels of a pathogen usually diminish the chances of infection of the host's population (Tanada & Kaya, 1993). Ultimately, the low number of conidia recovered in

maize can probably be attributed to the architecture and low density of its canopy which subsequently may result in a low level of "collection efficiency"[8] (Mierzejewski *et al*., 2007). But it is also probable that the architecture of the canopy may result in a reduced number of conidia per unit area due to runoff.

The relatively low efficiency of *Bb* for mite control on tomato was also observed in previous experiments (Chapter 3). A similar observation was made by Chandler *et al*. (2005). It has been reported that tomato plants possess allelochemicals, which can be inhibitory to entomopathogenic fungi (Costa & Gaugler, 1989; Poprawski *et al*., 2000; Santiago-Álvarez *et al*., 2006), thus resulting in a low control efficacy of the target pest. For example, Costa & Gaugler (1989) reported that the alkaloid tomatine significantly reduced the formation of colony development and growth of conidiophores of *Bb* on growth medium, but would also inhibit germination of the fungus on insects when ingested by the insects. Tomatine was also reported to inhibit *in vitro* colony formation and growth of *Nomuraea rileyi* (Gallardo *et al*., 1990).

Conversely, host plants can also increase the susceptibility of invertebrate herbivores to entomopathogens, i.e. through dietary stress (Tanada & Kaya, 1993; Mayer *et al*., 2002). For example, the triterpene cucurbitacin found in cucumber plants and related cucurbit species has been reported to reduce mite survival and fitness (Agrawal, 2000), which can influence their susceptibility to *Bb* infection. The fitness of TSM can also be influenced by the host acceptance and performance (Wermelinger *et al*., 1991; Agrawal, 2000). However, the five crops used in the trials in this study are all suitable hosts of TSM. The mortality of mites assessed in the control treatment was low and not different among the crops. Therefore, where *Bb* was sprayed, the differences in mortality were due to the fungal effect, taking into account minor crop effect.

The efficacy of control of *Bb* to TSM was partially host-dependent, which would explain the discrepancies in control levels of mites observed in previous experiments. The lack of correlation between persistence of conidia and mortality suggested that several sprayings are required for efficient control of TSM. To determine the optimal

[8] "Collection efficiency is the ratio of the number of drops that impact on the collecting surface to the total number of drops approaching surface"(Mierzejewski *et al*., 2007)

frequencies for effective application of the fungus, another study was conducted, results of which are presented and discussed in Chapter 5 of the present thesis.

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Chapter 5

Field evaluation of *Beauveria bassiana* efficacy for the control of the two-spotted spider mite

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Abstract

To evaluate the field efficacy of *Beauveria bassiana* applications against *Tetranychus urticae*, trials with eggplants were carried out at Ukulinga University Research Farm, during the spring of 2006 and 2007. In the 2006-Trial, 1g l^{-1} of *B. bassiana* conidia (*ca.* 0.7×10^{12} conidia ha^{-1}) were sprayed onto plants at different intervals (1 wk, 1 wk, 3 wk and 4 wk spray interval). In the 2007-Trial, *B. bassiana* conidia were sprayed at concentrations of 1 g l^{-1} and 2 g l^{-1} (*ca.* 0.8×10^{12} and 1.6×10^{12} conidia ha^{-1}) each at two intervals (1 wk and 2 wk), the *Bb* concentration and spraying interaction was checked. Based on population densities of mites and leaf damage assessed throughout the trial, and the relative efficacies computed, the *Bb* sprays made with an interval of one week or two weeks showed better control than those of three or four weeks. In both trials there was no statistical difference between 1 wk and 2 wk spray interval for all parameters measured. Furthermore, *Bb* at 2 g l^{-1} performed significantly better than 1 g l^{-1}, regardless of the spraying interval applied. No interaction was found between the spraying interval and the concentration. Ultimately, to suppress the population density of *T. urticae* and prevent eggplant damage in spring, one spray every two weeks at the rate of 2 g l^{-1} was most effective.

5.1 Introduction

The strain PPRI 7861 of *Beauveria bassiana* (*Bb*) (Balsamo) Vuillemin has the potential to control the two-spotted spider mite (TSM), *Tetranychus urticae* Koch in greenhouse applications (Chapter 3). However, it was not very effective against juveniles and eggs of TSM (Chapter 2 & 3). Additionally, because of the short life cycle of TSM and the time delay for *Bb* to infect mites, the female mites may continue to lay eggs during the *Bb* incubation period (Wekesa *et al*., 2006). Therefore, a single application of the fungus may not be sufficient to suppress the mite population in crops. Generally, efficient control by *Bb* depends primarily on its persistence on plant surfaces (Ignoffo, 1992).

In field application, persistence of *Bb* has been a concern because of environmental conditions such as ultraviolet light (Daoust & Pereira, 1986; Inglis *et al*., 1993), extreme temperatures (Inglis *et al*., 1997) and rain (Wraight & Ramos, 2002). Despite significant numbers of viable conidia observed on plants in greenhouse trials three weeks after application (Chapter 4 of the present thesis), there was no evidence of an association between the persistence of conidia and the mortality of mites. Moreover, *Bb* controls pests by contact. It is, therefore, important that emerging mites pick up a lethal dose of conidial inoculum previously deposited on leaf surfaces.

However, there has been no conclusive evidence of the effectiveness of this method of exposure (Fernandez *et al.*, 2001). The limited movement of mites on the leaf surfaces also reduces the likelihood of their contact with conidial residues (Kennedy & Smitley, 1985), and indicates that conidia in spray droplets need to hit the target pest to be more useful. In this case, the control of mites would rely on repeated applications to target the emerging cohorts in the crop. Hence, a spray interval time less than the generation time of TSM should provide more control than that of long spraying intervals. The objectives of the present study were to compare four different spraying intervals and to evaluate the field efficacy of two concentrations of *Bb* application against *T. urticae.* Field data on microbial pesticide applications are essential as initial steps towards evaluating the commercial potential of these microbes as biocontrol agents.

5.2 Materials and methods

5.2.1 Plant materials and artificial inoculation of TSM

Eggplant seedlings, *Solanum melongena* L. var. Black Beauty, were prepared in a greenhouse[1] and transplanted into the field 4-5 weeks after emergence for trials (seedlings were *ca.* 12mm high). Seeds were provided by McDonald Seeds[2]. There were two trials, one established on 01 September 2006 and the other on 27 August 2007. Both trials were carried out at Ukulinga, a research farm of the University of KwaZulu-Natal (UKZN), (29º40'S-30º24'E; alt.: 775m.a.s.l.) in the section allocated to the Plant Breeding Department[3]. Fertilizer [2N-3P-2K (22)] was broadcast (600kg ha^{-1}) and incorporated before planting. Seedlings were irrigated three times per week until establishment, thereafter for two to three weeks; irrigation was done once a week when it was necessary. After plant establishment, 2-3 wk after transplanting, plants were inoculated with *T. urticae* obtained from a culture maintained on tomato in the greenhouse at the Faculty of Science and Agriculture, UKZN. Plants were inoculated by placing infested leaf discs containing about 40 *T. urticae* females on the youngest fully expanded leaf for each plant in the trial. In the first trial, however, the first inoculation failed, probably because of heavy rain which occurred immediately after inoculation. A second mite inoculation was therefore initiated. Cypermethrin was sprayed *ad libitum* in the 2006-Trial to suppress eggplant flea beetle infestation, *Epitrix fuscula* Crotch (Coleoptera: Chrysomelidae), which occurred early in the crop. No other chemical pesticides were used. Trial maintenance (trimming, weeding, irrigation, monitoring of pests other than TSM and presence of mite predators) was done on a routine basis.

[1] Controlled Environment Facility, University of KwaZulu-Natal
[2] McDonald Seeds (Pty) Ltd, Pietermaritzburg, South Africa
[3] Faculty of Science and Agriculture, Pietermaritzburg campus

5.2.2 Fungal applications and environmental field conditions

The strain PPRI 7861 of *Bb* was used for the trials. Formulated conidial powder (*ca.* 2.1×10^9 conidia g^{-1}) was provided by Plant Health Products[4] (PHP). A conidial powder, 1 or 2 g l^{-1}, was suspended in 0.01% aqueous Break-thru® (polyether-polymethylsiloxane-copolymer) and sprayed onto plants at different intervals of time using 2.5 l Solo 402 hand-held sprayers[5]. Plants were sprayed to run off, corresponding approximately to 0.7 l per plot (*ca.* 1.47×10^9 and 2.94×10^9 conidia per plot respectively for the concentrations of 1g and 2g l^{-1}). All the sprays were applied late in the evening (4.00-6.00 pm) when the wind speed and the solar irradiation were at low levels.

Environmental conditions of the field during the study (Fig. 5.1 & 5.2) were determined from the daily weather data recorded at Ukulinga station[6] *ca.* 200 m from the trial locations. Data were kindly provided by the Agro-Climatology Department, (UKZN, Pietermaritzburg Campus, South Africa). The two trials were carried out during the spring weather of 2006 and 2007. In the 2006-Trial, the daily maximum and minimum temperature ranged from 15.4ºC to 38.8ºC and from 6ºC to 19.5ºC respectively. The mean daily RH ranged between 52.9% and 94.1%, with daily maxima between 86.5% and 97.1%. The daily total irradiation varied between 3.7 and 31.3 MJm^{-2}. During the 2007-Trial, the daily maximum and minimum temperature varied from 12.1-37.4ºC and from 9.2-21.1ºC respectively. The mean daily RH ranged from 21% to 87% with daily maximum between 81.8% and 96.7%. In both trials, temperatures below 10ºC and above 30ºC occurred only for a few days. The total solar irradiation varied between 1.9 and 31.33 MJm^{-2}.

[4] Plant Health Products (Pty) Ltd, Nottingham Road, Pietermaritzburg, South Africa
[5] Solo Kleinmotoren GmbH, Germany
[6] The station is managed under a joint project by the Council for Scientific and Industrial Research and the University of KwaZulu-Natal

5.2.3 Experimental design and trial layouts

2006-Trial – A suspension of 1g conidia l^{-1} (*ca.* 0.7×10^{12} conidia ha^{-1}) was sprayed once a week at four different time intervals (1 wk, 2 wk , 3 wk and 4 wk) making five treatments, including the control blank:

1) *Bb* sprayed every week (1 wk spray)
2) *Bb* sprayed every two weeks (2 wk spray)
3) *Bb* sprayed every three weeks (3 wk spray)
4) *Bb* sprayed every four weeks (4wk spray)
5) Control (Break-thru® solution alone sprayed every week)

Table 5.1 Spraying schedule and assessment of mite density in the 2006-Trial.

Date	Wind speed ($m\ s^{-1}$) [(a)]	1 wk spray	2 wk spray	3 wk spray	4wk spray	Control
23 Oct.	2.1 - 3.0	+	+	+	+	–
30 Oct.	2.4 - 4.0	+	–	–	–	–
06 Nov.	2.6 - 3.8	+	+	–	–	–
14 Nov[(b)]	1.9 - 3.3	+	–	+	–	–
20 Nov.	1.7 - 2.2	+	+	–	+	–
27 Nov.	1.5 - 3.6	+	–	–	–	–
04 Dec.	2.1 - 4.0	+	+	+	–	–
11 Dec.	2.5 - 3.9	+	–	–	–	–
Number of *Bb* sprays		**8**	**4**	**3**	**2**	**0**

[(a)] Range of wind speed between 4 and 6 pm period during which sprays were applied, data recorded every 10 minutes at Ukulinga weather station about 200 m from the trial location

[(b)] The spray was planned for 13 Nov. but there was heavy rain immediately after application, so it was then reapplied the following day

(+) *Bb* spray application; **(–)** Sprays with Break-thru® alone

Sprays were initiated on 23 October 2006 and subsequently applied on different occasions. There were 8 occasions in total. Treatment (1) was sprayed with *Bb* on each occasion while treatment (4) had only two *Bb* sprays (Table 5.1). On each occasion, where *Bb* was not sprayed in the plot, Break-thru® solution was sprayed to homogenize the effect of adjuvants in the treatments. Treatments were arranged in a randomized complete block design (RCBD) with four replications. Blocking was done to account for the fertility gradient in the field.

The field trial was 16 m wide and 25 m in length (400 m^2). There were 20 plots, five in each block. Each plot comprised two rows of five plants each (ten plants per plot), and the six plants in the middle were evaluated for the study. In each plot, plants within a row were spaced by 0.5 m and ther was 1 m between rows; plots within and between blocks were separated by a 3 m distance. This spacing was necessary to control the interference of treatments due to spray drift during *Bb* application.

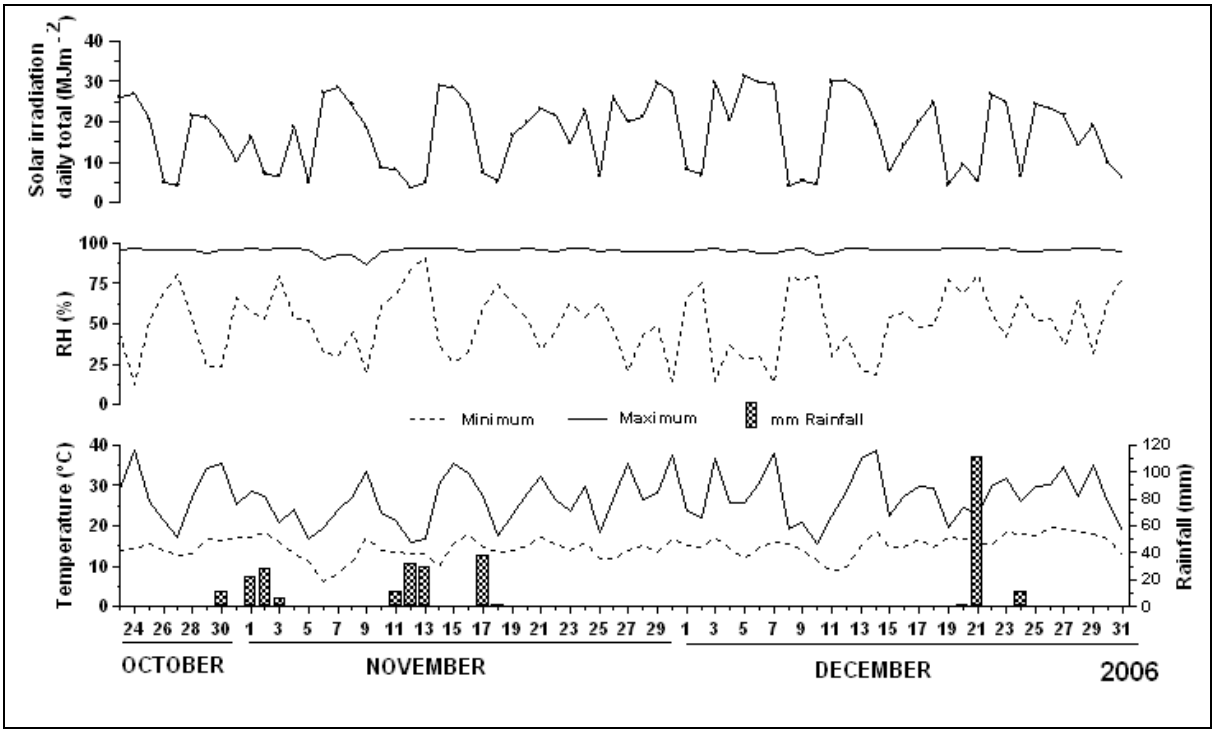

Figure 5.1 Daily weather during the 2006-Trial period (data recorded at Ukulinga station-CSIR in 2006) *ca.* 200m from the trial location. Heavy rain occurred on 21 December and no data on mite density were recorded after that.

2007-Trial – A concentration factor was included: *Bb* was sprayed at 1 and 2 g l^{-1} (*ca.* 0.8×10^{12} and 1.6×10^{12} conidia ha^{-1}). The sprays were applied once a week, every week or every two weeks. For the control treatment, Break-thru® solution was sprayed without *Bb*. It was investigated in this trial a) whether there was a difference between the two spraying intervals; b) whether there was a difference as a result of the *Bb* concentrations; and c) whether the differences as a result of the spray intervals depended on whether *Bb* was sprayed at 1 g or 2 g l^{-1}. Because the effect of TSM damage on yield was also assessed, a

positive control treatment where plants were not infested with mites was included in the trial, the number of treatments was eventually six: (1) *Bb* sprayed at 1 g l^{-1} every week; (2) *Bb* sprayed at 1 g l^{-1} every two weeks; (3) *Bb* sprayed at 2 g l^{-1} every week; (4) *Bb* sprayed at 2 g l^{-1} every two weeks; (5) the negative control (Break-thru® sprayed without *Bb*); (6) the positive control (TSM-free plants). The spray applications started on 8 October and were applied for 10 weeks (Table 5.2). As in the 2006-Trial, at each occasion of spraying, when *Bb* was not applied in the plots, Break-thru® alone was sprayed to control the variation in the treatments which may have been due to an adjuvant effect. However, plots in the positive control treatment were not sprayed. The design structure was the same as in the 2006-Trial (RCBD with four replications). There were 24 plots, each with eight plants arranged in two rows. The four in the middle were evaluated for the study. As in the 2006-Trial, plants in each plot were separated by 0.5 m within the row and rows were 1 m apart. Plots within and between blocks were 3 m apart. The total area of the field was 432 m^2 (16 m×27 m).

Table 5.2 Spraying schedule and assessment of mite density in the 2006-Trial.

Date	Wind speed ($m\ s^{-1}$) (*)	*Bb* 1g l^{-1}		*Bb* 2g l^{-1}		Control
		1 wk-spray	2 wk-spray	1 wk-spray	2 wk-spray	
08 Oct.	1.5 - 3.3	+	+	+	+	–
15 Oct.	2.2 - 3.0	+	–	+	–	–
22 Oct.	2.1 - 2.8	+	+	+	+	–
29 Oct.	1.3 - 2.2	+	–	+	–	–
05 Nov.	0.5 - 2.7	+	+	+	+	–
12 Nov.	0.3 - 2.3	+	–	+	–	–
19 Nov.	1.3 - 2.4	+	+	+	+	–
26 Nov.	1.4 - 3.2	+	–	+	–	–
03 Dec.	0.3 - 1.7	+	+	+	+	–
10 Dec.	0.1 - 2.5	+	–	+	–	–
Number of *Bb* sprays		**10**	**5**	**10**	**5**	**0**

(*) Range of wind speed between 4 - 6 pm period during which sprays were applied, data recorded each 10 minutes at Ukulinga weather station, about 200 m from the trial location

(+) *Bb* spray applications

(–) Sprays with Break-thru® solution alone

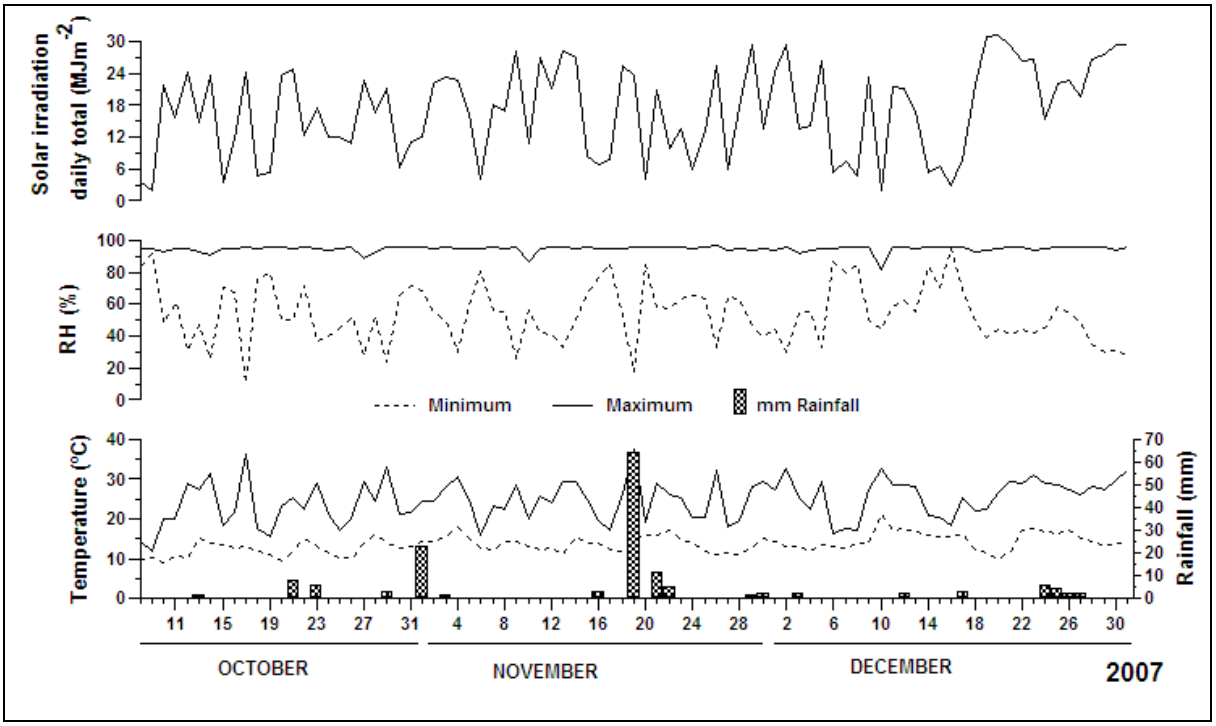

Figure 5.2 Daily weather during the 2007-Trial period (data were recorded at Ukulinga station *ca.* 200 m from the trial location).

5.2.4 Data collection and analysis

Mite population densities – The efficacies of different treatments were assessed based on *in situ* counts of living mites (Chandler *et al.*, 1979; Shi & Feng, 2006). Five leaves (one on the bottom, two in the middle and two on top of a plant) were randomly selected on each of the four middle plants in a plot for mite counts. Living adult mites were counted using a hand magnifier; the number counted in each plot was averaged and estimated per leaf. Counting was initiated before the first *Bb* spray on 22 October 2006 and 8 October 2007 for the 2006 and 2007-Trials respectively, and subsequently done every week for nine occasions in the 2006-Trial and eleven in the 2007-Trial.

The population of mites between two adjacent assessment points was estimated by computing individual mite-days as described by Ruppel (1983):

$$Mite-days = \left(X_{i+1} - X_i\right) \times \left(\frac{Y_i + Y_{i+1}}{2}\right)$$

where X_i and X_{i+1} are adjacent points of assessment times; Y_i and Yi_{+1} are the corresponding number of mites per leaf.

Change in population density and control efficacy – Based on population densities of mites assessed on the first assessment (before spraying) and the last assessment (at the end of the trial) the percentage change in mite densities and efficacy of control were estimated for each treatment. The percentage change for each treatment including the blank control (spray without *Bb*), was computed as follows:

$$\%\ change\ in\ mite\ density = \frac{Y_i - X}{X} \times 100$$

where X and Y_i are respectively the number of mites per leaf before the first spray and at ith time after the first spray.

The percentage efficacy of control was estimated relative to the control and computed using a modified Abbott's formula, as suggested by Henderson & Tilton (1955):

$$\%\, relative\ \ efficacy\ \ = (1 - \frac{T_i C_o}{T_o C_i}) \times 100$$

where: T_o = number of mites per leaf in a given treatment before the first spray

T_i = number of mites per leaf in a given treatment at ith time after the first spray

C_o = number of mites per leaf in the control treatment before at the first spray

C_i = number of mites per leaf in the control treatment ith time after the first spray.

Plant leaf damage by TSM and percentage infested leaves – Leaf damage was observed as chlorotic spots or dead leaf areas due to TSM feeding. This was assessed concurrently with mite densities on the four selected middle plants in the plot and all the leaves on each

plant were assessed. A zero to five rating scale adapted from Meyer (1996) was used (see also Chapter 3 of the present thesis):

0 = no damage;

1 = 1-20% of leaf area damaged

2 = 21-40% of leaf area damaged

3 = 41-60% of leaf area damaged

4 = 61-80% of leaf area damaged

5 > 80% of leaf area damaged

The following formulae were used to estimate the leaf damage:

$$LDI = \frac{\sum LDI_p}{N_p}, \text{ and } LDI_p = \frac{\sum LDI_f}{N_f}$$

Here, *LDI* is the mean leaf damage index per plot; LDI_p is the leaf damage index scored per plant; N_p is total number of scored plants per plot; LDI_f is the leaf damage index scored per leaf and N_f *is* the total number of leaves scored per plant. The mite-day computation was also adapted to evaluate the area under the curve of mite damage on time and the values were expressed as severity-days.

Yield assessment – In the 2007-Trial, eggplant fruits attaining marketable size (Rice *et al*., 1986) of the four selected plants in each plot were harvested and weighed immediately. The first fruits were picked on 24 November (93 days after transplanting) and subsequently every four days until 22 December, when fruits were no longer developing. The crop yield was recorded and evaluated in Kg per plot (18 m^2) for each treatment.

Statistical analyses – In the 2006-Trial, TSM did not develop in one of the plots after the second attempt of inoculation and an additional inoculation for the particular plot was not applicable since mites had already developed in other plots. It was then decided to disregard the plot resulting in an unbalanced design because one of the treatments had three replications, while the others were replicated four times. Using conventional ANOVA for this kind of data was not appropriate (Quinn & Keough, 2002). An alternative solution was to discard data for the whole block to which the plot belonged, resulting in three

replications for all the treatments and a balanced design (Underwood, 1997). However this option was regarded as unnecessary since it was possible to analyze, effectively, the data with general linear model procedures (Quinn & Keough, 2002). One of the general linear models is the restricted maximum likelihood estimation (REML), provided by the GenStat (2007) statistical package. This was adopted for analysis of the unbalanced data in the trial. The Tukey-Kramer method is recommended for comparison of means generated from unbalanced data (Hsu, 1996).

For the 2007-Trial, differences in treatment effects were determined by ANOVA after checking the underlying assumptions of this procedure (Snedecor & Cochran, 1989). When an F test was significant, means between treatments were separated by Tukey's Honestly Significant Difference with a 5% experiment-wise error rate (Hsu, 1996). Linear regression analyses were done to determine the relationship between mite-days and the severity-days values and how these variables affected the crop yield. GenStat (2007) was used for all analyses.

5.3 Results

5.3.1 2006-Trial

Initial population density was statistically the same in the trial plots at the time of the first *Bb* application ($p > 0.05$) and ranged between 18 and 23 mites per leaf (Table 5.3). On Day 7 after initial spray (AIS), the number of mites per leaf was still equal for all the treatments, but was significantly higher in the control on Day 14 AIS ($p < 0.001$). However, from Day 21 AIS throughout the trial, the number of mites per leaf varied significantly at each assessment time due different spray application intervals ($p < 0.001$ for each assessment time). The population density was consistently lower where *Bb* was sprayed at the interval of 1 wk and 2 wk compared to 3 wk and 4 wk sprays. Similar observations were also made with the overall cumulative mite-days computed for each treatment in the trial (Fig. 5.3) to give an indication of the intensity of infestation as affected by time interval of spraying.

The LDI values varied significantly among plots as a result of different spray applications (Table 5.3) from 21 d AIS onward ($p < 0.001$). *Bb* sprays applied the first week initially prevented the increase of mite damage in plants until 21 d after spraying (Table 5.3). Further decreases in mite damage were observed with subsequent sprays. The sprays made within 1 wk or 2 wk performed better than the 3 wk or 4 wk-spray intervals and the control. The overall cumulative severity-days ranged from 66.7 to 160.6 and varied significantly as a result of spray applications ($p < 0.001$, Fig. 5.3). Indeed, the damage to plant leaves was more severe where no *Bb* sprays were applied (control), with overall cumulative severity-days 1.43, 1.38, 2.12 and 2.41-fold higher than those observed in 4 wk, 3 wk, 2 wk and 1 wk-spray intervals respectively.

Table 5.3 Population densities (mean number of mites per leaf) and leaf damage indexes assessed in the 2006-Trial. Initial assessment (Day 0) was made before the first spray application for both LDIs and mite counts.

Treatments	Days after initial spray								
	0	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**
i) N_0 mites per leaf									
Control	18	57	115 a	155 a	156 a	174 a	196 a	214 a	212 a
1 wk-spray	23	48	58 b	65 c	45 d	47 d	41 c	32 d	27 c
2 wk-spay	21	49	48 b	68 c	63 c	68 c	50 c	50 c	38 c
3 wk-spray	20	51	58 b	103 b	131 b	139 b	123 b	116 b	111 b
4wk-spray	19	56	63 b	119 b	140 ab	150 b	136 b	112 b	110 b
Wald statistics (*)	2.3	1.47	4.9	99.1	124.8	178	166.6	193.9	140.3
P values	0.87	0.278	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ii) Leaf damage index									
Control	0.16	0.98	1.90	3.28 a	3.70 a	3.6 a	3.83 a	3.68 a	3.47 a
1 wk-spray	0.29	1.00	1.60	1.93 b	1.53 d	1.11 d	1.03 c	0.85 c	0.98 d
2 wk-spray	0.22	1.51	1.57	2.01 b	1.90 c	1.8 c	1.25 c	1.11 c	1.1 d
3 wk-spray	0.10	1.40	2.10	2.50 b	2.4 bc	2.63 b	2.80 b	2.3 b	1.75 c
4wk-spray	0.15	1.00	1.70	2.45 b	2.41 b	2.55 b	2.88 b	2.58 b	2.19 b
Wald statistics(*)	9.71	10.98	14.31	93.60	73.62	221.42	241.80	358.7	158.25
P values	0.203	0.152	0.074	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

(*) Wald statistics were produced by REML
In each column, mean mites per leaf with the same letter are not significantly different (Tukey-Kramer method, $\alpha = 5\%$)

Based on counts of living mites, the percentage change in mite population and relative efficacy was evaluated on each assessment occasion. The percentage change in mite densities was calculated relative to initial population assessed the day before the first spray. Positive values of the estimates implied an increase. Hence, mite densities increased in all treatments, but the increase was low where *Bb* was sprayed at a 1 wk or 2 wk interval, moderate at 3 wk and 4 wk spray intervals and high in the control (Table 5.4).

A decrease of mite density for 3 wk and 4 wk spray intervals was noticeable at Day 42 AIS, and occurred after a second spray was made for these treatments, following the initial spray. The control treatment, however, was characterized by a progressive increase in mite density until the end of the trial, suggesting that the weather conditions observed during the trial period had no direct effect on variation of mite populations.

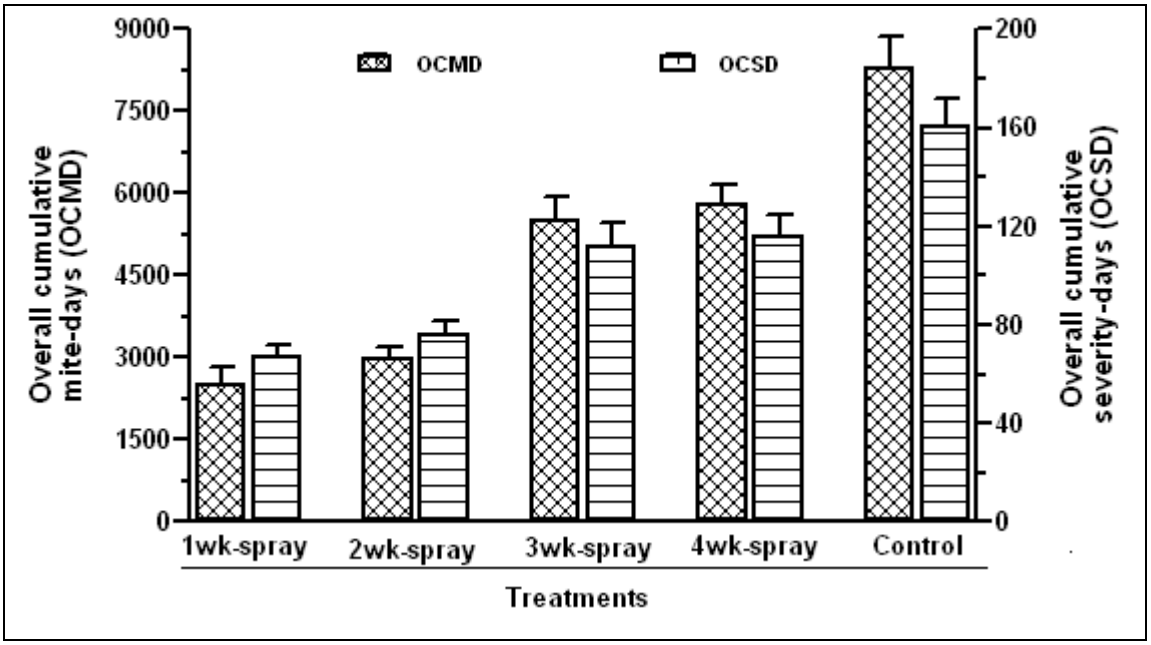

Figure 5.3 Overall cumulative mite-days per leaf (OCMD) and cumulative severity-days (OCSD) in the 2006-Trial. Treatment effects were highly significant for both parameters (Wald statistic = 242.9, $p < 0.001$ for OSMD, Wald statistic = 306.1, $p < 0.001$ for OCSD). The bars represent the standard errors (n = 3 for 4w-spray and n = 4 for the other treatments).

With regard to the efficacy control of *Bb* sprays, the effect of treatment varied significantly from Day 21 AIS onward (Table 5.4) with trends similar to those observed with mite

densities. The sprays made after a week or two weeks provided the same level of control throughout the trial, which was better than that provided by either 3 wk-spray or 4 wk-spray. For example, at the end of the trial (56 d AIS), a control efficacy of > 80% relative to the control blank was observed with the first two treatments, while it was less than 40% when *Bb* was sprayed at 3 wk or 4 wk intervals ($p < 0.01$, Table 5.4). Based on relative efficacy, density change, and cumulative mite-days and severity-days, the *Bb* sprays applied at one or two week-intervals performed consistently better in the 2006-Trial and were, therefore, considered for the 2007-Trial.

Table 5.4 Mite density change and relative control efficacy (percentage means) assessed over time in the 2006-Trial.

Treatments	Days after initial spray							
	7	**14**	**21**	**28**	**35**	**42**	**49**	**56**
i) Mite density change								
Control	218.3	558 a	772.9 a	799.1 a	910.9 a	1033.6 a	1128.9 a	1136 a
1 wk-spray	133.1	173.7 b	214.2 c	116.6 c	126.4 c	91.4 c	48 d	18.5 c
2 wk-spay	142.5	137.5 b	237 c	215 c	233.9 c	146.7 c	146.2 c	88.4 c
3 wk-spray	188	227.5 b	480 b	637.8 b	672.2 b	588.9 b	558.9 b	523.4 b
4wk-spray	218.3	307.2 ab	606.3 b	749 ab	802.9 ab	721.7 b	604.4 b	577.9 b
Wald statistics(*)	5.9	140.9	99.1	124.8	178.0	166.6	193.9	140.3
P values	0.27	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ii) % efficacy								
1 wk-spray	27.7	57.4	63.8 a	74.4 a	75.4 a	82.3 a	87.8 a	89.5 a
2 wk-spay	19.6	60.6	59.3 ab	62.3 a	62.3 a	76.3 a	79.6 a	83.1 a
3 wk-spray	8.7	46.7	31.2 bc	10.1 b	14.0 b	32.7 b	42.6 b	39.4 b
4wk-spray	0.3	29.2	12.5 c	4.2 b	1.6 b	19.3 b	42.4 b	47.0 b
Wald statistics(*)	0.18	2.43	11.52	19.6	22.96	23.5	17.5	20.1
P values	0.94	0.13	0.01	0.008	0.003	0.003	0.011	0.007

(*) Wald statistics were produced by REML
In each column, mean mites per leaf with the same letter are not significantly different (Tukey-Kramer method, $\alpha = 5\%$)

5.3.2 2007-Trial

Assessments of mite population densities and leaf damage indexes were stopped on 17 December when a decline in mite density was observed in the trial after 11 assessment events. Treatments and assessment time showed a significant interaction for both mite population and leaf damage index ($p < 0.001$ all the variables). Therefore, data were analyzed separately for each assessment.

In the first assessment (pre-spray), the number of mites per leaf ranged between 59 and 65 with no difference among treatments ($p > 0.05$, Table 5.5). Similarly, there were no differences between means of mite densities established one week after spray applications ($p > 0.05$). From Day 14 AIS onwards throughout the trial the number of mites per leaf scored in the control was significantly higher than in any of the *Bb* treatments (Table 5.5). From Day 21 AIS, mite densities were consistently lower where *Bb* was sprayed at 2 g l^{-1}, either at 1 wk or 2 wk intervals than where it was sprayed at 1 g l^{-1} (Tukey's HSD, at 5%). The highest number of mites (321 mites per leaf) was observed in the control treatment at Day 49 AIS and was about 4-11 times higher than that observed in the *Bb* treatments; then it leveled off to 317, 314 and 307 mites per leaf respectively at Day 56, 63 and 70 AIS. This reduction in the number of mites observed in the control treatment appeared one week after a heavy rain (68.5 mm) which occurred late in the trial on 19 November 2007.

Cumulative mite-days were computed for each assessment time and showed similar trends to those observed with the number of mites per leaf (Fig. 5.4). At the end of the trial (Day 70 AIS) the overall cumulative mite-days ranged between 2401 and 16996 and varied as a result of treatment applications ($F_{4,12} = 235$, $p < 0.001$; CV = 26.1%). As expected, the overall cumulative mite days was higher in the control treatment than in the *Bb* treatments combined ($p < 0.001$; Table 5.6). The two *Bb* concentrations were also significantly different in efficacy ($p < 0.001$), while the differences established as a result of the two spraying intervals were not ($p > 0.05$). No interaction existed between the *Bb* concentration and the spraying interval ($p > 0.05$).

Differences in LDI values due to treatment application were apparent 21 d AIS ($F_{4, 12}$ = 3.24; $p < 0.05$; C.V = 20.5%) and were observed throughout the trial (Table 5.5). The damage to leaves due to TSM feeding was consistently higher in the control and lower where *Bb* was sprayed at 2 g l^{-1}. However, this concentration was not significantly different from 1 g l^{-1} on many assessment occasions (Tukey's HSD, α = 5%). Similar trends were observed with the cumulative severity-days computed for the trial (Fig. 5.4).

Table 5.5 Number of living mites per leaf and leaf damage index (mean ± SE) assessed over time in the 2007-Trial.

	Days after the first application										
	0[a]	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**
i) N_0 mites per leaf[b]											
Control	61±6	72±3	123±8 a	225±21a	264±25 a	296±24 a	312±20 a	321±15 a	317 ±13 a	314±16 a	307±17 a
1g/wk	65±4	69±4	62±3 b	73 ±8b	84±5 b	105±6 b	93±7 b	99±4 b	78±6 b	71±7bc	73±3 b
1g/2 wk	59±5	66±5	68±7 b	70±10 b	98±10 b	125±20 b	112±11 b	110±6 b	83±11 b	82±11 b	80±9 b
2g/wk	64±7	67±3	54 ±5 b	28±5 c	24±4 c	25±4 c	27±5 c	28±7 c	24±3 c	25±3 c	19±4 c
2g/2 wk	62±4	70±8	69±6 b	34±8 c	22±5 c	23±7 c	39±6 c	37±9 c	28±5 c	29±5 c	20 ±3 c
$F_{4,12}$	0.9	1.3	23.8	10.1	15.4	34.4	60.4	55.5	50.4	91.2	56.6
Significance	ns	ns	***	***	***	***	***	***	***	***	***
C.V (%)	16.2	12.4	11.9	30.0	27	25	25.3	18.8	24.7	22.3	29.6
ii) Leaf damage index [b]											
Control	0.8±0.04	1.6±0.1	1.5±0.2	1.8±0.1 a	2.3±0.4 b	3.3±0.3 a	3.2±0.4 a	3.3±0.3 a	3.1±0.4 a	3.5±0.2 a	3.5±0.4 a
1g/wk	0.9±0.07	1.5±0.2	1.3±0.1	1.2±0.2 c	1.7±0.3ab	1.7±0.2 b	1.7±0.2 b	1.5±0.3 b	1.7±0.3 b	1.2±0.2 b	1.3±0.2 c
1g/2 wk	1.4±0.22	1.2±0.1	1.4±0.3	1.3±0.1 c	2.0±0.2 b	1.9±0.4 b	2.0±0.2 b	2.3±0.3 b	1.8±0.2 b	1.6±0.3 b	1.8± 0.1b
2g/wk	1.1±0.08	1.9±0.3	1.2±0.1	1.4±0.3bc	1.2±0.1 b	1.6±0.3 b	1.2±0.1 b	1.3±0.2 b	1.2±0.1 c	1.0±0.1 b	0.8±0.0 c
2g/2 wk	1.2±0.11	1.5±0.1	1.4±0.2	1.6±0.3ab	1.5±0.2 b	1.5±0.2 b	1.2±0.1 b	1.1±0.1 b	1.3±0.2 c	1.1±0.1 b	1.0±0.1 c
$F_{4,12}$	1.61	1.03	2.71	3.24	6.71	6.2	10.62	7.79	39.76	13.04	31.69
Significance	ns	ns	ns	*	*	**	**	**	**	***	***
C.V (%)	20.6	23.3	18.9	20.5	29.1	26.3	29.4	25.8	15.01	29.4	21.6

[a] Assessment done prior to spray application

[b] In each column, means of mites per leaf or leaf damage index bearing the same letter are not significantly different by Tukey's HSD test (α= 5%)

C.V (%): percentage coefficient of variation

ns: not significantly different

***: significantly different at $p < 0.001$; **: significantly different at $p < 0.01$; *: significantly different at $p < 0.05$

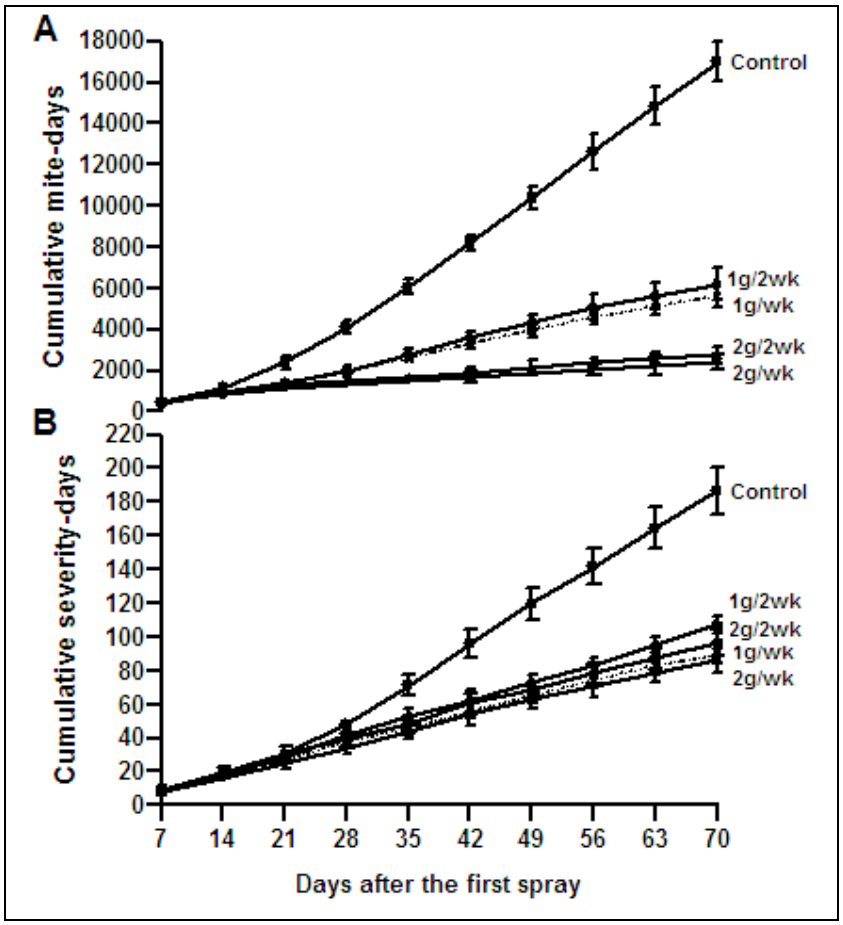

Figure 5.4 Cumulative mite-days (A) under the curve of mite number per leaf and cumulative severity-days (B) under the curve of leaf damage indexes on time, in the 2007-Trial. The bars are the standard errors of means (n=4). Where the error bars overlap for each assessment time, treatments are not significantly different (α = 5%).

The overall cumulative severity-days ranged between 86.18 (2 g / 1 wk) and 186.9 (control) with a significant difference between the control and the *Bb* treatments taken together ($F_{1,\ 12}$ = 138.69; $p < 0.001$, Table 5.6). In contrast to the findings with the cumulative mite-days, there was no significant difference established between the *Bb* concentrations ($F_{1,\ 12}$ = 4.39; $p > 0.05$). The two spraying intervals did not differ in their average means ($F_{1,\ 12}$ = 0.6; $p > 0.05$), nor was the interaction effect *Bb* concentration – spraying interval significant ($F_{1,12}$ =3.21; $p > 0.05$). Despite the differences in trends for mite densities and leaf damage indexes, the overall cumulative severity-days correlated positively with the overall mite-days per leaf ($\hat{y} = 47.69 + 0.009x$; $R^2 = 0.65$; $p < 0.01$).

Surprisingly, the levels of leaf damage by TSM observed with the different treatments including the control did not affect yield in the trial ($p > 0.5$; $R^2 = 0.21$); similarly, the yield assessed did not correlate with the population density of mites ($p > 0.05$, $R^2 = 0.37$). Yield ranged between 17.3 and 19.2 kg per plot (18 m^2) and was statistically the same for all the treatments including the two controls ($F_{5,15} = 3.71$; $p > 0.05$; C.V. = 24.2%, Fig. 5.5).

Table 5.6 Results of orthogonal contrasts of mean parameters in the 2007-Trial.

Contrasts	F value	P value	F value	P value
	Overall cumulative mite-days		Overall cumulative severity-days	
1. Control with other treatments	205.56	<0.001	138.69	<0.001
2. *Bb* concentrations $2gl^{-1}$ with $1gl^{-1}$	46.90	0.003	4.39	0.058
3. Spraying intervals 1 wk with 2 wk	3.97	0.117	0.60	0.45
4. 1 wk ($1gl^{-1}$ vs $2gl^{-1}$) with 2 wk ($1g^{-1}$ vs $2gl^{-1}$)	2.22	0.139	3.21	0.073
	% mite change at 49d		% mite change at 70d	
1.Control with other treatments	221.28	<0.001	156.27	<0.001
2. *Bb* concentrations $2gl^{-1}$ with $1gl^{-1}$	11.37	0.006	6.98	0.002
3. Spraying intervals 1 wk with 2 wk	4.56	0.058	2.24	0.061
4. 1 wk ($1gl^{-1}$ vs $2gl^{-1}$) with 2 wk ($1g^{-1}$ vs $2gl^{-1}$)	2.36	0.127	1.45	0.138
	% efficacy at 49d		% efficacy at 70d	
1.Control with other treatments	–	–	–	–
2. *Bb* concentrations $2gl^{-1}$ with $1gl^{-1}$	17.88	0.002	8.97	0.015
3. Spraying intervals 1 wk with 2 wk	1.64	0.232	1.17	0.071
4. 1 wk ($1gl^{-1}$ vs $2gl^{-1}$) with 2 wk ($1g^{-1}$ vs $2gl^{-1}$)	0.94	0.382	0.15	0.120

In Contrast 1, the control mean is compared to all other treatment means taken together
In Contrasts 2 and 3 *Bb* concentrations and spraying intervals are respectively checked for their main effects
In Contrast 4, the difference resulting from the two spraying intervals was evaluated relative to either concentration was applied (interaction effect)

The percentage change in number of mites per leaf (pre-spray versus post-sprays) and the relative control efficacy were computed for Day 49 AIS when the maximum number of mites was observed in the control, and Day 70 AIS at the end of the trial. For each of these assessment times, mite densities were significantly different as a result of different treatments ($F_{2,4} = 65.14$, $p < 0.001$ for 49 d AIS; and $F_{2,4} = 43.73$, $p < 0.001$ for 70 d AIS) with a remarkable shift of population relative to the pre-spray population. The shift was marked by a large increase in mite density in the control (Table 5.7), a moderate increase where *Bb* was sprayed at 1 g l^{-1} and a decline when *Bb* was sprayed at

2 g l^{-1} (Table 5.7). These findings demonstrated some level of control provided by the *Bb* sprays during the trial crop. This is underlined by the fact that no significant differences were established between the *Bb* treatments and the control when mites were counted on leaves before the first spray was applied ($p > 0.05$). By Day 49 AIS the control efficacy ranged between 64.5 and 91.6%, and between 74.2 and 93.8 % by Day 70 AIS (Table 5.7), with significant differences between the two concentrations of *Bb,* regardless the spraying interval applied (Table 5.6). Moreover, the two spraying intervals did not significantly differ in their main effect. The apparent increased control relative to Day 47 observed on Day 70 AIS was probably spurious because mite densities declined also in the control treatment before that day (Table 5.5) but showed similar trends to those of Day 49 AIS.

Table 5.7 Percentage change in mite density and relative efficacy (mean ± SE) evaluated at 49 d and 70 d after the first spray in the 2007-Trial.

Treatments	% mite density change(*)		% efficacy	
	49 d	70 d	49 d	70 d
Control	426.6 ± 24.5	403.2 ± 31.1	–	–
1g/wk	52.4 ± 8.4	12.4 ± 2.1	71.2 ± 3.0	78.6 ± 3.3
1g/2 wk	86.6 ± 11.3	36.0 ± 6.5	64.5 ± 2.4	74.2 ± 3.2
2g/wk	-56.3 ± 6.9	-71.6 ± 8.5	91.6 ± 4.5	94.3 ± 4.8
2g/2 wk	-40.3 ± 4.2	-68.9 ± 7.6	88.6 ± 3.3	93.8 ± 3.9

(*) The positive values indicate increase in mite density in the treatments and the negative values indicate that the number of mites declined in the treatments (refer to Table 5.6 for statistics)

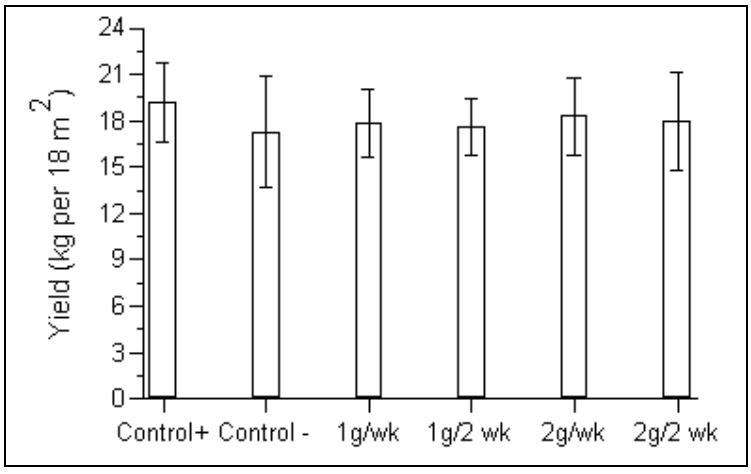

Figure 5.5 Yield parameter (mean ± SE) assessed in the 2007-Trial. No significant difference found between treatments ($F_{5, 15} = 1.71$; $p > 0.05$)

5.4 Discussion

The *Bb* strain tested here had shown potential for the control of TSM on eggplant under greenhouse conditions in other experiments (Chapters 3, 4, & 7). Under field conditions, however, microbial pesticides are known to be affected by weather conditions (Wraight & Ramos, 2002). The temperature and RH conditions of the 2006- and 2007-Trials appeared to favour disease development by *Bb* infection on TSM. In both trials, *Bb* applications provided some level of control of TSM. Findings in this study support previous reports on the field efficacies of *Bb* against citrus red mite, *Panonychus citri* McGregor (Shi & Feng, 2006) and recently against cotton spider mites, *Tetranychus* spp. (Shi *et al*., 2008). When the first spray was applied, population density assessed averaged 20 and 62 mites per leaf, in the 2006- and 2007-Trial respectively. This level of infestation is normally higher than that found in natural infestation in the field where eight mites per plant, three weeks after planting, is regarded as common in South Africa (Meyer & Craemer, 1999).

Generally, with the trends of mite population densities observed in the control treatments, the weather conditions did not directly impede the development of mites, which would have interfered with the activity of the *Bb* treatments. However, in the 2007-Trial, a noticeable decline of mite density in the control appeared after a heavy rain on 19 November (68.5 mm). Fortunately this occurred late in the crop when most of data was already taken. The impact of rain on mites has been reported previously (Pal *et al*., 1989; Leite *et al*., 2003). Pal *et al*. (1989) observed that a heavy rain (> 50 mm) washed mites from eggplant leaves. Rainfall on other days during the two trials occurred with low intensity and did not appear to affect the development of mites noticeably.

Multiple applications of microbial pesticides may improve availability of infectious inoculum, thereby providing efficient control of the target pest (James *et al*., 1995). Nevertheless, there was no conclusive evidence that control of TSM by *Bb* depended on persistence of conidia on crop foliage (Chapter 4), probably because of the limited ability of individual mites to acquire secondary conidia from the treated leaf surfaces (Chandler *et al.,* 1979; Kennedy & Smitley, 1985; Shi & Feng, 2006). Hence, repeated

applications of the fungus would directly target new emerging adults, thereby providing a better control. Based on population density, leaf damage and control efficacies, in the 2006-Trial more efficacious control was provided when *Bb* was sprayed at 1 wk or 2 wk intervals rather than 3 wk and 4 wk intervals. These results are likely reliable since they were mostly far from the critical level of significance (5%) stated for analysis (Quinn & Keough, 2002).

The lengh of the lifecycle of TSM (7-14 d) (Meyer, 1981, Gutierrez, 1994) may provide an explaination as to why sprays applied at an interval of 1 wk or 2 wk provided better control than those of 3 wk or 4wk intervals, as they could target emerging mites. Indeed, 1 wk and 2 wk intervals showed a consistently similar effect regardless whether 1g or 2 g l^{-1} of *Bb* was applied. Sprays made with 2 g l^{-1} were more effective than those of 1 g l^{-1}, suggesting that the field efficacy of *Bb* was concentration dependent. This supports the results of previous experiments in the greenhouse (Chapter 3, 4 & 7). Repeated spray applications with 2 g of *Bb* conidia per litre reduced the population of TSM by 40.7 to 56.3% at 49 d AIS and reduced the damage to leaf by 60 to 66% during the same time, but the difference between 1 g l^{-1} and 2 g l^{-1} was less evident when the leaf damage index was considered.

The greatest damage to leaves (41-60% of leaf area) was observed in the control blank. The effect of *Bb* treatments on leaf damage always appeared later than that of mite population trends. This was not surprising since the damage due to TSM feeding is only visible 2 to 3 days after mite injury has occurred (Park & Lee, 2002). On the other hand, the damage to leaves observed on plants did not adversely affect yield because the total weight of fruits harvested in each plot was statistically the same for all the treatments, including the TSM-free control. Generally, eggplants are known to be tolerant to TSM (Bostanian *et al*., 2003). The low impact of TSM on yield may also have been because severe damage to plants was observed in the crop only after flowering, and once flowers are set, fruits continue to grow. The fist set of flowers occureed 63 days after initial sprays (after 10 spray occasions). By analogy to what was observed on tomato (Stacey *et al*., 1985), it is probable that the yield losses may occur in the subsequent season. However, fruits were only harvested in one season in this study. In KwaZulu-Natal Province, eggplants produce from 10 to 15 ton ha^{-1} per season and can be grown for two

seasons; although the yield and quality of the fruits are poorer when plants are retained for a second season (Alleman & Young, 1993). Therefore, the suppression of mite populations by application of *Bb* sprays at 2 g l^{-1} should prevent loss of yield, as a result of TSM infestation, in the second season. Notably, this would be more important when a perennial crop is considered. From an economic perspective, spraying 2 g l^{-1} (*ca.* 1.6×10^{12} conidia ha^{-1}) every week may not be profitable, since no substantial difference in efficacy control existed between the one week and two-week spray intervals. Therefore it is proposed that applying 2 g l^{-1} of conidia every two weeks would be equally effective and would cost half as much to apply, in both product and labour costs.

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Chapter 6

In vitro tests and bioassays for the compatibility of two selected chemical fungicides with *Beauveria bassiana*

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Abstract

The fungicides azoxystrobin (a strobilurin) and flutriafol (a triazole) were tested *in vitro* for their effects on the germination of conidia and mycelial growth of *Beauveria bassiana*. The fungus was inoculated to Sabouraud dextrose agar + yeast (SDAY) containing each of the fungicides at three different concentrations [the recommended rate for field use (1×X) and the dilutions 10^{-1}×X and 10^{-2}×X]. The control consisted of SDAY medium without the chemicals. The fungicides were also tested, at the same concentrations, in a bioassay for their effect on *B. bassiana* activity against *Tetranychus urticae.* Flutriafol was the most harmful at all the concentrations tested *in vitro*. It drastically reduced the germination of *Bb* conidia (55.1- 92.4 % 24 h after inoculation) and showed a strong to a complete inhibition of mycelia after 14 d of incubation period. In bioassays, flutriafol also reduced the efficacy of *Bb* against mites. The inhibitive effect of azoxystrobin *in vitro* varied with the concentration applied. A significant effect was observed at 1×X and 10^{-1}×X concentrations on both the germination of conidia and mycelial growth. At 10^{-2}×X concentration, azoxystrobin showed little effect on *Bb*. However, when this fungicide was tested in bioassays none of the concentrations reduced the *Bb* activity against mites.

6.1 Introduction

Two-spotted spider mite (TSM) *Tetranychus urticae* Koch may occur together with other pests or pathogens in the crop. Notably, the increase of plant wetness due to regular spray applications to suppress TSM populations has been associated with outbreaks of fungal diseases (Dr. J.L Hatting, pers. com.). Since chemical pesticides can be applied for the control of fungal diseases or other pests, their effects on *Beauveria bassiana (Bb)* (Balsamo) Vuillemin are of particular concern, if this fungus is to be used as a biocontrol agent (BCA) for the control of TSM.

Although many of these chemicals have been reported to be compatible with *Bb* (Goettel *et al*., 2000), other studies have shown that conidial survival and efficacy of the fungus can negatively be affected by interaction with chemical pesticides (Clark *et al*., 1982, Anderson & Roberts, 1983; Todorova *et al.,* 1998; Shi *et al*., 2005). Fungicides are likely to have the most inhibitory effects (Jaros-Su *et al.,* 1999; Kouassi *et al*., 2003), diminishing its potential pesticidal activity. However, depending on the type of the chemical used, the effect of fungicides on *Bb* was reported to vary from a strong to non-inhibitory effect (Tedders, 1981; Majchrowicz & Poprawski, 1993; Jaros-Su *et al.*, 1999).

In this study two fungicides, flutriafol (Impact®) and azoxystrobin (Amistar®), were tested *in vitro* for their effects on conidial germination and mycelial growth of *Bb* and their effect on fungal infection of TSM. Most authors agree that spore germination and mycelial growth *in vitro* are useful criteria for testing the possible side-effects of fungicides on beneficial entomopathogenic fungi (i.e. Clark *et al*., 1982; Majchowicz & Poprawski, 1993), although inoculum survival has also been considered as an important factor for testing the fungicidal effects (Loria *et al*., 1983).

Flutriafol (a triazole) is an inhibitor of the biosynthesis of ergosterol in an important group of fungi and azoxystrobin (a strobilurin) inhibits electron transfer from cytochrome b to cytochrome c_1 in the mitochondrial membrane, resulting in inhibition of conidial germination (Sisler & Ragsdale, 1984; Stenersen, 2004). These fungicides are commonly used in South Africa for the control of fungal diseases such as powdery

mildews and rusts on various crops (Nel *et al*., 1999; Schutte *et al.,* 2003). Since they both have a broad spectrum action, it was suspected that they might inhibit the activity of *Bb* against TSM.

6.2 Material and methods

6.2.1 Fungicides

The commercial fungicides flutriafol ((RS)-2,4-difluoro- α -(1H-1,2,4-triazol-1-ylmethyl)-benzhydryl alcohol) and azoxystrobin (Methyl (E)-2-{2-[6-(2cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate) were provided in the form of suspension concentrates under the trade name Impact® and Amistar® respectively (Table 6.1).

Table 6.1 Fungicides tested for their compatibility with *Beauveria bassiana.*

Common name	Chemical class	A.I	Appl. rate (*)	Application volume	Trade name	Company
Flutriafol	triazole	125g l^{-1}	1 l ha^{-1}	300 l ha^{-1}	Impact®	Zeneca Agrochemical SA (Pty) Ltd.
Azoxystrobin	strobilurin	250g l^{-1}	1 l ha^{-1}	300 l ha^{-1}	Amistar®	Syngenta SA (Pty) Ltd.

A.I: Active ingredient content (pure)
(*) Appl.: Application rate of chemical for field use

6.2.2 *In vitro* test of *Beauveria bassiana* inhibition by the fungicides

Effect on germination of conidia – The *Bb* isolate PPRI 7861 inoculum was prepared by growing the fungus in Petri dishes containing Sabouraud dextrose agar[1] (40 g dextrose, 10 g mycological peptone and 15 g agar in 1 l of distilled water as directed by the manufacturer) and 1% yeast extract (SDAY) (Goettel & Inglis, 1997). After 21d of incubation at 25±1ºC, 3 ml of 0.01% Tween 80 in distilled water was added to the Petri dish and 1 ml of conidial suspension was pipetted into a 20 ml bottle containing 9 ml of distilled water. The concentration of the suspension in the bottle was determined using a

[1] Biolab Diagnostics (Pty) Ltd, Midrand, South Africa

Neubauer haemocytometer and adjusted to a final suspension with 10^6 conidia ml^{-1}. An aliquot of 0.1 ml of the final suspension was inoculated to SDAY Petri dishes containing the fungicides at different concentrations and spread using an L-shaped glass rod. The Petri dishes were incubated at 25±1ºC in the dark. Germinated conidia were counted at 6 h, 12 h, 18 h and 24 h after incubation.

The SDAY + fungicide medium was prepared by adding 1ml of the fungicide at the relevant concentration to a 500-ml Erlenmeyer flask containing 299 ml of SDAY liquid cooled to 45ºC. The mixture was agitated in a vortex mixer and poured into 90mm Petri dishes. Concentrations were prepared based on the recommended field application rates assuming a delivery volume of 300 l ha^{-1} (Table 6.1). Each of the fungicides was tested, at 1, $10^{-1}$and 10^{-2} times the recommend rate (X) for field use. Petri dishes (SDAY) without fungicides were also prepared for the control blank, making in total seven treatments.

Conidia were examined for germination under a compound light microscope at 400 × magnification. All conidia with an apparent germ tube were considered as germinated (Hywell-Jones & Gillespie, 1990). Counts were made at four different random locations per Petri dish and there were three Petri dishes per treatment. At each field of view all conidia were counted and percentage germination calculated. Germination inhibition was computed by subtracting the proportion of conidia germinated in the fungicide treatments to that of control blank (*Bb* without fungicide) divided by the proportion of germinated conidia in the control. The test was done on three different occasions over a week and the data were combined for analysis after checking the homogeneity of variance between the three testing occasions with Bartlett's test (Snedecor & Cochran, 1989). Data were analysed by one way analysis of variance (ANOVA) and treatment means were separated by Tukey-Honestly Significant Difference (HSD) test at a 5% experimental error wise rate using GenStat (2007).

Effect on linear growth of mycelium – The treatments were the same as for the germination test. The SDAY- fungicide Petri dishes were prepared in the same manner as in the germination test (above). However, *Bb* inoculation was done by placing a 5 mm diameter disc of SDAY with *Bb* mycelia, cut from the 21 d old *Bb* colony (see

above), in the centre of a Petri dish containing SDAY and the fungicides (Hokkanen & Kotiluoto, 1992). Care was taken to reverse the disc so that the mycelium came into contact with the SDAY- fungicide medium. There were five Petri dishes per treatment. Inoculated Petri dishes were incubated in the dark at 25±1ºC and the diameter of mycelial growth, minus the diameter of the disc, was measured on Day 4, Day 6, Day 8, Day 10 and Day 14 post-inoculation. Inhibition of mycelial growth as a result of fungicide treatments relative to the control was calculated using the same procedure as the inhibition of germination. The test was done on two different occasions and data were combined for analysis between treatments and testing occasion. Data were analysed by repeated measures ANOVA. Treatments means were separated using the Tukey-HSD test (α=5%). A regression analysis of data was also performed to estimate the rate of the mycelial growth over time. All analyses were done using GenStat (2007).

6.2.3 Effect of flutriafol and azoxystrobin on the activity of *Beauveria bassiana* against *Tetranychus urticae*

Preparation of *Bb* suspensions – Conidial powder (0.1 mg) was suspended in 100ml distilled water containing 0.01% Tween 80. The concentration of conidia in the suspension was determined using a Neubauer haemocytometer and adjusted to a final concentration of 10^8 conidia ml^{-1}, after the viability was checked (Chapter 2, Section 2.2.1.3), by diluting the conidial suspension with 0.01% Tween 80 in water or adding more powder. The final suspension prepared was immediately used for bioassays. The conidial power (Isolate PPRI 7861) used in this test was previously produced on SDAY medium as indicated in Chapter 2 (Section 2.2.1.2).

***Tetranychus urticae* bioassays** – Mites were obtained from a single-age population cultured on bean plants in the greenhouse (Chapter 2, Section 2.2.1.1). Thirty female mites were placed on a bean leaf disc (*Phaseolus vulgaris* L. var. Tongati) in a Petri dish (90 mm-diameters) and sprayed with suspension samples using a Burgerjon spray tower (Burgerjon, 1956). Preparation of Petri dishes containing leaf discs, and spraying techniques, were described in Chapter 2 (Section 2.2.1.5). Based on the recommended rate for field use (Table 6.1), suspension samples with three different concentrations were prepared for each of the fungicides. Suspensions were prepared in distilled water

at 1, 10^{-1} and 10^{-2} times the normal rate (X). The concentrations below the normal rates were used to check the effect of the fungicides even at low concentration that would likely occur in the field after chemical breakdown or washing off. Treatments consisted of mites sprayed with *Bb* alone, *Bb* + each of the fungicides at each of the concentrations (six treatments), each fungicide sprayed alone at each of the concentrations (six treatments) and the control (Tween 80 solution sprayed alone), making a total of 14 treatments. There were four Petri dishes with leaf discs and mites per treatment. The *Bb* suspensions were first sprayed on leaf discs and spray deposits were allowed to dry before the fungicide preparations were applied. When fungicides were sprayed, water was applied to the *Bb* treatment to minimize the variation due to spray. Petri dishes were kept in the laboratory at room temperature (22 ± 1°C) with a 12 h light period. The bioassay was repeated once and data were combined for analysis.

Dead mites were recorded every day for 5 days from Day 6 after treatment application. Percentage mortalities of TSM in the treatments were corrected relative to their corresponding controls, using Abbott's formula (Abbott, 1925). Dead individuals were counted every day for each treatment and were then removed, placed on water agar and incubated at 25 ± 1°C to check for the outgrowth of fungal mycelia from the mite body, to confirm mortality due to the fungus. Data were transformed (angular transformation) prior to analysis and submitted to one-way ANOVA with no blocking (GenStat, 2007); treatment means were compared using Tukey-HSD test at a 5% level of significance. Probit analysis was also performed to determine the median lethal time (LT_{50}) of mites due to treatment application (Finney, 1971). Treatments with smaller LT_{50} values were considered more efficient.

6.3 Results

6.3.1 *In vitro* test of *Beauveria bassiana* inhibition by the fungicides

The two fungicides affected the germination of conidia, with flutriafol being more inhibitory. At 6 h and 12 h, 3% and 17% of conidia respectively had germinated in the control while there were no germinated conidia in either of the Petri dishes containing the fungicides. At 18 h and 24 h after incubation there were germinated conidia in all

the Petri dishes, with a low proportion on the SDAY-fungicide media (Table 6.2). The levels of inhibition in conidial germination depended upon the concentrations and the type of the fungicide in the SDAY medium (Table 6.2). At 10^{-2}×X concentration, the fungicides were usually less inhibitory. Of the two fungicides, flutriafol was the more inhibitory on conidia even at 10^{-2} dilution level of its normal rate for field application, while azoxystrobin had no effect at that concentration 24 h.

Table 6.2 Percentage germination and germination inhibition (mean ± SE) of *Beaveria bassiana* conidia 18 h and 24 h after inoculation on SDAY medium alone (control) or mixed with fungicides at different concentrations.

Treatments	Germinated conidia (%)		Inhibition of conidial germination (%)	
	18h	24h	18h	24h
Control	79.3 ± 5.3 a	96.3 ± 0.8 a	–	–
Azoxystrobin 1×X	21.0 ± 2.1 c	58.3 ± 4.1 b	73.5 ± 3.1 b	39.3 ± 4.4 c
Azoxystrobin 10^{-1}×X	23.1 ± 3.2 c	62.3 ± 2.0 b	71.2 ± 4.5 b	35.2 ± 2.6 c
Azoxystrobin 10^{-2}×X	41.6 ± 3.7 b	83.0 ± 5.2 a	48.2 ± 3.9 c	13.8 ± 4.6 d
Flutriafol 1×X	5.2 ± 1.2 c	7.3 ± 5.9 d	93.4 ± 4.9 a	92.4 ± 6.4 a
Flutriafol 10^{-1}×X	7.2 ± 1.9 c	11.1 ± 3.5 d	90.8 ± 7.6 a	88.5 ± 4.1 a
Flutriafol 10^{-2}×X	39.3 ± 5.3 b	43.2 ± 2.8 c	50.6 ± 5.2 c	55.1 ± 5.8 b
F values	19.83 (df = 6, 36)	11.83 (df= 6, 36)	11.83 (df= 5, 30)	5.10 (df= 5, 30)
P values	<0.001	<0.001	0.003	<0.001
Coefficient of variation (%)	11.4	8.9	23.6	28.3
HSD (α=5%)	15.7	18.4	13.3	11.5

Means in each column followed by the same letter are not significantly different according to Tukey's HSD test (α=5%)

With regards to mycelial growth, treatment effects were significant ($p < 0.001$). There was also a significant difference between the times of assessment ($p < 0.001$), and the interaction between treatment and time was significant ($p < 0.001$), suggesting that the *Bb* developed on the prepared media, to some extent, except where flutriafol was added at the recommended rate for field use (Fig. 6.1). Moreover, the area under the growth curve (AUGC) calculcualted (Fig. 6.2) was signicantly different among treatments ($F = 127.1$; d.f = 6, 36; $p < 0.001$) with less AUGC values where fungicides were included in the medium. Taken all together, the fungicides inhibited the growth of *Bb* at all the concentrations tested *in vitro*.

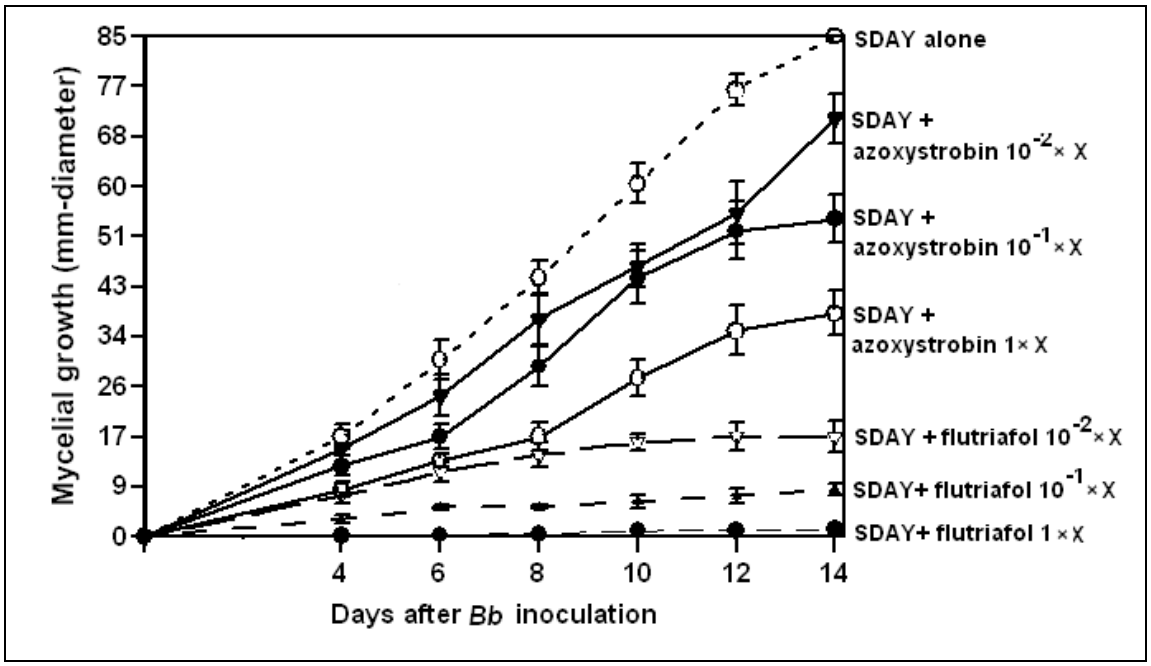

Figure 6.1 Effects of azoxystrobin and flutriafol at different concentrations on the linear growth of *Beauveria bassiana* (mean diameter ± SE) on Sabouraud dextrose agar supplemented with yeast (SDAY). The diameter (5 mm) of the SDAY disc for inoculation was subtracted during the measurements.

As for germination of conidia, flutriafol was also the most inhibitory (Tukey's HSD test at α=5%). During the 14-day period of assessment, the percentage inhibition ranged from 58.8 to 80% at the 10^{-2} dilution level, from 82.7 to 90.5% at the 10^{-1} dilution and to a complete inhibition at the recommended rate for field use (Fig. 6.1). Azoxystrobin used at the normal rate (1×X) caused a 52.1-61% level of inhibition. The fungicide caused a moderate level of inhibition at the 10^{-2} dilution level (11.8 to 16.5%) and at the 10^{-1} dilution (15.3 to 36%). However, the two levels of diluted azoxystrobin were not much different in their effect in the first twelve days of assessment, and the difference was only significant after 14 d. *Bb* tended to overcome inhibition by this fungicide at the 10^{-2} dilution in the SDAY medium.

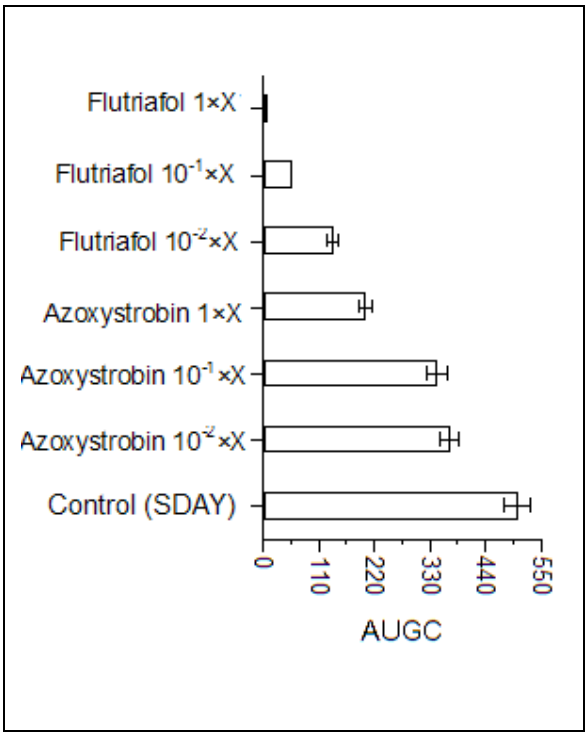

Figure 6.2 Area under the growth curve (AUGC) of mycelia of *Beauveria bassiana* over time on Petri dishes with SDAY medium or SDAY mixed with the fungicides at different concentrations. The bars are standard errors.

6.3.2 Effect of the fungicides on the activity of *Beauveria bassiana* against *Tetranychus urticae*

Effect of azoxystrobin – When azoxystrobin was sprayed without *Bb* at the concentrations of 10^{-2}×X, 10^{-1}×X and 1×X the mean percentages of dead mites were 3.7%, 6% and 4.9% respectively. These mortalities were not significantly different from that of the control blank (Tween solution alone). This suggested that the fungicide on its own was not toxic to TSM (Fig. 6.3). Where the fungicide was sprayed together with *Bb* the corrected mortality ranged between 82.1% and 89.7 %; these mortalities demonstrated that none of the levels of azoxystrobin impaired the activity of *Bb* as no significant difference was established from that caused by *Bb* sprayed alone (Tukey's HSD, α=5%; Table 6.3). Further, more than 90% of mite cadavers in the *Bb* and *Bb*-fungicide treatments showed external mycelial development after two to four days of incubation at 25±1ºC. External growth of mycelia observed confirmed that mite death was caused by the fungus.

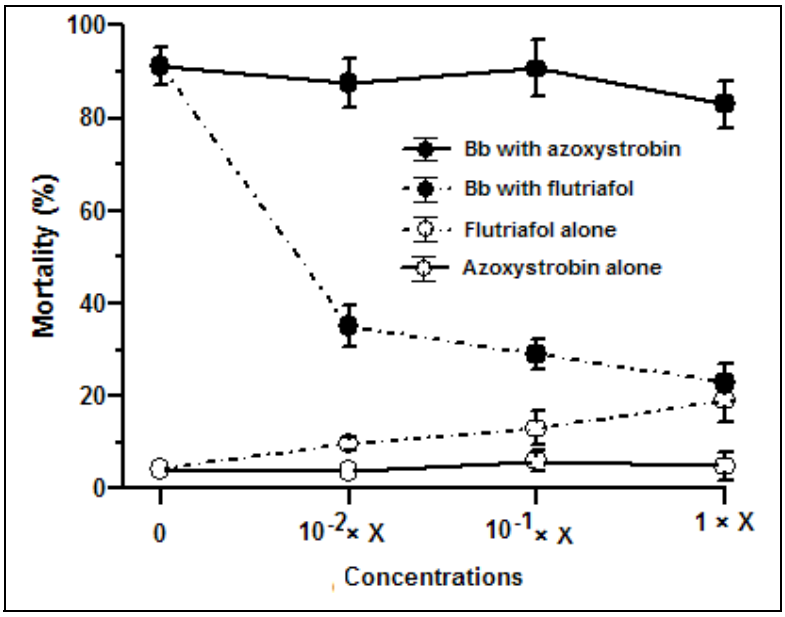

Figure 6.3 Total mortality of *Tetranychus urticae* (means ± SE) due to treatment applications after 10 days. The recommended rate for field application (X) was prepared assuming a delivery rate of 300 l ha^{-1}, see Table 6.1.

Table 6. 3 Corrected mortality and overt mycosis (% means ± SE), and the median lethal time (LT_{50}) estimates with their 95% confidence intervals (CI) in the bioassay.

Treatments	Corrected Mortality[(a)]	Apparent mycosis [(b)]	LT_{50} in days [(c)]	95% confidence intervals	
				Lower limit	Upper limit
Bb alone	91.0 ± 4.3 a	90.5 ± 5.9 a	6.1	5.3	7.4
Bb + azoxystrobin (1×X)	82.1± 4.1 a	81.1 ± 8.7 a	6.3	4.9	8.3
Bb + azoxystrobin (10^{-1}×X)	89.7± 5.4 a	88.4 ± 4.9 a	5.9	5.1	6.8
Bb + azoxystrobin(10^{-2}×X)	85.8 ± 6.1 a	85.1 ± 5.1 a	6.0	5.5	8.1
Bb + flutriafol (1×X)	4.5 ± 0.3 c	3.7± 0.5 c	–	–	–
Bb + flutriafol (10^{-1}×X)	18.3 ± 2.1 b	15.3± 2.4 b	–	–	–
Bb + flutriafol (10^{-2}×X)	25.5 ± 3.3 b	17.8± 2.6 b	–	–	–
F (df=6, 36)	21.12	20.9			
P values	<0.001	<0.001			
Coefficient of variation	12.9 %	21 %			

[(a)] For *Bb* suspension sprayed without chemical, the mortality was corrected from that observed in the control blank (Tween solution alone), while the mortalities due to *Bb*-fungicide treatments were corrected from those of fungicides sprayed alone at their corresponding concentrations;
[(b)] The apparent mycosis was estimated based on the total number of mites in the treatment;
[(c)] The LT_{50} values in *Bb*-flutriafol treatments were not determined as the mortality of mites observed at the end of the bioassays, in each of the treatments, was less than 50%;
In each column means followed by the same letter are not significantly different by Tukey's HSD test at a 5% significance level.

Effect of flutriafol – The mortalities of TSM caused by flutriafol alone at the levels of 10^{-2}×X, 10^{-1}×X and 1×X were 9.7%, 13.0% and 19.1% respectively. These mortalities were statistically higher than that observed in the control blank (4.3%), suggesting that the fungicide was toxic to TSM; and the toxicity increased as the concentration of the fungicide was increased in the spray suspensions (Fig. 6.3). Where *Bb* was sprayed together with flutriafol at the three levels of concentration, the total mortality of mites assessed 10 days after treatment applications ranged between 23.1% and 35.3% (Fig. 6.3) with a corrected mortality between 4.5% and 25.5% (Table 6.3). The percentage Abbott's corrected mortalities due the fungal treatments were not far from the percentage overt mycosis. These mortality parameters proved that the fungicide significantly reduced the control efficacy of *Bb* against TSM at all the levels tested.

6.4 Discussion

Flutriafol is a demethylation inhibitor (DMI) in the triazole class of fungicides. It disrupts the biosynthesis pathway of ergosterol, a compound vital for the fluidity and biological functions of the cell wall in the higher fungi (Sisler & Ragsdale, 1984; Berg, 1986; Stenersen, 2004). Azoxystrobin belongs to the strobilurin class. Fungicides of this class interfere with the fungal mitochondrial respiration pathway and act as potent inhibitors of spore germination and mycelial growth (Wood & Holloman, 2003; Stenersen, 2004). Both flutriafol and azoxystrobin have a broad spectrum action against plant pathogenic fungi and were expected to affect the beneficial entomopathogenic fungus *B. bassiana.*

Flutriafol was shown to be highly inhibitory to *Bb* at all the concentrations tested *in vitro*. Even at its one-hundredth of recommended rate, the fungicide significantly reduced the germination of conidia and inhibited the growth of mycelia. This suggested that even after chemical breakdown or dilution by rain, the fungicide may still be harmful to *Bb*. At its recommended field rate, it showed more than 90% inhibition of conidial germination and a complete inhibition of mycelial growth. With *in vitro* tests, using other DMIs, previous studies have demonstrated varying effects on the fungus. For example, bitertanol, a triazole foliar fungicide, was less harmful (Hassan *et al.*, 1991), while triadimefon, another triazole, significantly reduced the mycelial growth of

Bb on SDA when it was applied at the recommended field rate (Majchrowicz & Poprawski, 1993). In assays to investigate the effect of commonly used fungicides for the control of stripe rust, *Puccinia striiformis* f. sp. *tritici* in South Africa, on *Conidiobolis thromboides* and *Beauveria bassiana,* Naudé & Hatting (1998) observed a high inhibition by tebuconazole (a triazole). Similarly, Shah *et al.* (2009) found that the myclobutanil (triazole) completely inhibited the germination of conidia and strongly reduced mycelial growth when it was tested *in vitro* at the recommended rate. Usually, most of the DMI fungicides have similar mode of action (Berg, 1986), but their effects may vary depending on the strain of fungus tested (Shapiro-Ilan *et al*., 2002). For example, when they tested seven strains of *Bb* for their resistance to fenbuconazole, Shapiro-Ilan *et al*. (2002) observed that six of them were less susceptible while the commercial strain GHA was highly inhibited on SDAY. At all the concentrations tested, flutriafol also reduced the virulence of *Bb* against TSM in the bioassays when the two agents were sprayed together. This suggests that even if the fungicide chemical breaks down or is diluted by rain it could still compromise the mite infection process and reduce the efficacy of control of the BCA.

In contrast to flutriafol, azoxystrobin was less harmful to *Bb* germination but reduced mycelial growth by more than 50% when it was applied at the recommended rate. However, this fungicide was anticipated to be highly inhibitory to conidial germination, given its mode of action against fungal pathogens (Stenersen, 2004). Azoxystrobin has also been shown to strongly inhibit the spore germination of *Lecanicillium longisporum* (formerly, *Verticillium lecanii* and *L. lecanii*), another entomopathogenic Hyphomycetes, but to not affect its mycelial growth, when applied at the recommended field rate (Kim *et al.*, 2001). However, in a recent research work, Shah *et al*. (2009) demonstrated that azoxystrobin was less harmful to *Bb* germination, even at the recommended field rate. Similar to the findings in this study, these authors demonstrated that the fungicide significantly inhibited the growth of *Bb* on SDA at the recommended rate.

When *Bb* was applied alone or together with the fungicide in bioassays against TSM, the means of mortality were statistically the same and ranged between 83% and 91.3%. Together with the LT_{50}s observed, these results demonstrated that the fungicide did not

affect the activity of *Bb* on mites, at any of the concentrations. Furthermore the low mortality of mites observed in the controls including where fungicides were applied alone reflect the efficacy of the bioassay and the reliability of results. External mycelial growth observed was a good indication that mite mortality was due to the application of *Bb*. It also demonstrated that the fungicide residues had little effect on the growth of *Bb* inside mite bodies.

Although not the focus of the study, it was also noteworthy that the two fungicides, on their own, affected the survival of mites differently. Flutriafol showed toxicity to the mites, while azoxystrobin did not. There have been several reports on acaricidal effect of fungicides on *T. urticae* (Ball, 1982; Fungo & Curry, 1983; Alston & Thomson, 2004). In bioassays on detached bean leaves, Alston & Thomson (2004) demonstrated that the strobilurin and triazole fungicides were less toxic to *T. urticae* females. However, they tested only the effect of fungicide residues and not the effects of direct contact exposure.

The current study demonstrated that two fungicides commonly used in South Africa varied in their toxicity to *Bb*. Flutriafol was the most inhibitory *in vitro* to germination of conidia and mycelial growth even when diluted, and affected the virulence of *Bb* in bioassays. It may, therefore, jeopardize the efficacy of the Bb control of strategy against mites. This must be taken into consideration if these two agents are applied together in the field. However, more tests may be needed to confirm this. On the other hand, azoxystrobin proved to be less harmful and may be applied within an integrated pest management program (IPM) against TSM and fungal plant pathogens.

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Chapter 7

Combining applications of soluble silicon and *Beauveria bassiana* to four crops to control the two-spotted spider mite

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Abstract

A possible synergy between the biological control agent *Beauveria bassiana* (*Bb*) and the element silicon (Si) for the control of the two-spotted spider mite (TSM), *Tetranychus urticae* was investigated. Greenhouse experiments were carried out on cucumber, eggplant, bean and maize plants. Silicon was added to nutrient solutions in the form of potassium silicate at concentrations ranging from 0 - 160 mg l^{-1}. Conidia of *Bb* (Isolate PPRI 7861) were suspended in Break-thru® and sprayed onto plants 7 d after TSM inoculation. In other experiments, foliar and root applications of Si were compared. Plants treated with Si at higher concentrations, 80 and 160mg l^{-1}, were less damaged by TSM. However, Si alone did not cause the death of mites. Higher mortalities of adult mites, as well as juveniles, were caused by *Bb* and this was more efficient when the fungus was combined with soluble silicon. It was also found that the *Bb* efficacy increased more when Si was applied to roots than sprayed onto leaves, although both methods of application resulted in a reduction in initial damage of plants by TSM. These results indicate that the applications of Si as a plant nutrient may provide some protection of the plants against TSM infestations in both monocotyledonous and dicotyledonous plants, and enhances the biocontrol efficacy of *Bb*.

7.1 Introduction

Although occurring at the highest concentration of inorganic constituents within plant tissues (0.1 to 10% dry weight), the element silicon (Si) has been regarded as non-essential for plant growth, and its deficiency in crops went unrecognized for many years (Jones & Handreck, 1967). There is, however, increasing evidence of its role as a 'functional' plant nutrient (Epstein, 1999). In soil solution, Si occurs mainly as monosilicic acid (H_4SiO_4) and is taken up by plants in this form, which may polymerize and accumulate in epidermal tissues as amorphous silica ($SiO_2.nH_2O$), in the form of phytoliths, (Bakber & Shone, 1966; Ma & Takahashi, 2002). Both silicon phytoliths and silicic acid in plants play an important role in enhancing their resistance against pests and diseases (Yoshihara *et al*., 1979; Epstein, 1999; Fawe *et al*., 2001; Ma, 2004).

For plant pests, application of soluble Si has been reported to suppress insects such as folivores, borers and sap suckers, and non-insect pests such as spider mites (Savant *et al,* 1997; Laing *et al.,* 2006; Keeping & Kvedaras, 2008). Resistance to pest enhanced by the presence of Si in the plants results in feeding deterrence to the herbivorous arthropods and reduce their performance and survival (Moraes *et al*., 2005; Massey *et al.,* 2006). Therfore, application of Si would synergize with the fungal biocontrol agent *Beauveria bassiana* (*Bb*) (Balsamo) Vuillemin for controlling the two-spotted spider mite (TSM), *Tetranychus urticae* Koch in plants. Indeed, it was hypothesized in the current study that both juvenile and adult stages of TSM would become more susceptible to *Bb* infections if they fed on Si-treated plants.

Silicon has been observed to synergize with *Bb* for control of stored grain beetles, *Oryzaephilus surinamensis* Linnaeus and *Rhyzopertha dominica* Fabricius (Lord, 2001), red flour beetle, *Tribolium castaneum* Herbst (Akbar *et al*., 2004) and bean bruchid, *Acanthoscelides obtectus* Say (Dal Bello *et al*., 2006). In these studies, however, Si directly affected the cuticle of the target insect, which predisposed it to *Bb* infection. This chapter concerns a number of experiments performed under greenhouse conditions on four crops consisting monocotyledonous and dicotyledonous, in order to investigate (a) the effect of Si on mite control; (b) its interaction with *Bb*; and (c) the effect of spraying Si on leaves versus root application.

7.2 Materials and methods

7.2.1 Plant materials and *Tetranychus urticae* inoculation

Cucumber (*Cucumis sativus* L.), eggplant (*Solanum melongena* L.), bean (*Phaseolus vulgaris* L.) and maize (*Zea mays* L.) were used, and all the trials were carried out in greenhouses[1]. Bean seed was provided by Pro-Seed[2], and cucumber, eggplant and maize seeds were provided by McDonald Seeds[3]. Seedlings were prepared in trays and transplanted into 2.5 l pots (one plant per pot) containing composted pine bark, commercially prepared, as the growing medium (0.57% N, 0.18% P, 0.38% K, 0.82% Ca, 16.53% C, 0.14% Mg, 73 mg kg^{-1} Zn, 12.29 mg kg^{-1} Fe, 75% water-holding capacity, 60% air-filled porosity, 650 kg ton^{-1} bulk density and a pH of 5.5). Maize seeds were directly sown in pots. The varieties of cucumber, bean, eggplant and maize used were Ashley, Tongati, Black Beauty and Colorado, respectively.

Twenty adult females of TSM, from a stock culture in a greenhouse, were artificially inoculated onto plants at transplanting by placing them onto the youngest fully expanded leaf of each plant. Maize plants were inoculated 4 wk after plant emergence.

7.2.2 Soluble silicon and fungal application

Potassium silicate (K_2SiO_3) as product K2550 (SiO_2: 20.5-20.9%; KO_2: 8-8.15%) was provided by PQ Corporation[4], and used as the source of soluble silicon (Si). It was included in the nutrient solution system or sprayed onto leaves, where applicable.

A conidial powder containing 2.1×10^9 conidia g^{-1} of *Bb* Strain PPRI 7861 were formulated and provided by Plant Health Products[5]. For all the trials, conidia were suspended in a Break-thru® solution (0.01% v/v) and sprayed onto plants one week after mite inoculation using a 2.5 l Solo 402 hand-held sprayer[6].

[1] Controlled Environment Facility, University of KwaZulu-Natal Pietermaritzburg, South Africa
[2] Pro-Seed cc (Pty) Ltd, Pietermaritzburg, South Africa
[3] McDonald Seeds cc (Pty) Ltd, Pietermaritzburg, South Africa
[4] PQ Corporation (previously named Ineos Silicas) South Africa (Pty) Ltd
[5] Plant Health Products (Pty) Ltd, Nottingham Road, South Africa
[6] Solo Kleinmotoren GmbH, Germany

7.2.3 Experimental designs

7.2.3.1 Combined effect of soluble silicon and *Beauveria bassiana* at multiple concentrations

Trials on cucumber, eggplant, bean and maize were run concurrently in the greenhouse with the same protocol. All the plants are susceptible to TSM but have different capacities to take up and accumulate Si. Maize, as a grass (monocot), accumulates Si actively (Ma & Takahashi, 2002). While eggplant and bean are believed to have a poor capacity in accumulating Si (Ma *et al*., 2001), cucumber plants can accumulate higher levels of Si (Liang *et al*., 2005a). For irrigation and fertilisation, plants were provided with a constant supply of nutrient solution in 8L buckets. Si was added to the nutrient solution at 0, 20, 40, 80 and 160 mg l^{-1} (0, 0.7, 1.43, 2.86 and 5.7 mM Si, respectively). Solutions in the buckets were changed every 3 days. *Bb* conidia were applied at 0, 0.5, 1 and 2 g l^{-1}, making a factorial trial design with twenty treatments.

Treatments were replicated three times in a randomized complete block design (RCBD). There were five plants per treatment in each replication (regarded as an experimental unit or plot) fed with nutrients from one bucket. In each crop trial, Si treatment started immediately at transplanting for cucumber, bean and eggplant, and two weeks after emergence for maize. Soluble silicon was constantly available to plants for the duration of the experiment. The pH in the nutrient solutions was checked every day and was maintained between 5.5 and 6.0 using hydrochloric acid. To compensate for the additional amount of potassium applied in K_2SiO_3, appropriate adjustments were made to the nutrient solution with potassium chloride (KCl). For each crop, the trial was conducted twice and data were combined for analysis.

7.2.3.2 Effects of root and foliar applications of silicon

Trials were performed on eggplant and bean. Plants were drip irrigated three times a day for five minutes and nutrients were supplied through the irrigation system. Soluble silicon was drenched into pots or sprayed to run off on leaves at the rate of 80 mg l^{-1}. *Bb* was sprayed onto plants at the concentration of 1 g l^{-1}.

The trial for each crop comprised six different treatments: the control (water spray instead of Si and no *Bb* applied), application of *Bb* without silicon (*Bb*), Si applied to roots but *Bb* was not sprayed (Si to roots), Si applied to roots and *Bb* sprayed (Si to roots + *Bb*), a foliar spray with Si without *Bb* (Si leaves) and a foliar spray of Si and *Bb* sprayed (Si leaves + *Bb*). Each treatment was replicated five times and there were five plants in each plot. Plots were arranged in a RCBD.

Silicon was applied four times a week, starting immediately after seedling transplanting, *Bb* was sprayed one week after TSM inoculation (two weeks after transplanting) and every subsequent week for four weeks. Where Si was sprayed, pots were covered with polyethylene plastic to avoid Si run-off onto the roots. Mortality of mites and leaf damage indexes were assessed in plots one week after each *Bb* spray. At each time of assessment, plant leaves were collected for Si analysis. The trial was conducted twice for each crop and data were combined for analysis.

7.2.4 Assessment of *Tetranychus urticae* mortality and plant leaf damage

Mortality of TSM and leaf damage indexes were assessed as described in Chapter 3 (Section 3.2.3) of the present thesis.

7.2.5 Determination of silicon content of plant leaves

7.2.5.1 Inductively coupled plasma emission spectrometry (ICP-ES) analysis

Three to five leaves were randomly collected per plant and washed or not washed in distilled water for analysis of Si content. Collected leaves were allowed to dry in an oven at 70°C for 48 h. Dried material was ground and kept in plastic bottles until analysis. Silicon content was analysed with ICP-ES. Plant samples (ground material) were weighed into polytetrafluoroethylene (PTFE-Teflon) vessels and digested with nitric and hydrochloric acids (Feng *et al*., 1999) in a Mars_Xtration microwave digester[7]. The digestate was diluted in ultra-pure water and the Si content was

[7] CEM Corporation, USA

determined with a Varian 720 ES-ICP Optical Emission Spectrometer[8] after calibration with silicon standards. The concentrations of Si in leaves were then estimated as percentage of dry mass (% DM).

7.2.5.2 Energy dispersive X-ray microanalysis

Energy dispersive X-ray microanalysis (EDX) was performed to pre-determine the silicon levels within the leaves resulting from the different treatments. Five leaf samples were collected from different treatments and freeze dried. Silicon was then detected with an EDAX detecting unit[9] connected to a Philips XL 30 environmental scanning electron microscope (ESEM)[10] operating at high vacuum and a voltage of 15 kV. X-ray mapping was also performed with the same samples. The EDX levels facilitated estimation of the range of Si standards that were prepared for ICP analysis.

7.2.6 Statistical analyses of data

Data on mortalities of TSM, LDIs and concentration of Si within leaves were analysed by an ANOVA procedure with the GenStat (2007) computer package. Means were compared by Tukey's Honestly Significance Difference (HSD) test at a 5% significant level.

7.3 Results

7.3.1 Combined effect of soluble silicon and *Beauveria bassiana* at multiple concentrations

7.3.1.1 Mortality of *Tetranychus urticae*

In the *Bb* × Si factorial ANOVA, each effect was tested with a mean square error (MSE) of 62.69 in eggplant, 70.74 in cucumber, 67.35 in bean and 43.98 in maize (Table 7.1).

[8] Varian Australia (Pty) Ltd., Australia
[9] EDAX Inc, New Jersey, USA
[10] Philips Electron Optics, Eindhoven, Holland

In the eggplant trial, there was a significant interaction effect (at the 5% significance level) between *Bb* and Si ($p < 0.05$; Table 7.1). In particular, application of *Bb* was more effective when Si rates were increased. A low mortality of mites was observed in the treatments without *Bb* applications (Fig. 7.1a), suggesting that Si alone did not affect the mortality of TSM. The Si main effect was also not statistically significant ($p > 0.05$). The Si application had an effect on *Bb* efficacy only when it was applied at 80 and 160mg l^{-1} and these two concentrations were not significantly different in their effects. Analysis of Si content of eggplant leaves also showed no statistical difference between plants treated with soluble silicon at 80 and 160 mg l^{-1} (Table 7.2). As expected, mite mortality was high with high concentrations of *Bb* ($p < 0.001$); the use of *Bb* at 2 g l^{-1} resulted in more than 60% mite mortality. On the other hand, when *Bb* was applied at 1 g l^{-1} in combination with Si at high rates, it provided the same level of control as an application of *Bb* alone at 2 g l^{-1} (Fig. 7.1a).

In the bean trial, significant differences in mortality of TSM occurred as a result of the different concentrations of *Bb* applied ($p < 0.001$; Table 7.1). The Si main effect was significant ($p < 0.05$) and more dead mites were found where Si was applied at 80 and 160 mg l^{-1} (Fig. 7.1b). The *Bb* × Si (4 × 5) interaction effect fell short of statistical significance ($p > 0.5$). The presence of a significant interaction effect between *Bb* and Si was further evaluated by reducing the *Bb* levels to two for analysis. This showed that there was a significant interaction effect between *Bb* and Si when *Bb* was applied at 1 or 2g l^{-1} compared to the control ($F_{4;\,18} = 3.67$; $p = 0.013$; CV = 1.2 %). As shown in Fig. 7.1b, application of Si at 80 and 160 mg l^{-1} increased the effect of *Bb* at 1g l^{-1}; the respective mean mortalities of mites of the combined treatments were higher than those of the application of *Bb* alone at 1 g l^{-1} or combined with 20 or 40 mg l^{-1} of Si. The combination of *Bb* at 1 g l^{-1} with Si at 80 or 160 mg l^{-1} was as effective as the application of *Bb* alone at 2 g l^{-1} for the control of mite populations.

Results of the cucumber trials were similar to those found in the bean trials, with more than 80% mite mortalities occurring as a result of the best treatments (Fig. 7.1c). A significant interaction effect was also found between Si and *Bb* ($p < 0.05$; Table 7.1). Application of *Bb* resulted in greater mite mortality when it was combined with Si at 80 and 160 mg l^{-1}. On the other hand, Si at 20 and 40 mg l^{-1} had no effect on *Bb* efficacy.

Table 7.1 Results of the two-way ANOVA of percentage mortalities of TSM in the trials.

Source of variation	Eggplant				Beans			
	DF	**MS**	**F value**	**P value**	**DF**	**MS**	**F value**	**P value**
Bb	3	16178.41	258.07	< 0.001	3	19916.74	295.72	< 0.001
Si	4	44.51	0.71	0.43	4	267.38	3.97	0.031
Bb × Si	12	329.12	5.25	0.028	12	63.31	0.94	0.519
Error	38	62.69			38	67.35		
	Cucumber				Maize			
Bb	3	20281.16	286.7	< 0.001	3	2001.09	45.5	< 0.001
Si	4	52.35	0.74	0.54	4	28.59	0.65	0.629
Bb × Si	12	396.14	5.6	0.02	12	51.90	1.18	0.334
Error	38	70.74			38	43.98		

DF: degree of freedom; MS: mean squares; F statistics and P values are provided for trials; the analysis was done at 5% level of significance.

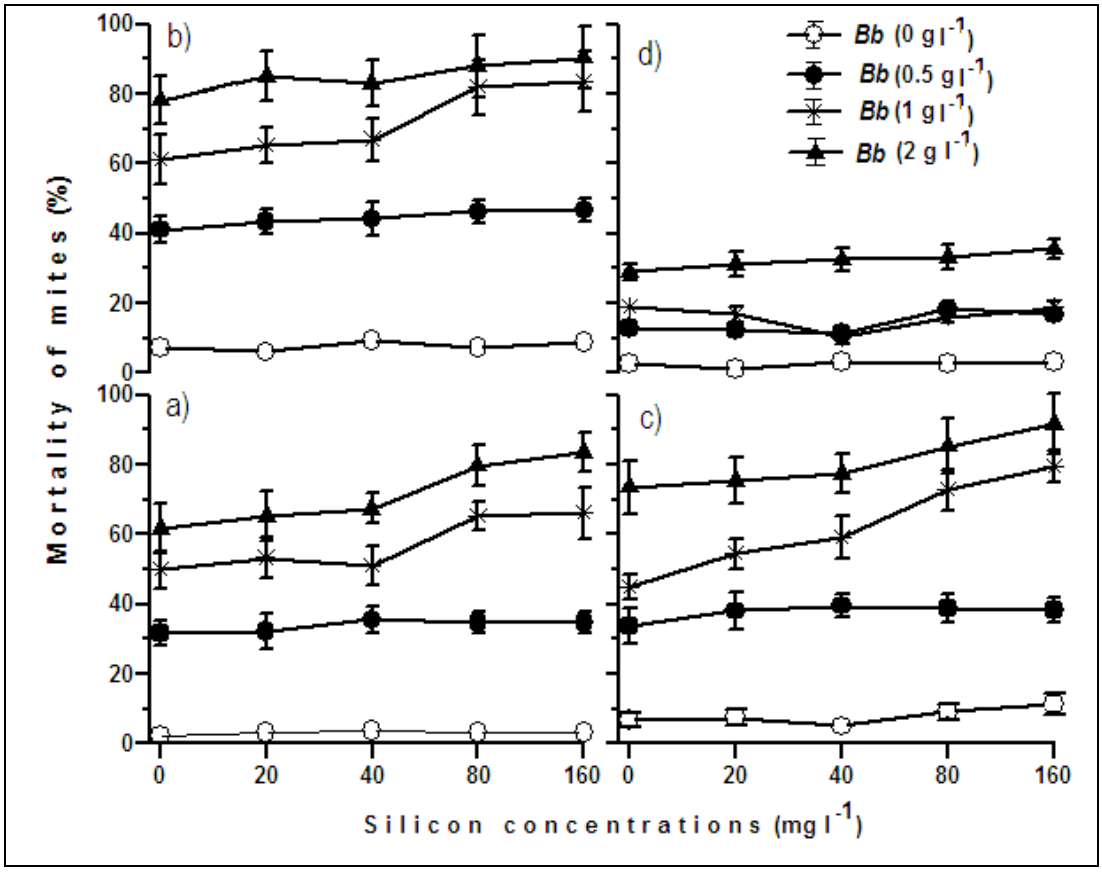

Figure 7.1 Mortalities of TSM (mean ± SE) assessed 14 d after treatment applications in eggplant (a), bean (b), cucumber (c), and maize (d) trials. Polynomial contrasts showed significant *Bb* linear effects for all the plant trials ($p < 0.001$) and a significant Si linear effect for bean trial ($p < 0.05$). A significant *Bb*-Si linear-linear interaction effect was found as well for cucumber ($p < 0.05$) and eggplant ($p < 0.05$) trials.

As in the eggplant and bean trials, the Si rates of 80 and 160 mg l^{-1} applied to cucumber plants were not significantly different in their effects on mite mortality, whether applied alone or in combination with *Bb*.

In the maize trials, a significant effect was found for the main effect of *Bb* ($p < 0.001$; Table 7.1). But there was no significant result for the Si main effect ($p > 0.05$) nor was the *Bb* × Si interaction ($p > 0.05$) significant. Mean mortalities of mites were high with the increase of *Bb* concentrations, regardless of the concentrations of Si applied. When *Bb* was sprayed at 2 g l^{-1}, the mortality of mites was much higher compared to other treatments (Fig. 7.1d). It was noteworthy that the application of the fungus, *per se*, resulted in relatively poor control of TSM in the maize trials, with less than 40% mean mortality from the best treatments.

7.3.1.2 Leaf damage indexes and Si content of plant leaves

The TSM leaf damage was assessed once, 2 wk after the *Bb* spray. Surprisingly, this parameter did not identify any effects for the *Bb* × Si interaction or the *Bb* main effect for all the trial crops. However, significant differences were found between the LDI means for the main effect Si (Table 7.2). With increased levels of soluble silicon, there was an improvement in plant resistance to TSM damage (Fig. 7.3).

Amendment with 80 mg l^{-1} of K-silicate substantially reduced TSM damage by 32.6, 35.7 and 52.1% in eggplant, bean and cucumber, respectively. The values of LDI estimated in Si-treated plants at 160 mg l^{-1}were not much different from those of plants treated with 80 mg l^{-1} of Si. In maize, low values of LDI were observed in plants that received more than 40 mg l^{-1}. Silicon applied at the rates of 40, 80 and 160 mg l^{-1} did not result in different LDI means for maize (Table 7.2).

For all the crops, the Si content (% DM) was significantly different as a result of the levels applied (Table 7.2). In eggplant, bean and cucumber, the mean percentages of Si content of leaves were not statistically different between the plants treated with 80 mg l^{-1} and 160 mg l^{-1} of Si. As was expected, maize leaves had a higher Si content than the other crops. The Si content of plant leaves was proportional to the

concentration of Si supplied to plants. By contrast, the positive increase of the Si content of leaves with the increase of soluble silicon in the nutrient solution did not necessarily result in equivalent reduction in mite damage. For example, in maize plants, the Si amendment with 40 mg l^{-1} was the optimum concentration for TSM damage reduction (46.1% of leaf mite damage reduction), but a higher Si content was found in maize leaves when Si was supplied at 160 mg l^{-1} (Table 7.2).

Table 7.2 Mean leaf damage indexes for main effect Si and silicon content in leaves.

	Si rates (mg l^{-1})	Eggplant	Bean	Cucumber	Maize
LDI[(a)]	Control	2.91 a	1.50 a	3.13 a	2.54 a
	20	2.83 a	1.30 ab	2.71 a	1.94 b
	40	2.75 a	1.23 ab	2.63 a	1.37 c
	80	1.87 b	1.01b	1. 5 b	1.40 c
	160	1.76 b	0.93 b	1.12 b	1. 38 c
	F (df = 4;38)	19.8	11.3	13.9	9.8
	P value	0.023	0.004	0.013	0.037
	C.V. (%)	27.6	15.1	29.8	16.7
Si content (% DM)[(b)]	Control	0.001 c	0.004 c	0.003 c	0.003 d
	20	0.008 c	0.006 c	0.009 c	0. 061 c
	40	0.016 b	0.010 b	0.389 b	1.065 b
	80	0.068 a	0.021 a	0.878 a	1.167 b
	160	0.057 a	0.024 a	0.945 a	1.654 a
	F (df = 4; 38)	3.35	3.01	16.61	12.49
	P value	0.018	0.028	<0.001	<0.001
	C.V. (%)	26.2	29.3	30.1	28.8

[(a)] LDI means in each column with the same letter are not significantly different by Tukey's HSD (α=5%)
[(b)] Si content means in each column with the same letter are not significantly different by Tukey's HSD (α=5%)

7.3.2 Effects of root and foliar application of silicon

7.3.2.1 Eggplant trial

a) Mortality of TSM – At each of the four different assessments, mite mortalities were significantly different among the different treatments (Table 7.3). At Week 1, application of *Bb* alone was not significantly different from its combination with Si, whether applied to roots or to leaves. The mortality of adults and juveniles TSM was mainly due to *Bb*. The combination treatment *Bb* + Si applied to roots was better than

the combination *Bb* + Si applied to leaves at Week 2, Week 3 and Week 4 of assessment times for juvenile mites and at Week 4 only for adult mites (Table 7.3). Application of Si alone (to roots or leaves) did not cause any significant effect on mite mortality throughout the assessment.

Table 7.3 Mean percentages (mean ± SE) of mite mortality for four week eggplant trial assessment.

	Treatments	1 wk	2 wk	3 wk	4wk
Adults	Control	5.1 ± 0.6 b	7.5 ± 1.2 b	12.7 ± 2.3 c	9.1 ± 2.1 c
	Bb	51.3 ± 7.4 a	57.1 ± 8.8 a	59.7 ± 4.6 b	65.5 ± 6.7 b
	Si to roots	7.1 ± 1.2 b	10.5 ± 3.0 b	13.2 ± 2.1 c	16.4 ± 2.6 c
	Si to roots + *Bb*	50.2 ± 5.3 a	65 ± 10.1 a	72.2 ± 7.4 a	81.3 ± 9.4 a
	Si to leaves	8.1 ± 0.7 b	11.5 ± 2.7 b	12.9 ± 1.7 c	10.1 ± 1.9 c
	Si to leaves + *Bb*	52.2 ± 6.9 a	63.5 ± 7.3 a	63.4 ± 6.1 ab	68.6 ± 8.5 b
	$F_{5, 20}$	27.29	38.3	36.4	32.15
	P value	<0.001	<0.001	<0.001	<0.001
	C.V. (%)	25.1	30	30.1	27.8
Juveniles	Control	1.2 ± 0.3 b	2.7 ± 1.0 c	2.9 ± 1.2 c	3.1 ± 1.2 c
	Bb	18.4 ± 3.4 a	23.0 ± 2.1 b	27.3 ± 5.1 b	26.1 ± 5.7 b
	Si to roots	1.3 ± 0.2 b	2.5 ± 0.7 c	3.5 ± 3.0 c	5.6 ± 3.0 c
	Si to roots + *Bb*	20.2 ± 4.3 a	36.0 ± 4.3 a	53.0 ± 6.6 a	58.0 ± 7.1 a
	Si to leaves	2.1 ± 0.7 b	3.7 ± 0.8 c	4.5 ± 2.7 c	4.5 ± 2.7 c
	Si to leaves + *Bb*	22.2 ± 3.9 a	24.5 ± 7.3 b	31.2 ± 7.3 b	33.2 ± 4.6 b
	$F_{5, 20}$	8.11	13.3	17.5	11.6
	P value	<0.001	<0.001	<0.001	<0.001
	C.V. (%)	29.1	17	19.2	23.1

For adults and juveniles, means within a column, followed by the same letter are not significantly different by Tukey's HSD at 5% level of significance.

b) Leaf damage indexes – Leaf damage index means presented in Table 7.4 show significant differences in the treatments for all the times of assessment. At Week 1, low levels of leaf damage were observed where Si was sprayed onto leaves, regardless of whether *Bb* was sprayed on or not. In Week 2, except for the application of *Bb* alone, all other treatments were significantly different from the control in their LDI means.

Applying Si to roots together with spraying on *Bb* resulted in low levels of mite leaf damage, but it was not significantly different from the combination of *Bb* + Si applied to leaves or Si alone applied to leaves. All the treatments in Week 3 and Week 4 caused significantly lower LDI means compared to the control, and the combined treatments of *Bb*-Si were the best. On its own, *Bb* was also better than application of Si alone at Week 4, but in the plots where Si was sprayed onto leaves the damage of TSM was still low.

Table 7.4 Mean leaf damage indexes (mean ± SE) for four week assessment in the eggplant trial.

Treatments	1 wk	2 wk	3 wk	4wk
Control	1.6 ± 0.40 ab	2.4 ± 0.23 a	2.8 ± 0.15 a	3.7 ± 0.30 a
Bb	1.7 ± 0.42 a	2.3 ± 0.34 a	1.5 ± 0.33 b	1.2 ± 0.20 d
Si to roots	1.8 ± 0.29 a	1.7 ± 0.24 b	2.3 ± 0.15 b	2.4 ± 0.24 b
Si to roots + *Bb*	1.6 ± 0.40 ab	1.1 ± 0.31 c	0.9 ± 0.16 d	0.9 ± 0.15 d
Si to leaves	1.1 ± 0.21 c	1.3 ± 0.20 c	1.5 ± 0.16 c	1.7 ± 0.30 c
Si to leaves + *Bb*	1.2 ± 0.19 bc	1.2 ± 0.15 c	1.1 ± 0.15 d	1.1 ± 0.27 d
$F_{5, 20}$	2.35	11.99	25.83	29.64
P value	0.041	<0.001	<0.001	<0.001
C.V. (%)	25.6	29	31.7	28.1

Within a column, means followed by the same letter are not significantly different by Tukey's HSD test (α=5%)

Table 7.5 Means of Si concentration (%) in eggplant leaves assessed for four weeks.

	Treatments	1 wk	2 wk	3 wk	4wk
Unwashed leaves	Control	0.001 c	0.002 c	0.001 c	0.001 c
	Si to roots	0.021 b	0.032 b	0.301 b	0.390 b
	Si to leaves	0.049 a	0.800 a	1.130 a	1.900 a
	F (df = 2; 8)	19.6	14.6	11.6	18.9
	P values	<0.001	<0.001	<0.001	<0.001
	C.V. (%)	24.9	23.1	26.3	27.8
Washed leaves	Control	0.001 c	0.003 c	0.005 c	0.006 c
	Si to roots	0.026 a	0.047 a	0.052 a	0.084 a
	Si to leaves	0.013 b	0.014 b	0.015 b	0.017 b
	F (df = 2; 8)	13.96	10.65	6.88	10.95
	P values	<0.001	<0.001	<0.001	<0.001
	CV (%)	19,1	21	23.5	29.1

For unwashed or washed leaf samples, means within a column followed by the same letter are not significantly different by Tukey's HSD at 5% level of significance.

With regards to LDI trends throughout the assessment, *Bb* alone, *Bb* + Si applied to leaves and *Bb* + Si applied to roots reduced the TSM damage by 8.3; 9.2 and 49.4% respectively, from Week 1 to Week 4 (Fig. 7.2). On the other hand, application of Si alone delayed the progress of mite damage in the trial plots compared to the control, although it did not stop the damage. For example, the level of leaf damage observed in the control at Week 1 was only observed in the Si treate-plants at Week 4 (Table 7.4). A severe infestation of TSM in the control plots resulted in defoliation and death of plants (Fig. 7.4).

Silicon content of leaves – Silicon content of leaves differed as a result of the two methods of Si application to plants. With unwashed leaf samples, a higher concentration of Si was found where Si was supplied in sprays, compared to root applications. However, the reverse was true with washed samples (Table 7.5). All the leaf samples collected from Si treated through root applications had a higher Si content than the control. In the leaves collected at the first and second intervening assessment, where Si was applied to the roots, the leaf Si content was similar for both washed and unwashed samples. Unexpectedly at the third and fourth week, there was about five times more silicon in unwashed than washed leaves (Table 7.5), it was thought that these leaves had Si on surface, probably from accumulated drifts when adjacent plots were sprayed in the trial, though caution was taken during spraying, however this could also haved been observed on the controls.

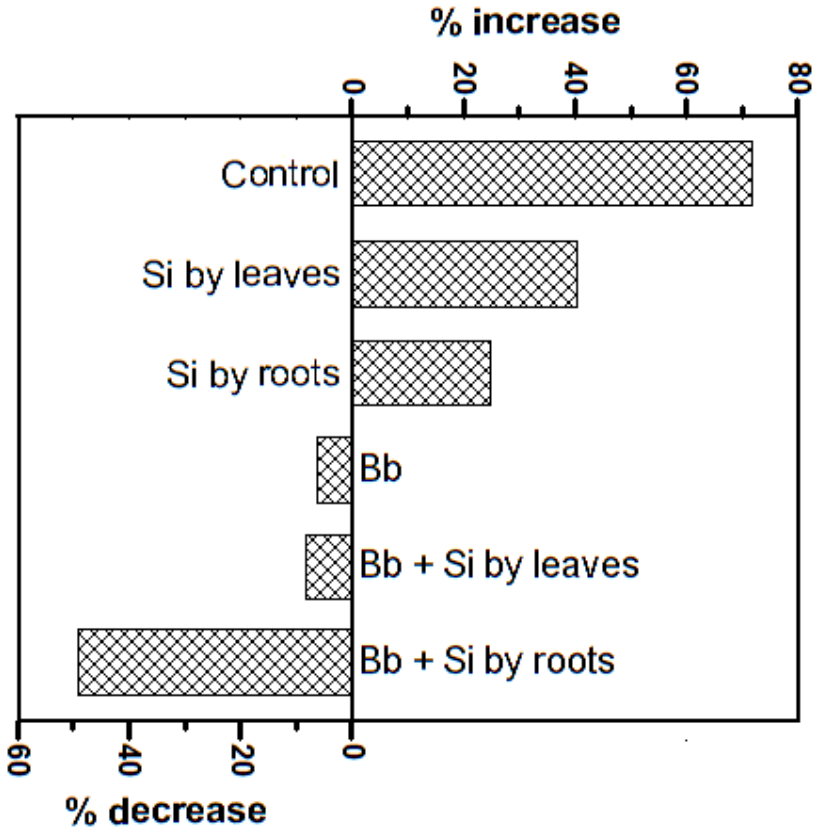

Figure 7.2 Change in leaf damage as result of treatments throughout the assessment in the eggplant trial. Percentage change for each treatment was calculated as follows: % change in damage $=\sum(\frac{LDI_{i+1}-LDI_i}{LDI_{i+1}} x100)$ **; where LDI_i is the leaf damage index mean for the i^{th} intervening assessment (i = 1, 2 and 3); and LDI_{i+1}, the leaf damage index mean for the consecutive assessment. The positive values reflected increases in leaf damage at the end of the experiment while the negative values reflected a reduction in leaf damage.**

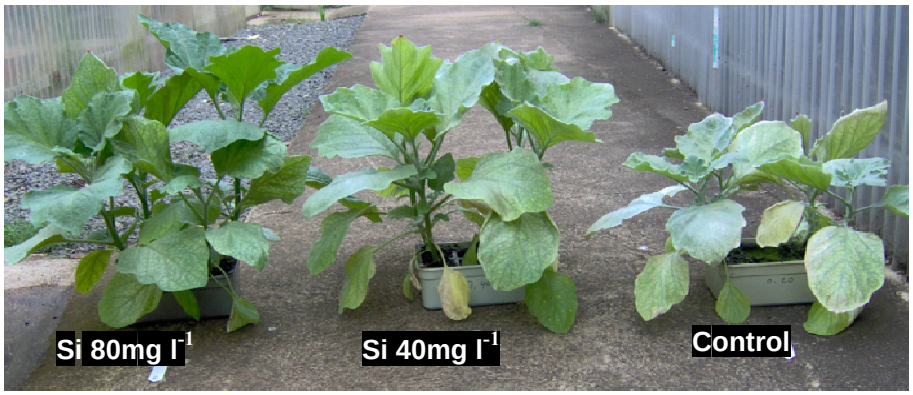

Figure 7.3 Eggplant plants showing different levels of TSM damage four weeks after infestation. In the control treatment, all the leaves were bronzed and covered by mite webs, and plants were stunted as a result of high infestation levels. Where Si was applied at 40 mg l^{-1}, all the leaves showed symptoms of mite infestation but webs were not observed. In the plant treated with 80 mg l^{-1} of Si, mite damage was localized on a few old leaves at the bottom of the plant.

Figure 7.4 Eggplant plants showing different levels of *Tetranychus urticae* damage six weeks after mite infestation. +Si is where soluble silicon was supplied to plants through roots at 80 mg l^{-1}. *Bb* conidia were sprayed at 1 g l^{-1}. In the control treatment (-*Bb*; -Si) there was severe damage, with leaf bronzing and defoliation. Where Si was applied alone (-*Bb*; +Si), the plants were healthier than the control but still showed high infestation of mites with a layer of webs and bronzing of leaves. Where *Bb* was applied alone (+*Bb*; -Si) or in combination with Si (+*Bb*; +Si), only a few leaves at the bottom of the plants had stippling symptoms due to mites and yellowing, which is typical of leaf senescence.

7.3.2.2 Bean trial

a) Mortality of TSM – As in the eggplant trial, the bean trial showed significant differences in mortalities of TSM as a result of various treatments at each of the four assessments (Table 7.6). Spraying *Bb* on plants treated with Si to their roots was the best treatment with > 90% of mite adult cadavers assessed at Week 4. Si applied to roots increased the effect of *Bb* on mite (both adults and juveniles) mortality but Si sprayed onto leaves did not because the control provided by *Bb* combined with foliar sprays of Si was not significantly different from *Bb* alone (Table 7.6). As was expected, higher mortalities of TSM were observed in Week 4 and applications of Si alone to roots or leaves were not significantly different from the control for any assessment.

Table 7.6 Percentages (mean ± SE) of mite mortality for four week bean trial assessment.

	Treatments	1 wk	2 wk	3 wk	4 wk
Adults	Control	4.4 ± 0.2 b	5.1 ± 1.1 b	6.1 ± 1.6 c	11.1 ± 3.0 c
	Bb	58.1 ± 6.1 a	60.4 ± 7.5 a	70.8 ± 7.6 b	75.5 ± 12.1 b
	Si to roots	3.9 ± 1.0 b	5.5 ± 1.4 b	5.3 ± 1.1 c	12.3 ± 2.6 c
	Si to roots + *Bb*	67.3 ± 7.3 a	69.5 ± 10.1a	82.6 ± 12.4 a	93.4 ± 11.8 a
	Si to leaves	4.1 ± 0.7 b	5.1 ± 2.9 b	7.3 ± 1.7 c	14.4 ± 2.1 c
	Si to leaves + *Bb*	63.2 ± 10.8a	64.1 ± 11.2 a	66.7 ± 9.7 b	78.9 ± 10.4 b
	$F_{5, 20}$	24.6	27.5	31.3	29.1
	P value	< 0.001	< 0.001	< 0.001	< 0.001
	C.V. (%)	26.2	19.1	14.2	12.7
Juveniles	Control	2 ± 0.2 b	3.1 ± 1.1 b	3.7 ± 1.6 c	5.1 ± .3.0 c
	Bb	33.1 ± 6.1 a	40.2± 7.5 a	43.8 ± 7.8 b	42.5 ± 8.9 b
	Si to roots	1.7 ± 1.0 b	3.5 ± 1.4 b	4.3 ± 1.3 c	5.6 ± 1.6 c
	Si to roots + *Bb*	37.4 ± 7.3 a	49.5 ± 10.1 a	56.0 ± 9.4 a	61.1 ± 8.7 a
	Si to leaves	2.2± 0.7 b	2.1 ± 0.3 b	5.1 ± 1.0 c	4.4 ± 1.2 c
	Si to leaves + *Bb*	36.1 ± 7 a	44.1 ± 11.2 a	41.4 ± 6.9 b	44.9 ± 9.3b
	$F_{5, 20}$	14.1	23.8	11.4	9.8
	P value	< 0.001	< 0.001	< 0.001	< 0.001
	C.V. (%)	31.2	22.5	29.6	23.7

For adults and juveniles, means within a column, followed by the same letter are not significantly different by Tukey's HSD at 5% level of significance.

b) Leaf damage indexes – Mean LDIs varied among treatments (Table 7.7). Spraying Si prevented plants from initial damage, as shown by the low values of LDI scored in Week 1 and Week 2. However, in Week 4, an increase in leaf damage was observed where Si was applied alone, whether to roots or to leaves, although the LDI means remained low compared to the control (Table 7.7). In Week 4, mite damage was

reduced by the combined treatments *Bb*-Si applied on leaves and *Bb*-Si applied to roots by 75 and 78.6% respectively. These two treatments were not significantly different from one another and were better than *Bb* applied alone. Application of *Bb* only, on the other hand, was better than the control and Si alone, reducing mite damage by 64.3%. Damage to plants was reduced throughout the assessment period by *Bb* alone or *Bb* combined with soluble silicon (Fig. 7.5). Conspicuous effects were observed with the combined treatment of Si applied to roots + *Bb*, where the mite leaf damage was reduced by 59.8% from Week 1 to Week 4. Ultimately, trends in the effect of soluble silicon supplied to roots or sprayed to leaves in this trial were similar to those found in eggplants, although there was less damage caused by TSM in the bean trial than in the eggplant trial.

Table 7.7 Leaf damage indexes (mean ± SE) over four weeks of assessment on bean leaves.

Treatments	1 wk	2 wk	3 wk	4 wk
Control	1.3 ± 0.13 a	1.4 ± 0.22 a	2.1 ± 0.3 a	2.8 ± 0.23 a
Bb	1.3 ± 0.20 a	1.3 ± 0.62 a	1.1 ± 0.25 bc	1.0 ± 0.14 c
Si to roots	1.1 ± 0.12 a	1.0 ± 0.17 b	1.1 ± 0.24 b	1.5 ± 0.29 b
Si to roots + *Bb*	1.2 ± 0.18 a	1.1 ± 0.06 b	0.9 ± 0.36 c	0.6 ± 0.05 d
Si to leaves	0.8 ± 0.10 b	0.9 ± 0.10 b	1.2 ± 0.23 b	1.6 ± 0.25 b
Si to leaves + *Bb*	0.7 ± 0.11 b	0.6 ± 0.01 c	0.7 ± 0.22 c	0.7 ± 0.12 d
$F_{5, 20}$	3.35	5.66	8.31	13.91
P value	0.021	0.003	0.002	< 0.001
C.V. (%)	18.7	25.2	21.9	17.4

Within a column, means followed by the same letter are not significantly different by Tukey's HSD test (α=5%)

c) Silicon content of leaves – The means of Si content of leaves increased from Week 1 to Week 4, and differences were observed between washed and unwashed leaf samples. Si content of leaf samples where Si was applied to roots was fairly consistent in both washed and unwashed samples (Table 7.8; Fig. 7.6). As was also observed in eggplant, unwashed samples had a high Si content where soluble silicon was sprayed on leaves (Table 7.8; Fig. 7.6), as a result of spray deposits and not Si uptake. In the washed samples, the mean values of Si content of sprayed leaves were not significantly different from the control (Table 7.8). X-ray mapping performed with washed samples showed also a higher Si content where Si was applied to roots than when Si was sprayed onto leaves (Fig. 7.7).

Table 7.8 Si concentration (means % DM) in bean leaves analysed with ICP

	Treatments	1 wk	2 wk	3 wk	4 wk
Unwashed leaves	Control	0.003 c	0.001 c	0.002c	0.004c
	Si to roots	0.022 b	0.042 b	0.23 b	0.27 b
	Si to leaves	0.067 a	0.790 a	1.01 a	1.81 a
	F (df = 2; 8)	11.53	13.21	18.70	14.52
	P values	< 0.001	< 0.001	< 0.001	< 0.001
	CV (%)	15.1	19.7	23.5	22.4
Washed leaves	Control	0.002 b	0.001 b	0.004 b	0.003 b
	Si to roots	0.032 a	0.047 a	0.197 a	0.336 a
	Si to leaves	0.001 b	0.002b	0.007 b	0.008 b
	F (df = 2; 8)	7.87	8.61	9.52	15.12
	P values	< 0.001	< 0.001	< 0.001	< 0.001
	CV (%)	19.1	26.1	17.5	27.8

For unwashed or washed leaf samples, means within a column followed by the same letter are not significantly different by Tukey's HSD at 5% level of significance.

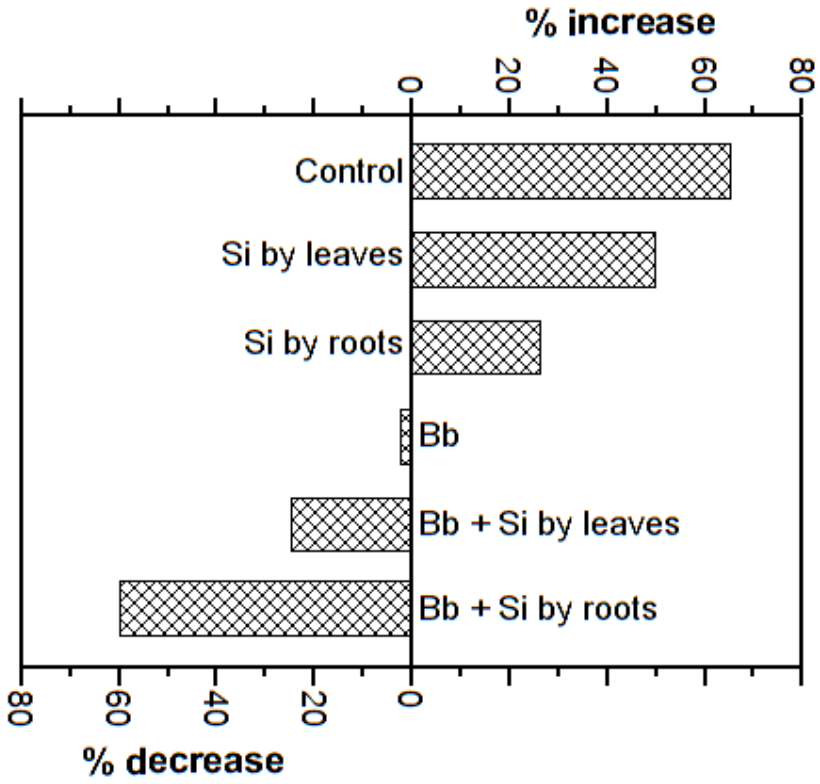

Figure 7.5 Change in leaf damage as result of treatments in the bean trial. Percentage change as a result of each treatment was calculated as follow: % change in mite damage =$\sum(\frac{LDI_{i+1}-LDI_i}{LDI_{i+1}} \times 100)$; where LDI_i is the leaf damage index mean for the i^{th} intervening assessment (i = 1, 2 and 3); and LDI_{i+1}, the leaf damage index mean for the consecutive assessment. The positive values reflected increases in leaf damage while the negative values reflected a reduction of damage due the treatments applied.

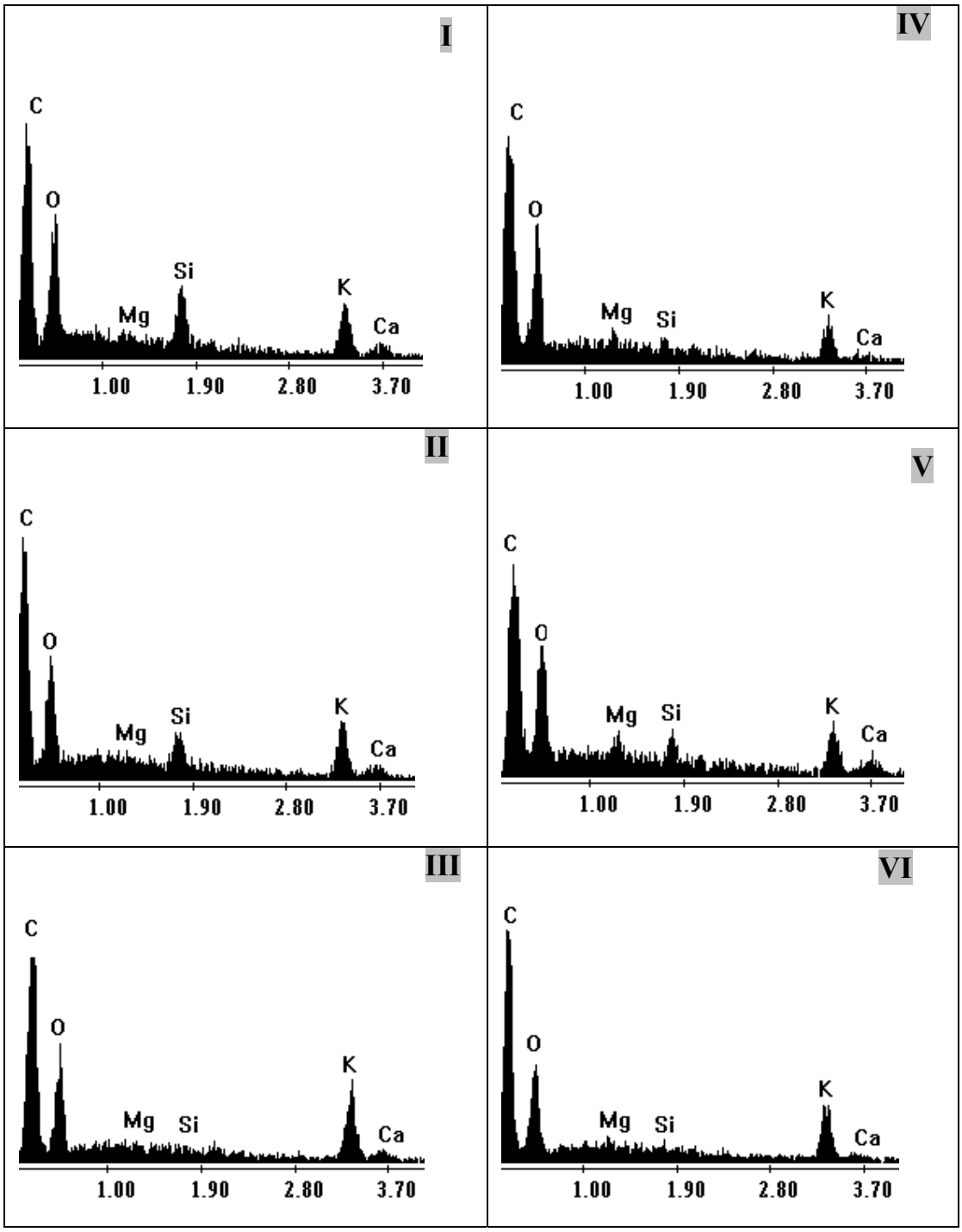

Figure 7.6 EDX graphs of Si content of bean leaves in different treatments with soluble Si: I, II, III were unwashed samples respectively where Si was sprayed, applied on roots and the control. IV, V, VI are respectively Si sprayed on leaves, Si applied on roots and the control in the washed samples. The x axis values are the energy levels of the x-rays and Si was detected at 1.7398 Kev. The peaks show the level of the concentration of the elements. For the control treatment, neither washed (III) nor unwashed leaves showed peak for Si content. Similarly, no apparent peak for Si appeared in the washed sample where Si was sprayed on leaves.

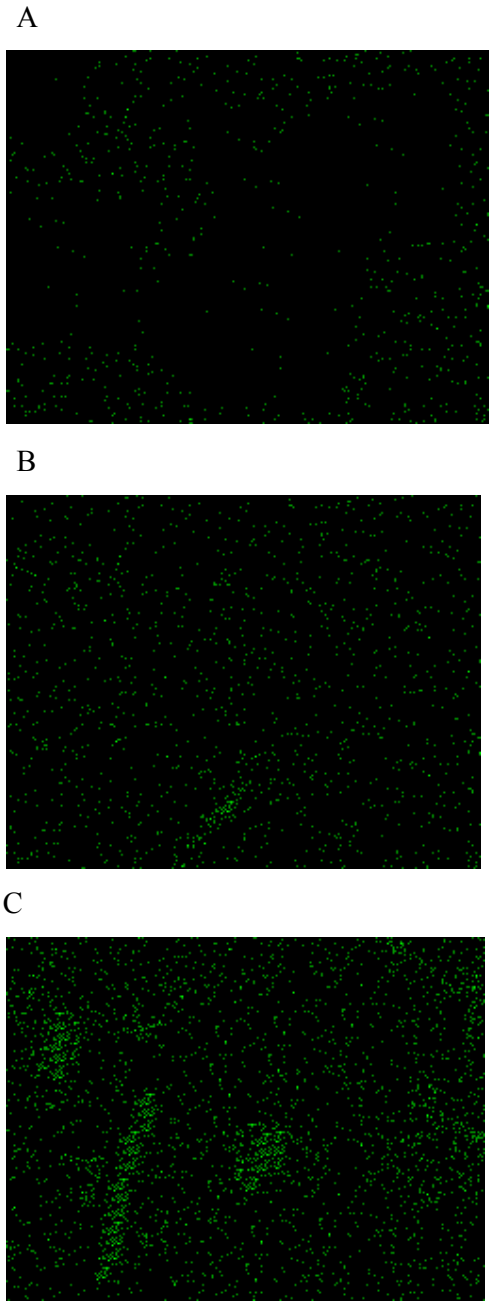

Figure 7.7 X-ray mapping micrographs of Si content of washed leaf samples of beans. A: control (leaves sprayed with water only); B: Si sprayed on leaves and C: Si applied to roots. The green dots are pixels representing the Si element. Matrix: 256×200. The black area is where Si was not present. The arrows show the areas of high concentration of Si. A high concentration of Si is observed in C.

7.4 Discussion

7.4.1 Combined effect of soluble silicon and *Beauveria bassiana* at multiple concentrations

The Si content of leaves showed that all four crops tested absorbed silicon, in particular maize, and that the total level of Si content increased as the Si concentration was increased in the nutrient solutions. This is similar to the observation of Ma & Takahashi (2002) who found that Si content of cucumber leaves increased with the increase of Si concentration in the medium. The absorption of Si had a significant effect on TSM control. The most striking effect of Si was that this element enhanced the control efficacy of *Bb* in the trials, as measured by higher mortalities of TSM observed when *Bb* was applied with Si. The combined application of *Bb* and Si resulted in synergistic control in cucumber, eggplant and bean trials. On the other hand, Si on its own did not directly affect the mortality of TSM, because, at all the concentrations, and in all the trials carried out; the mortality of TSM as a result of Si applied alone was not significantly different from that observed in the control treatment.

The synergistic effect of Si was evident when this element was applied at the two highest concentrations, 80 or 160 mg l^{-1}. Both concentrations had similar effects on the efficacy of *Bb*. Similarly, the interaction effect with Si was found when *Bb* conidia were applied at 1 g l^{-1} except for maize. The efficiency of *Bb* at this rate on mite mortality was improved to the level resulting from applications of *Bb* alone at 2 g l^{-1}. The mortality of TSM as a result of 0.5 g l^{-1} of *Bb* was less than 50% in all the trials and control did not improve whether silicon was applied or not, no matter at what rate it was applied. In previous experiments (Chapter 4), the amount of *Bb* recovered from maize plants immediately after spraying 2 g conidia l^{-1} was not much different from that recovered from cucumber, eggplant and beans plants when 0.5 g l^{-1} was sprayed. Consequently, it seemed plausible that the low mortality of mites observed in the maize trial in this study, even with the best treatments, was due to relatively little inoculum of *Bb* being deposited on maize plants as a result of spray applications.

The interaction of *Bb* and Si, in the form of amorphous silicon dioxide (a sorptive dust) and diatomaceous earth (an abrasive dust), against stored grain beetles *Oryzaephilus surinamensis* Linnaeus and *Rhyzopertha dominica* Fabricius has been reported previously (Lord, 2001). Diatomaceous earth was also reported to synergize with *Bb* in controlling red flour beetle, *Tribolium castaneum* Herbst (Akbar *et al*., 2004) and bean weevil *Acanthoscelides obtectus* Say (Dal Bello *et al*., 2006). In these reports, results were ascribed to the direct action of the desiccant dusts on insect cuticles by absorbing lipids, and by cuticular abrasion. Lord (2001) suggested that the significant cuticle abrasion by the desiccant dusts or removal of surface lipids could make underlying nutrients more available, which would enhance germination of the fungus and thereby contribute to increased infection of the stored grain beetles.

In contrast, the role of Si in enhancing *Bb* efficacy in this study was plant-mediated rather than by direct effect on the TSMs. As exemplified by the LDI values assessed two weeks after *Bb* sprays (three weeks after mite inoculation), application of Si increased resistance of plants to mite damage. For example, application of > 40mg l^{-1} of Si to maize reduced the TSM feeding injury of leaves by 46.1% compared to the control (-Si-*Bb* untreated plants) while application of Si alone at 80mg l^{-1} resulted in a reduction in TSM numbers of 32.6, 35.7 and 52.1% in eggplant, beans and cucumber respectively.

There is increasing evidence of feeding deterrence against herbivore arthropods by Si-reated plants. For example, Djamin & Pathak (1967) found that the growing of rice in silica impeded feeding and boring by the striped rice borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), and reduced larval survival and the number of dead hearts. Similarly, Goussain *et al.* (2002) found that fall armyworm larvae, *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae), displayed inhibited feeding on maize plants fertilized with Si, suffering increased mandibular wear, higher mortality and cannibalism, reflecting increasing plant resistance to this species. Application of Si to sugarcane reduced damage by the African stalk borer, *Eldana saccharina* Walker (Keeping & Meyer, 2002; Kvedaras & Keeping, 2007). For phloem feeders, the adverse effects of plant Si were reported by Brazilian researchers, who found that the greenbug aphid, *Schizaphis graminum* Rondani, displayed a non-preference for, and lower longevity and fecundity on leaves from Si-fertilized wheat (Basagli *et al*., 2003; Moraes

et al., 2004) and sorghum plants (Carvalho *et al*., 1999). The corn leaf aphid, *Rhopalosiphum maidis* Fitch, was also observed to have reduced preference for leaves from Si-treated maize leaves (Moraes *et al.,* 2005).

These observations support the hypothesis of this study that Si increased the resistance of plants against TSM which may have reduced the feeding of TSM, and made it more vulnerable to *Bb* infections. With regards to the mechanism whereby Si enhances resistance in plant against the mite, this is not known. In the works cited above (Djamin & Pathak, 1967; Carvalho *et al.*, 1999; Goussain *et al.,* 2002; Keeping & Meyer, 2002; Basagli *et al*., 2003; Moraes *et al.,* 2004; Moraes *et al,*. 2005; Kvedaras & Keeping, 2007), a mechanical barrier resulting from silica deposition in epidermis plant cells was reported to be responsible for plant resistance. It is, however, difficult to confirm whether silicon conferred a mechanical defense against mites in this study, especially because dicotyledonous plants such as eggplant and bean are unlikely to deposit much Si (Epstein, 1999; Ma & Takahashi, 2002). In the case of cucumber, soluble silicon in the plant has been associated with enhanced levels of chemical defense to infections by pathogens (e.g. Chérif *et al*., 1994; Rodriguez *et al*., 2004; Caia *et al.,* 2008) or infestations by the whitefly, *Bemisia tabaci* Gennadius (Correa *et al*., 2005). Furthermore, soluble silicic acid in plants can inhibit feeding by sucking arthropods (Yoshihara *et al.,* 1979). Hence, it is hypothesized in the current study that Si might have primed biochemical defenses in the plants, which then interfered with the feeding behaviour of TSM. Increased levels of defense enzymes such as peroxidase, polyphenoloxidase and phenylalanine ammonia-lyase have been reported as implicated in enhanced resistance in plants following herbivorous attack (Karban & Meyers, 1989; Karban & Baldwin, 1997). To determine whether Si application increased the activity of these enzymes in response to TSM damage, other trials were carried out and results are presented and discussed further in Chapter 8 of this thesis.

7.4.2 Effect of root and foliar application of silicon on *Beauveria bassiana*

Analyses of Si content of leaves revealed a consistent absorption of Si in eggplant and bean plants following root application of potassium silicate and that foliar application resulted in negligible absorption of Si. These results corroborate those of Guével *et al*.

(2007), who found that foliar application of soluble silicon resulted in insignificant absorption of Si in wheat.

Following the evaluations of Si uptake, the relative impact of the two methods of Si application on TSM control and the synergy between Si and *Bb* were compared using the mortality of mites and leaf damage assessed in the treatments. Results on mortality of juveniles and adults were consistent in the two crops (eggplant and bean) and showed that *Bb* worked better with Si applied to roots than with Si applied to leaves. Notably, a high mortality of juvenile TSM was observed as a result of the combined treatment *Bb* + Si, indicating that soluble silicon amendments improved the efficacy of *Bb* against the juvenile stage of the pest. In previous experiments (Chapter 3 of the present thesis), it was observed that *Bb* was not very effective against the juvenile populations of TSM. One of the reasons was the intense moulting of larvae and the short ecdysis intervals, which may impede *Bb* from establishing on the juvenile mite bodies (Vey & Fargues, 1977). It has been previously reported that application of Si to host plants increased the duration of the nymphal stage of the whitefly *B. tabaci* on cucumber plants (Correa *et al*., 2005). Hence, application of Si to plants may have resulted in an extended juvenile period, thereby predisposing them to higher levels of *Bb* infection, as observed in the trials in this study.

With regard to leaf damage, varying levels of control were found. Leaf damage index values with application of Si were low at the beginning of the trial (first and second assessment), regardless of *Bb* application. Both methods of Si application caused a substantial reduction of initial TSM damage in the bean and eggplant trials. In previous reports, foliar sprays of Si were also observed to reduce whitefly, *B. tabaci* biotype B, on cucumber (Correa *et al.,* 2005) and the corn leaf aphid, *R. maidis*, on maize (Moraes *et al.,* 2005).

However, there is little information on the effect of Si foliar spray on herbivorous arthropods, most of the research having been directed towards the effect of Si sprays on disease suppression, particularly powdery mildew (Menzies *et al*., 1992; Liang *et al.*, 2005b; Guével *et al*., 2007). For example, spraying Si on wheat leaves reduced powdery mildew incidence as a result of direct action on the fungus rather than one mediated by

the plant (Guével *et al*., 2007). Liang *et al.* (2005b) also found that Si sprays on cucumber leaves could effectively control powdery mildew only via a physical barrier of Si deposited on leaf surfaces but could not enhance the induced resistance. Based on these reports and the findings in this study, e.g. the high Si leaf content for Si sprayed treatment only in unwashed leaf samples, it is plausible that Si deposits on leaf surfaces as a result of spraying interfered with mite feeding, thus protecting plants from damage.

Secondly, applications of Si alone, whether by foliar or root application, slowed the progression of mite damage in the trials compared to the control (Fig. 7.2 & 7.5), but a much greater reduction was due to *Bb* application or its combination with Si, particularly when Si was applied to roots. This suggested that Si synergized better with *Bb* when it was applied to root than to leaves, probably because the uptake of Si was mainly through roots as observed when the Si content of leaves was analysed. The higher efficiency of the combined treatment became clear late in the trials. This may be explained by the slow effect of *Bb* in reducing leaf damage due to the fact that, *Bb* indirectly reduces the damage caused by TSM on plants. Its effect was observed progressively all the tested crops and was associated with a reduction in mite population (see Chapter 5 of the present thesis).

Ultimately, this study showed that Si applied to host plants at sufficiently high levels (> 80 mg l^{-1}) had a positive effect against TSM. These findings were consistent and conclusive for the four different crops tested including three dicotyledons. A combination of Si and *Bb* provides far better control against *T. urticae,* which is often a goal in integrated pest management (IPM) programs.

7.5 References

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Chapter 8

Effect of soluble silicon on biochemical defense responses induced in bean leaves by the two-spotted spider mite

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Abstract

Soluble silicon (Si) may act as a priming agent to enhance the induction of a plant resistance process against pathogen and herbivore invasions. Peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) enzymes are among the biochemical responses generated in plants after induction of pest resistance. To investigate whether Si enhanced the synthesis of these enzymes in green beans following *Tetranychus urticae* infestations, two experiments were carried out in the greenhouse. Soluble silicon was added to plant nutrient solutions 1 wk after plant emergence, while *T. urticae* was inoculated onto plants 3 wk after their emergence. Enzyme activity was measured in the bean leaf samples collected at different days after mite inoculation. Their activity was high in plants infested with mites but such increases were not found in non-infested plants, suggesting that these enzymes were only expressed in bean plants as a response to mite infestations. Infested plants treated with Si had significantly high levels of the enzymes. Moreover, accumulation of the enzymes was faster in these plants than in infested plants without Si. These results were consistent and suggested that soluble silicon enhanced the release of the defense enzymes POD, PPO and PAL and accelerated the resistance of bean plants to the phytophagous mite *T. urticae* but did not directly induce the resistance response.

8.1 Introduction

The role of silicon (Si) in plant protection against pests and diseases is well documented (Fauteux *et al*., 2005; Laing *et al*., 2006; Keeping & Kvedaras, 2008). However, there is ongoing speculation on the mechanism of Si-mediated plant resistance to pest stressors. The theory of a physical barrier was initially dominant, particularly in monocotyledons because of their capacity to accumulate high levels of Si in epidermal tissues (Yoshida *et al*., 1962; Djamin & Pathak, 1967; Goussain *et al*., 2002). Nevertheless, there is increasing evidence, particularly in the field of disease management, that Si may enhance resistance to plants by accelerating biochemical defense response, (Chérif *et al*., 1992; 1994; Fawe *et al*., 1998). There are however few reports on the impact of soluble silicon on induced plant resistance to pest attacks (Correa *et al*., 2005; Gomes *et al*., 2005).

Previous experiments (Chapter 7 of the present thesis) indicated that Si applied synergized with *Beauveria bassiana,* a biocontrol agent, for the control of two-spotted spider mite (TSM) *Tetranychus urticae* Koch by increasing resistance of four crops, including monocotyledons as well as dicotyledons. It was speculated that a possible enhancement of the induction of defense chemicals, catalyzed by the presence of soluble Si in the plants took place rather than mechanical defense alone. This speculation was therefore, the impetus of the research reported in the present chapter.

In the defense system of the plants, enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD) are involved. Their role in signaling pathways in wound- and arthropod herbivory-induced resistance was reviewed by Constabel (1999), Gatehouse (2002), and recently by Bernards & Bastrup-Spohr (2008) and Constabel & Barbehem (2008). PAL catalyses the deamination of phenylalanine to cinnamic acid, the entry and key regulatory step in the biosynthesis of phenylpropanoids from which phenolic compounds are derived (Constabel & Barbehem, 2008). Phenolic compounds are involved in induced resistance against herbivore and pathogen attacks (Bi & Felton, 1995). POD and PPO are oxidative enzymes; they are involved in oxidation of phenolic compounds to quinones and in production of lignin and hydrogen peroxide or

other phenoxy radicals that may prevent efficient feeding by arthropod herbivores (Felton *et al.,* 1989; 1994; Hao *et al.* 1996).

Increased activity of these enzymes in plant tissues has been associated with increased resistance to pests and pathogens (Cipollini, 1997; Quin & Tian, 2005; Shi-ze *et al*., 2008). POD, PPO and PAL are inducible by mechanical wounding; insect wounding; pathogens; and abiotic stressors such as UV light (Hahlbroch & Scheel, 1989; Constabel, 1999). They may also be expressed in plants in response to spider mite attacks (Karban & Baldwin, 1997; Trevisan *et al*., 2003). However, spider mite feeding did not trigger the release of PPO in tomato plants (Constabel *et al*., 1995; Thaler *et al.,* 1996). The present study investigated whether TSM feeding can trigger the release of these three enzymes in bean plants and whether application of soluble silicon can accelerate their release.

8.2 Materials and methods

8.2.1 Plant growth and soluble silicon application

Bean seeds[1] (*Phaseolus vulgaris* L. var. Tongati) were directly sown into 2.5 L pots containing composted pine bark, commercially prepared, as the growing medium (0.57% N, 0.18% P, 0.38% K, 0.82% Ca, 16.53% C, 0.14% Mg, 73 mg kg^{-1} Zn, 12.29 mg kg^{-1} Fe, 75% water-holding capacity, 60% air-filled porosity, 650 kg ton^{-1} bulk density and a pH of 5.5). Pots were placed into small plastic troughs with a constant supply of water circulating from 8 l buckets at a flow rate of 2.5 l min^{-1}. The nutrient solution contained as mg l^{-1} (before potassium silicate application): 147 N, 28 P, 80 K, 20 Mg, 25 S, 61 Ca and 1 B made up from Hortichem Water Soluble Fertilizer 3:1:3(38) and Calcium Magnesium Nitrate[2].

[1] Bean seed were provided by Pro-Seed cc (Pty) Ldt, Pietermaritzburg, South Africa
[2] Hortichem 3:1:3 (38) and Calcium Magnesium Nitrate were provided from Ocean Agriculture (Pty) Ltd, Gauteng, South Africa

Silicon, in the form of potassium silicate[3] (product K2250; 20.5-20.9% SiO_2, 8-8.15% K_20), was added to the nutrient solution at a concentration of 80 mg l^{-1} 1 wk after plant emergence. It was constantly available to plants for the duration of the trials. Plants without Si treatment received potassium chloride (KCl) to compensate for the additional amount of potassium applied in K_2SiO_3. The nutrient solution in the buckets was changed every 3 d. The pH was maintained between 5.5 and 6.0 using hydrochloric acid.

8.2.2 *Tetranychus urticae* inoculation

Two-spotted spider mite populations were mass reared and maintained in a greenhouse[4] on the bean variety Tongati. About twenty female adult TSMs were artificially inoculated onto plants 3 wk after emergence, by placing an infested leaf disc, from a stock culture, on the third trifoliate leaf from the new emerging leaf of each plant in the trial. The number of mites per leaf disc was checked under a dissecting microscope prior to inoculation.

8.2.3 Treatments

Two trials were carried out in the greenhouse under natural light conditions. The first trial consisted of four treatments: (1) no TSM - no Si: neither mites nor Si were applied to plants (control), (2) no TSM - with Si: plants without mites but treated with Si, (3) with TSM - no Si: plants inoculated with mites and not Si-treated, and (4) with TSM - with Si: plants inoculated with mites and treated with Si. All the treatments were replicated three times in a randomized complete block design (RCBD). There were five plants per plot fed with nutrients from the same recycling system. In each plot, the three middle plants were evaluated.

In the first trial, plants not inoculated with TSM did not accumulate enhanced levels of the enzymes being studied. Therefore, in the second trial all the plants were inoculated with mites. Comparison was made between plants without Si treatment versus plants with Si.

[3] Potassium silicate was provided by PQ Corporation (previously INEOS Silicas) South Africa (Pty) Ltd
[4]Controlled Environment Facility, University of KwaZulu-Natal , Pietermaritzburg, South Africa

The two treatments were replicated five times in a completely randomized design (CRD) in the greenhouse. As in the first trial, each experimental unit consisted of five plants but all of them were evaluated.

8.2.3 Enzymatic activity assay

In the first trial, leaf samples for enzyme assays were collected every day from the day of mite inoculation until 6 d later. In the second trial, samples were collected every three days from the day of mite inoculation until 21 d later. Because it was not possible to analyse samples at the time of collection, freshly collected leaves were ground in liquid nitrogen using a chilled mortar and pestle. The ground samples were kept in a freezer at -20°C until the enzyme analyses were conducted.

Polyphenol oxidase (PPO) and Peroxidase (POD) – The extraction of PPO and POD was done following the methodology of Chen *et al*. (2000) with a slight modification. Ground tissue (1 g) was added to 10 ml of sodium phosphate buffer (0.1M, pH 6.4) containing 1.5% polyvinyl pyrrolidone (PVP) in centrifuge tubes. The mixture was homogenized for 1min twice, using an Ika® T25 Digital Ultra-turrax[5]. Homogenates were then agitated periodically for 1h using an ultrasonic water bath and centrifuged at 4500 x g for 30min at 4°C. The supernatant was collected and filtered using 0.2 µm pore Acrodisc Syringe filters[6].

The activity of PPO was assessed with a Du 800 UV/Vis spectrometer[7] using catechol as the substrate. An aliquot of 1 ml of enzyme extract was added to 2 ml catechol and the change was immediately measured in absorbance at 398 nm (A_{398}) for 1min according to Quin & Tian (2005). The activity was expressed as A_{398} per minute per mg of protein. The protein

[5] Polychem Supplies, Durban, South Africa
[6] Pall Corporation, USA
[7] Beckman Coulter, VWR LabShop, IL, USA

content of each enzyme extract was determined by the method of Bradford (1976), using Bradford dye with bovine serum albumin[8] used as the protein standard.

The POD activity was analysed using guaiacol as the substrate, following the method of Cipollini (1997). Enzyme extracts (10 µl) were added to 990 µl of a guaiacol solution containing 0.25% guaiacol (v/v) in 10 $mmol^{-1}$ sodium phosphate buffer at pH 6.4 and 1.25% hydrogen peroxide (v/v). The change in POD activity after 1 min was measured by absorbance at 460 nm and was expressed as A_{460} per minute per mg of protein.

Phenylalanine ammonia-lyase (PAL) – For PAL extraction, a 1 g ground tissue sample was homogenized with an Ika® T25 Digital Ultra-turrax twice for 0.25 min at medium speed in 15 ml of a sodium borate buffer (0.1M, pH 8.8) containing 2 mM β-mercaptoethanol and 3 mM ethylenediatetraacetic acid (EDTA) (Miklas, 1993). The mixture was centrifuged at 13,000 x g for 15 min at 4°C (Miklas, 1993). The supernatant was collected and filtered for the enzyme assay. The PAL activity was assayed following the method of Hughes & Dickerson (1989) with some modifications. Two hundred and fifty micro-litres of enzyme extract were added to 1ml extraction buffer and 1 ml of 50 mM L-phenylalanine as the substrate in the same buffer. The reaction mixture was incubated in a water bath at 37°C for 1h and the reaction was stopped by the addition of 0.1 ml of 6 N HCl. The PAL activity was determined by the production of cinnamate, measured spectrophotometically at 290nm, and was expressed as nanomoles of trans-cinnamate acid formed per hour per mg of protein (nmol h^{-1} mg^{-1} protein). The blank was a crude enzyme extract mixed with L-phenylalanine with zero time incubation.

8.2.4 Leaf damage assessment and the area under the curve (AUC) calculation

In Trial 2, the leaf damage index (LDI) was also assessed per plot by scoring mite damage on plant leaves with a zero to five rating scale adapted from Meyer (1996):

0 = no damage;

[8] Sigma Chemical Co., St Louis Mo, USA

1 = 1-20% of leaf area damaged leaf

2 = 21-40% of leaf area damaged;

3 = 41-60% of leaf area damaged;

4 = 61-80% of leaf area damaged

5 > 80% of leaf area damaged.

The following formulae were used to estimate the LDIs in the trials:

$$\mathrm{LDI_p} = \frac{\sum \mathrm{LDI_f}}{\mathrm{N_f}};\ \mathrm{LDI} = \frac{\sum \mathrm{LDI_p}}{\mathrm{N_p}}$$

Where LDI is the mean leaf damage index per plot; LDI_p: the leaf damage index scored per plant; N_p: total number of scored plants per plot; LDI_f: the leaf damage index scored per leaf and N_f: the total number of leaves scored per plant. The area under curve (AUC), expressed in severity-days, was calculated based on the LDIs estimated over time and using the following formula (adapted from Jeger & Viljanen-Rollinson, 2001):

$$AUC = \sum_{\mathrm{i}}^{\mathrm{n-1}} \left(\frac{\mathrm{LDI_i} + \mathrm{LDI_{i+1}}}{2} \right) \mathrm{x}(\mathrm{t_{i+1}} - \mathrm{t_i})$$

Where n is the number of assessment times (n=7), LDI_i is the damage at the i^{th} assessment time (i = 1, 2, 3 ... n-1). The rating intervals ($t_{i+1} - t_i$) were equally spaced and were 3d. Thus, the AUC above formula was:

$$AUC = \sum_{\mathrm{i}}^{\mathrm{n-1}} \left(\frac{\mathrm{LDI_i} + \mathrm{LDI_{i+1}}}{2} \right) \mathrm{x3}$$

8.2.5 Statistical analyses

Data collected for enzymatic activity were analysed separately for the different assessment times by ANOVA procedure with the GenStat computer package (GenStat, 2007). Means were compared by Tukey's Honestly Significance Difference (HSD) test at the 5% significant level. Repeated measures ANOVA were used in the second trial to determine the interaction effect between time and treatment on enzyme activity; the repeated factor was time in the analysis. A polynomial regression was also used to examine the effect of

LDI on the enzymatic activity in the two treatments. Because the biochemical defences induced in the plant following herbivore attacks increase at moderate levels of damage and decrease when damage levels are high (Karban & Baldwin, 1997), we speculated that a quadratic relationship could fit the model with the equation: $Y= \beta o + \beta_1 LDI + \beta_2 LDI^2 + Є$, where β_o is the intercept, β_1 and β_2 are the slopes; and $Є$ is the residual. Where the quadratic relationship did not fit the model, the term "$\beta_2 LDI^2$" was removed to check for a linear relationship.

8.3 Results

Trial 1 – Plants without mites did not show any significant accumulation of the three enzymes, whether Si was applied or not. In mite-stressed plants, on the other hand, accumulation of the enzymes was significant. For POD and PAL enzymes, levels increased from Day 2 after mite inoculation. The level of POD in plants treated with Si was significantly higher than that of untreated plants from Day 3 after mite inoculation (Table 8.1). Similarly, the PAL activity was higher in Si-treated than in untreated plants, although the difference was not significant (Table 8.1). With regards to PPO, a significant accumulation was observed from Day 1 after mite inoculation. However, the difference between Si-treated and untreated plants was only observed at Day 5 and Day 6 after mite inoculation (Table 8.1).

Table 8.1 Change in peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activity over time in bean plants in Trial 1.

	Treatments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
POD (A_{460} min^{-1} mg^{-1} protein)	no TSM - no Si	0.478	0.481	0.574 a	0.722 a	0.910 a	0.954 a	0.902 a
	no TSM - with Si	0.526	0.555	0.662 a	0.858 a	0.830 a	0.934 a	0.804 a
	with TSM - no Si	0.538	0.759	0.910 b	1.493 b	1.560 b	1.680 b	1.962 b
	with TSM - with Si	0.502	0.646	1.101 b	1.921 c	1.943 c	2.354 c	2.460 c
	$F_{3,12}$	0.5 ns	0.9 ns	7.5*	43.2***	40.2***	42.9***	32.4***
	LSD (5%)	-	-	0.25	0.29	0.37	0.34	0.38
	% C.V.	12	23.9	19.1	15.6	18.2	16.4	17.4
PPO (A_{398} min^{-1} mg^{-1} protein)	no TSM - no Si	0.0047	0.0044 a	0.0050 a	0.0064 a	0.0070 a	0.0073 a	0.0071 a
	no TSM - with Si	0.0053	0.0055 a	0.0052 a	0.0070 a	0.0065 a	0.0076 a	0.0066 a
	with TSM - no Si	0.0055	0.0078 b	0.0124 b	0.0145 b	0.0177 b	0.0157 b	0.0162 b
	with TSM - with Si	0.0054	0.0104 b	0.0128 b	0.0191 b	0.0214 b	0.0217 c	0.0317 c
	$F_{3,12}$	0.1 ns	4.5*	8.9**	15.1**	31.3***	46.2***	49.7***
	LSD (5%)	-	0.0043	0.0049	0.0052	0.0043	0.0033	0.0023
	% C.V.	18.1	20.6	22.7	29.3	23.9	17.0	16.9
PAL (nmol h^{-1} mg^{-1} protein)	no TSM - no Si	0.513	0.558	0.582 a	0.676 a	0.608 a	0.678 a	0.752 a
	no TSM - with Si	0.654	0.546	0.603 a	0.601 a	0.640 a	0.614 a	0.716 a
	with TSM - no Si	0.561	0.676	0.788 b	0.878 b	1.048 b	1.250 b	1.373 b
	with TSM - with Si	0.559	0.732	1.056 b	1.132 b	1.286 b	1.421 b	1.512 b
	$F_{3,12}$	0.2 ns	1.1 ns	4.0*	5.9**	4.9*	2.6*	3.99**
	LSD (5%)	-	-	0.33	0.32	0.44	0.313	0.182
	% C.V.	7.6	10.6	11.3	13.9	13.9	16.2	13.4

Means with the same letter within a column are not significantly different by Tukey-HSD at 5% level of significance; ns: not significantly different ($p > 0.05$); *** Significantly different at $p < 0.001$; **Significantly different at $p < 0.01$; *Significantly different at $p < 0.05$

Trial 2 – As in the first trial, application of Si resulted in significantly increased levels of POD, PPO and PAL in plants attacked by TSM. The increase in POD activity was significant from Day 6 after TSM inoculation (Fig. 8.1). However, the highest levels of POD were observed on Day 9, Day 12, Day 15 and Day 18 of assessment. At these assessment times, the POD levels of Si - treated plants were increased by 51.5%, 29.5%, 29.6% and 21.5%, respectively, compared to Si-untreated plants. The assessment on Day 21 did not show any significant difference between the two treatments ($p > 0.05$), although the Si-treated plants showed a 13.8% increase in POD level.

The levels of PPO were higher in Si-treated plants in the trial ($p < 0.001$) with an increase of 31.5% and 33.3% respectively on Day 18 and 21 after TSM inoculation. The maximum levels of PPO were found from Day 12 onwards in Si-treated plants. In the control, the maximum activity was found on Day 15, then the activity dropped down on Day 18 and 21 of assessment times (Fig. 8.2). In the Si-treated plants however, the activity of the enzyme was maintained until Day 21 of the assessment period.

The trends in PAL activity in the Si-treated plants and the control observed in the trial (Fig. 8.3) were similar to those of POD activity. The levels of PAL observed were significantly higher in Si-treated plants than those in the control from Day 6 of assessment until the end of the trial ($p < 0.001$ for all cases). The maximum levels of activity were reached at Day 12 of assessment in Si-treated plants with an increase of 46.8 % compared to the control.

The repeated measures ANOVA showed a significant interaction between time x treatment for POD ($F_{7, 56} = 4.25$; $p < 0.05$), PPO ($F_{7, 56} = 35.1$; $p < 0.01$) and PAL activity ($F_{7, 56} = 3.32$; $p < 0.05$). These results indicated that the effect of treatments on the enzyme activity changed over time. Indeed, the levels of all the enzymes increased in both Si-treated and Si-untreated plants, however, all the three enzymes accumulated faster in Si-treated plants than in the control (plants without Si).

Table 8.2 Leaf damage index (mean ± SE) assessed over time and the area under curve (AUC).

	Days after mite inoculations							AUC
	3	**6**	**9**	**12**	**15**	**18**	**21**	
No Si	0.6 ±0.02	1 ±0.07	1.1 ±0.13	1.3 ±0.14	1.8 ±0.12	2.3 ±0.11	2.9 ±0.07	27.87 ± 1.77
With Si	0.9 ±0.01	0.9 ±0.05	1 ±0.03	1.2 ±0.08	1.2 ±0.13	1.5 ±0.1	2 ±0.11	21.75 ± 1.17
$F_{1,8}$	0.6 ns	0.23 ns	1.81 ns	0.34 ns	4.95 ns	17.38 *	20.95 *	8.27 *
% C.V.	24.7	20.4	15.6	21.5	28.1	15.8	12.9	13.56

ns: not significantly different ($p > 0.05$)
* Means significantly different at $p < 0.05$

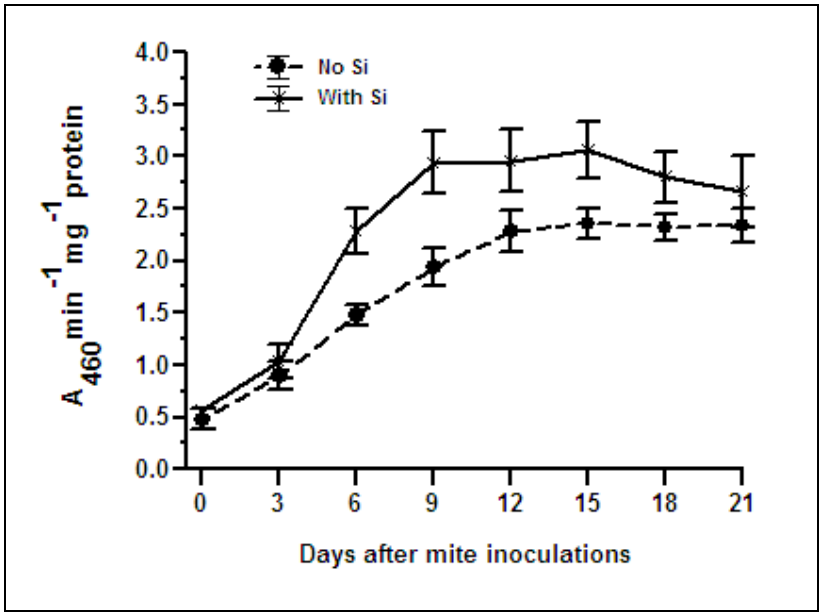

Figure 8.1 POD activity assessed every three days eight times in leaves of bean plants infested with *Tetranychus urticae* and treated or not treated with Si. The bars represent the standard errors of means (n=5). The points on the curves are POD mean values from five replications.

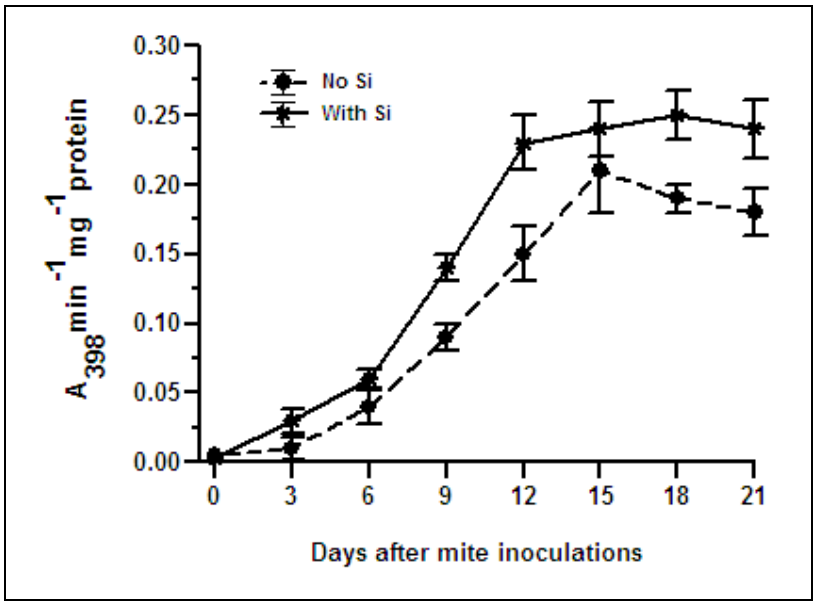

Figure 8.2 PPO activity assessed every three days eight times in leaves of bean plants infested with *Tetranychus urticae* and treated or not treated with Si. The bars represent the standard errors of means (n=5). The points in the curves are PPO mean values from five replications.

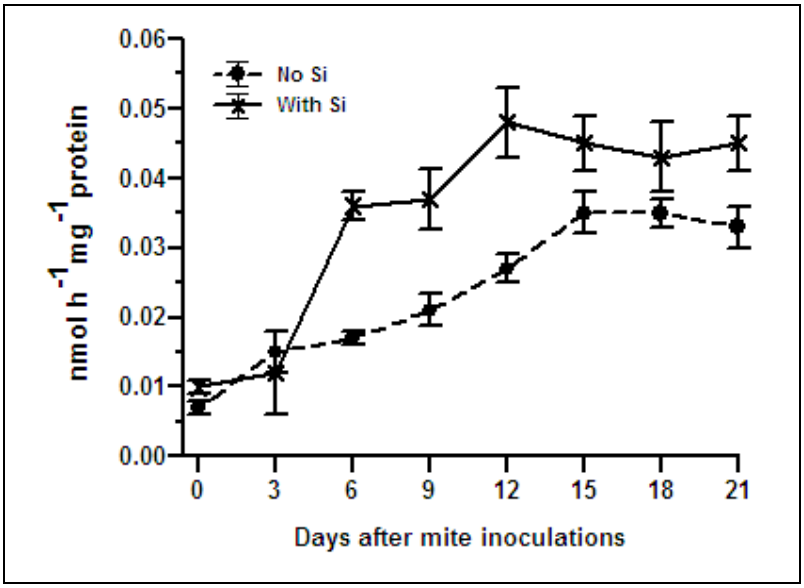

Figure 8.3 PAL activity assessed every three days eight times in leaves of bean plants infested with *Tetranychus urticae* and treated or not with Si. The bars represent the standard errors of means (n=5). The points in the curves are PAL mean values from five replications.

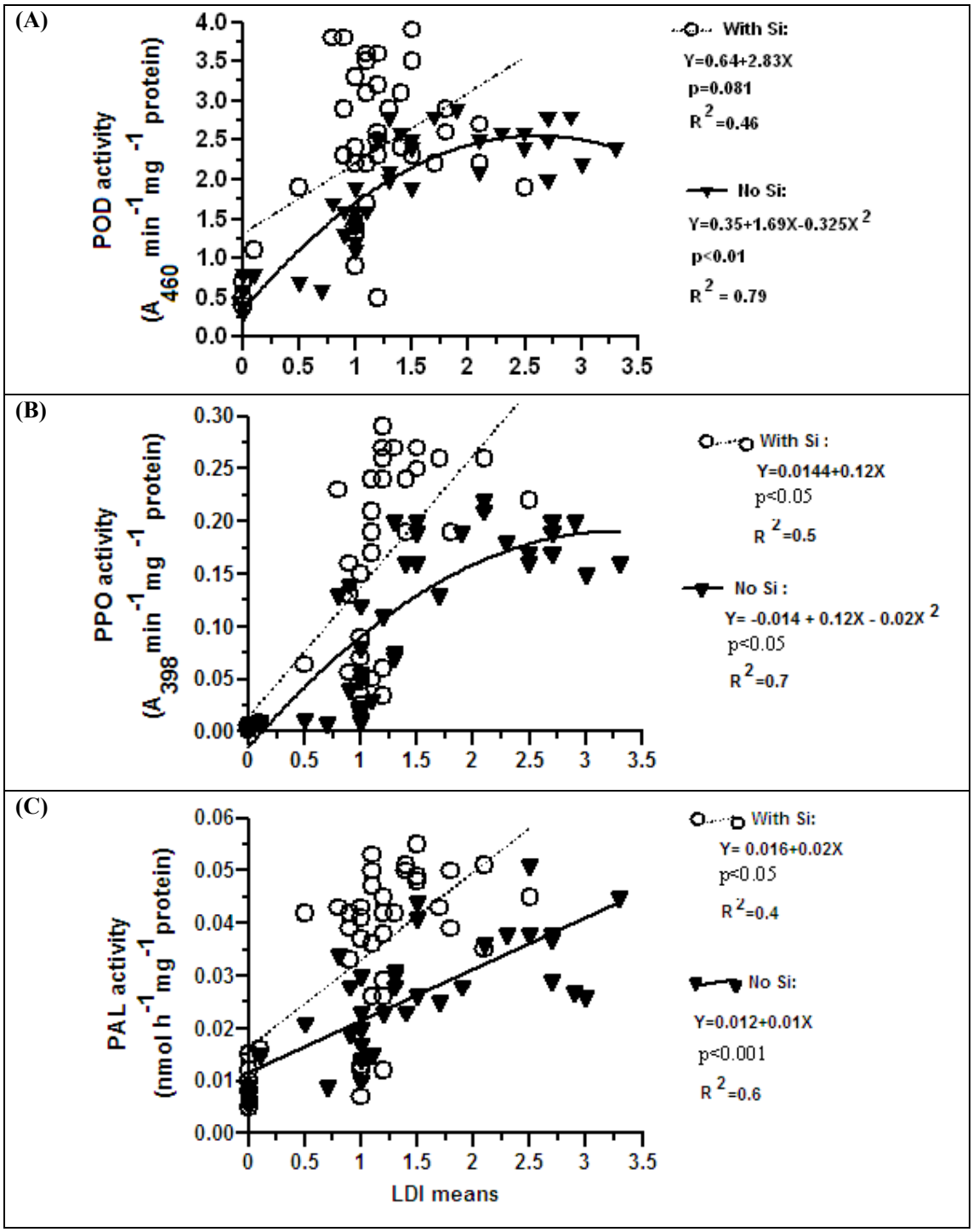

Figure 8.4 Changes in peroxidase (POD) (A), polyphenoloxidase (PPO) (B) and phenylalanine ammonia-lyase (PAL) (C) activity as a function of leaf damage index means in plants treated or not treated with Si. Symbols represent pooled data of each of the eight assessment times with mean values from five replications. Where a quadratic relationship did not fit the model, the quadratic term was dropped to fit a linear relationship.

The mean damage indexes (Table 8.2) were not significantly different between the Si-treated and untreated plants in the first two weeks of assessment ($p > 0.05$). On Day 18 and Day 21 high levels of damage occurred on plants in the control ($p < 0.05$). The AUC data analysed confirmed a high severity of TSM damage on plants without Si. Conversely, the polynomial performed showed that the levels of TSM damage to leaves influenced the activity of the enzymes (Fig. 8.4). In the control treatment, the enzyme activity increased progressively as the damage levels increased but tended to decline at high levels of damage, resulting in a significant quadratic relationship LDI-enzyme activity for POD ($p < 0.01$) and for PPO ($p < 0.05$). Such a relationship was not found for LDI-PAL activity ($p > 0.05$). Moreover, in the Si-treated plants there was no quadratic relationship between the LDI levels and the activity of PPO and PAL enzymes ($p > 0.05$). The high activity of these two enzymes corresponded to LDI values between 1 and 1.5 and no significant decline was observed at high levels of LDIs. However, at LDI values > 1.5, the POD activity tended to decline but there was neither a significant quadratic nor a linear relationship between the LDIs and the POD activity ($p > 0.05$).

8.4 Discussion

Enzymes such as POD, PPO and PAL are expressed in plants as part of cascade of defense reaction responses to herbivore attacks (Karban & Baldwin, 1997). The present study demonstrated that these enzymes were expressed in bean leaves infested with TSM. It was also investigated whether activity of POD, PPO and PAL was different in Si-treated and Si-untreated plants. In the two experiments, it was shown that bean plants fertilised with soluble silicon accumulated higher levels of the enzymes than plants without Si. The mechanism whereby Si enhanced the activities of POD, PPO and PAL enzymes is not clear. Some researchers suggested that Si may act as an elicitor in inducing defense responses in the plants (Correa *et al*., 2005; Gomes *et al.*, 2005). For example, Correa *et al*. (2005) reported that Si induced resistance in wheat plant to the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) in a manner similar to that of Benzothiadia-zole (BTH) a synthetic analogue of salicylate, the natural plant elicitor.

Conversely, it was found in the present study that application of Si alone to bean plants (without the stress of mite feeding) did not result in increased activity of the defense

enzymes. This finding is supported by previous work of Chérif *et al.* (1992), on induced resistance in cucumber against *Pythium ultimum,* and recently by the work of Caia *et al.* (2008) who reported a significant increased activity of PPO, POD and PAL in Si-treated rice plants only after the plants were inoculated with *Magnaporthe grisea* (Hebert) Barr. The evidence here is conclusive that Si was not an elicitor by itself, but primed the plants to respond to the elicitors released by TSM feeding.

The three defense enzymes monitored are involved in oxidation and biosynthesis of phytochemical defense compounds (Karban & Kuc, 1999). The enzyme peroxidase intervenes in the process of lignification and production of hydrogen peroxide or other phenoxy radicals that may interfere with herbivore digestion (Felton *et al.,* 1989, Hao *et al.* 1996). For instance, increased lignification in bean leaves due to the high activity of POD has been associated with enhanced resistance to *T. urticae* (Cipollini, 1997). The PPO enzyme is expressed at high levels in diseased and wounded plant tissues (Constabel, 1999). The proposed mode of action of PPO is based on the capacity of the enzyme to catalyse the oxidation of phenolic compounds to quinones, thus decreasing the nutritional quality of food and reducing its protein digestibility leading to plant protection (Felton *et al*., 1994). Quinones and reactive oxygen species (hydrogen peroxide) produced by the oxidation of phenolic compounds may also be absorbed by herbivores and have toxic effects (Constabel & Barbehem, 2008). On the other hand, PAL is positively associated with the biosynthesis of phenolic compounds that play an important role in the defense system of plants (Bi & Felton, 1995). Therefore, the increased activity of these enzymes observed in Si-treated plants may explain the lower severity of TSM on these plants, as measured by the low values of LDIs and the AUC compared to the Si-untreated plants (Table 8.2). High levels of POD, PPO and PAL have been found to be associated with increased resistance to *Bemisia tabaci* by cucumber plants (Shi-ze *et al*., 2008).

In both Si- treated and Si-untreated plants, accumulation of the enzymes was time dependent and was observed within 1-2 d after mite inoculation onwards. This period for activation of defence responses was also observed on tomato plants infested with spider mites (Kant *et al*., 2004). Because the level of mite damage increases as the population grows (Hussey & Parr, 1963), it seems plausible that the increased

accumulation of the enzymes observed over time was related to the increased levels of LDI (Fig. 8.4). Increased induced resistance as a result of increased levels of damage was previously reported in soybean plants infested by spider mites (Brown *et al.*, 1991) or by the Mexican bean beetles (Underwood, 2000). Conversely, high levels of damage reduced the strength of induced resistance (Karban & Baldwin, 1997). It was observed in the present study that the levels of POD and PPO in plants without Si increased as a result of moderate mite damage but decreased at higher levels of damage, as shown by the significant quadratic relationship between LDI and enzymes (Fig. 8.4). In Si-treated plants, on the other hand, the relationship between LDI and enzyme activity was positively linear (Fig. 8.4). This is probably because in these plants, the LDIs were kept at moderate levels, thereby maintaining the activity of the enzymes. Soluble silicon is known to maintain the production of defense chemicals in plants in response to diseases (Chérif *et al.*, 1992).

However, the trends of the change in enzyme activity in Si- treated plants showed not only higher levels of accumulation but also that the release of these defense enzymes was faster than in Si-untreated plants, with maximum levels reached within the second week after mite inoculation. For example, the same levels of enzyme activity measured for Day 6 after mite inoculation in Si-treated plants were observed in samples of Day 9, Day 12 and Day 15, respectively for PPO, POD and PAL in plants without silicon treatment. This effect of silicon is of paramount importance because the very rapid synthesis of biochemical defenses is a key element in effective plant resistance (Matern & Kneusel, 1988).

This study demonstrated that soluble silicon applied as a fertilizer may enhance plant resistance to the phytophagous mites *T. urticae* by accelerating and heightening the defense responses in the plant. This was shown by the rapid and high levels of accumulation of the defense enzymes POD, PPO and PAL measured in Si-treated bean plants infested with the pest. The production of these enzymes resulted in accumulation of biochemical defense compounds which resulted in reduced feeding by TSM. This confirms the hypothesis developed in Chapter 7 of the present thesis that Si enhanced the control efficacy of *Beauveria bassiana* by enhancing induced plant resistance to

TSM. It also implies that adding soluble silicon to plant fertilizers is a valuable option for the control of TSM and perhaps other arthropod-herbivores.

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Research overview

Introduction

The two-spotted spider mite (TSM) can inflict serious damage to plants; its importance in agriculture is reviewed in the first chapter. Problem of resistance to chemical acaricides is one of the main reasons why these mites are a key pest of greenhouse and vegetable crops. The use of biorational pesticides including entompathogenic fungi is expected to reduce the reliance on these chemicals.

In the present study, the possibility of using *Beauveria bassiana* (*Bb*) in TSM management was investigated. Laboratory bioassays were conducted to screen *Bb* strains for their virulence against the pest. The most potent was further studied in greenhouse and field trials on various crops. The research work was conducted at the University of KwaZulu-Natal, Pietermaritzburg and the findings are summarized as follows:

- All the *Bb* strains tested were infectious to TSM in laboratory, however the degree of virulence varied among strains, the most virulent being PPRI 7861 and PPRI 7315;
- Application of *Bb* conidia with Break-thru® as an adjuvant significantly improved the efficacy of the biocontrol agent in greenhouse conditions on various crops;
- Host plant type influenced the control efficacy of *Bb*;
- Field application of *Bb* with Break-thru® suppressed the populations of TSM in eggplants;
- When two fungicides, azoxystrobin (a strobilurin) and flutriafol (a triazole), were tested for their toxicity to *Bb*, azoxystrobin was found to be less harmful while flutriafol was highly toxic to germination of conidia and growth of mycelia;

- Application of soluble silicon as a plant nutrient enhanced the control efficacy of *Beauveria bassiana* through enhancement of induced plant resistance to *T. urticae.*

Implications of research findings

Different strains of *Bb* have been formulated and registered in different countries for use against insect pests. Various strains have been used in the control of mites, with varying results (Chandler *et al*., 2000; Irigaray *et al*., 2003; Chandler *et al*., 2005). So far, very few strains have been developed into commercial acaricides (Faria & Wraight, 2007). In South Africa, only *Bb* Plus (Strain PPRI 5339) developed by Biocontrol Products SA (Pty) Ltd., is registered for use against TSM. However, its efficiency, availability and adoption by growers are not well known. The most reliable control method of TSM, commonly used by growers in South Africa, is the application of abamectin, a chemical acaricide (Nel *et al*., 1999). However, due to its intense and frequent application, it is likely that mites will develop resistance to it. Resistance to abamectin has been reported in other countries (Campos *et al*., 1996; Beers *et al*., 1998; Stumpf & Nauen, 2002; Sato *et al.,* 2005). During the screening of *Bb* strains in the laboratory, the strains PPRI 7861 and PPRI 7315 killed > 80% of mites in 9 d post-inoculation and were far better than the commercial strain PPRI 5339. The control efficacy of these strains was also confirmed in a greenhouse on bean plants. This outcome is of great importance since it may lead to the development of a more efficient myco-acaricide for commercial use in South Africa.

Break-thru® was a better adjuvant for efficient application of *Bb* than an oil emulsion. Indeed, conidia of *Bb* are hydrophobic and cannot easily suspend in water, so surfactants are always needed for efficient application of these propagules. Moreover, fungal epidemics largely depend on specific weather conditions; usually these are associated with high humidity and moderate temperatures (Ferron *et al*., 1991; Tanada & Kaya, 1993). Unfortunately, the conditions for optimum infection are not always similar to those found in the environment of the pest. Hence, application of a good adjuvant is important for improving the infection rates of *Bb* and to help in reducing dependence on favourable weather conditions. Mineral oils have been widely tested and

have shown promissing results in protecting the microbial control agents from harmful environmental conditions (Feng *et al*., 1994; Prior *et al*., 1988; Shi *et al*., 2008). Since TSM colonies occur primarily on the lower surfaces of leaves and are protected by webs it was hypothesized that the silicon wetter Break-thru® might be useful for integration into the application of a fungal biocontrol agent such as *Bb* for the management of the pest. Break-thru® has not been tested much with microbial pesticide applications. This adjuvant has been mainly developed for the application of chemical pesticides. In South Africa, it is marketed by Evonik Industries Africa (Pty) Ltd. (formerly Degussa). It is applied at very low concentrations, which is important from an economic perspective. In most of the trials, *Bb* conidia (4×10^6 conidia ml^{-1}) suspended in 0.01% (w/w) aqueous Break-thru® solutions were twice as efficient in controlling adult populations of TSM as conidia suspended in an oil emulsion. It was then suggested that this adjuvant could be used in the application of *Bb* for the management of *T. urticae*. However, because of the high risk of excessive run-off of the spray suspension, the concentration of Break-thru® used in solution needs to be determined for each crop to which the BCA is to be applied.

The host plant affected the control efficacy of *Bb*. Previous studies have reported similar findings (Inglis *et al*., 1993; Kouassi *et al*., 2003). This implies that the host plant must be considered for a success of foliar applications of the BCA. The best control was achieved on cucumber, bean and eggplants, while there was a poor control on maize and tomato. The primary problem reported has been the short persistence of conidia on some host plant species (Kouassi *et al*., 2003), and presence of chemical compounds inhibitory to the BCA (Costa & Gaugler, 1989). It was demonstrated in this study that the amount of inoculum deposited following spray application also had a significant effect on control efficacy of *Bb* against TSM, and that it varied with crop types and the concentration of conidia in the suspensions. Direct application of conidia onto mites may be a requirement because they do not easily pick up conidia from treated leaf surfaces.

Field application of *Bb* significantly suppressed the population of TSM on eggplants. These findings concur with those of Shi *et al.* (2008), who reported that *Bb* controlled cotton spider mite, *Tetranychus* spp., under field conditions. Due to the effects of

fluctuating temperatures, low relative humidity and ultra-violet irradiation, field tests of *Bb* formulation applied against arthropod pests often provide variable results. However, field data are necessary as initial steps for assessing the commercial potential of biocontrol agents. The *Bb* strain and formulation tested in this study showed potential for mite control in the field. The efficiency of different spraying frequencies was also determined. This corresponded to one or two week intervals from one spray to another. When 2g l^{-1} of *Bb* conidia (*ca.* 1.6×10^{12} conidia ha^{-1}) was sprayed on eggplants at one or two week intervals a reduction in mite density of 68.9 and 71.6%, respectively was observed. A field application of higher concentrations than that applied would not be economical due to higher costs associated with production of these conidia (Feng *et al*., 1994; Ye *et al*., 2006). There were no significant differences between the two spraying intervals. Therefore one spray every two weeks would be as effective and half the cost of a weekly spray. However, this needs to be confirmed on other crops.

When azoxystrobin (a strobilurin) and flutriafol (a triazole) were tested for their toxicity to *Bb*, azoxystrobin was less harmful while flutriafol was highly toxic to germination of conidia and growth of mycelia. There have been many studies on the effects of fungicides on *Bb*. However, because the effects vary with the type of chemical and the strain of fungal species tested (Jaros-Su *et al.,* 1999; Shapiro-Ilan, 2002), compatibility of a specific strain with the more common fungicides is required. Azoxystrobin and flutriafol are commonly used in South Africa for the control of fungal diseases on various crops. There were no available reports on the effect of flutriafol on *Bb*. However, other triazole fungicides tested *in vitro* have shown varying effects. In this study, flutriafol was shown to be highly toxic to *Bb* strain R444, affecting germination and growth on a culture medium and the control efficacy of the BCA in bioassay against mites. On the other hand, azoxystrobin was shown to be less harmful to the germination of conidia and did not affect the control of mites in bioassays. Usually, chemical products found to be harmless to entomopathogenic fungi *in vitro* are likely to remain so in the field, while the harmful ones may not necessary remain so (Majchrowicz & Poprawski, 1993). Therefore, additional field or greenhouse studies are needed to confirm the effects of these fungicides within an integrated crop management system. If flutriafol is revealed to impede the activity of *Bb* even under field conditions, then strategies must be used to avoid contact between these agents. For example, flutriafol is

a systemic fungicide; therefore does not necessary have to be applied on leaves and could be applied as a drench instead.

Soluble silicon enhanced the control efficacy of *Bb* in the greenhouse. This was measured by an increased mortality of mites occurring where *Bb* was combined with soluble Si compared to where either of the two products was applied alone. This phenomenon was interpreted as a result of increased resistance in Si-treated plants synergizing with the biocontrol activity of *Bb*. Silicon has been widely documented for its role in enhancing plant resistance to pests and diseases. Initially, Si was thought to create a physical barrier in plants but there is also increasing evidence on its role in enhancing or priming of biochemical defense in plants. Most of these have been reported in the field of disease control. Our research reported in Chapter 8 showed that Si can play an important role in the biochemical defense system of plants against phytophagous arthropods. This agrees with previous reports by Correa *et al.* (2005) and Gomes *et al*. (2005). However, in these two reports it was suggested that Si acted as an elicitor in inducing plant resistance. In our study, we demonstrated that Si alone did not induce plant resistance to TSM but enhanced the production and accumulation of biochemical defenses only in plants infested with mites. The increased accumulation of these biochemicals in plants inhibits the feeding behavior of mites, which stresses the mites and increases in their susceptibility to *Bb* infections. The practical implications of such a phenomenon necessitate greater consideration of the actual crop to which silicon is applied, and the form and the concentrations of Si to be applied. In our trials, Si was applied in hydroponics in the form of potassium silicate. This synergized well with *Bb* at concentrations between 80 and 160 mg l^{-1} on cucumber, eggplant, bean and tomato. The beneficial effect of Si as biocontrol reported in this study is in support with the long-term objective at the University of KwaZulu-Natal, of including the element silicon in the integrated management systems of plant pests and diseases. For example, a new formulation of slow release silicate is being investigated for application to field crops.

Conclusion and further research

The susceptibility of TSM to *Bb* varied with the strains. The most virulent strains were PPRI 7861 and PPRI 7315. Based on this and its rapid growth and abundant sporulation on culture medium, the strain PPRI 7861 was selected for further investigations, including greenhouse and field tests. This strain has the potential to provide mite control, either as a stand-alone treatment or as a component of integrated pest management. Further investigations are required for it to be developed into a commercial myco-acaricide for field and greenhouse applications. These include more field trials to test the survival of the BCA under a challenging environment, to test its efficacy on more crops, to test its compatibility with other control agents and to test its ability to control other plant pests. However, it has to be noted that although the strain R444 significantly reduced the population of TSM adults and the damage to leaves of bean, eggplant and cucumber during the trials, in no case was 100% mortality observed, even with the best treatment. As such, there were always some living mites on plant leaves. This may cause a problem to some ornamental plants for which plant leaves, the plant parts mites attack, have a decorative value. In such cases, the combination of *Bb* with existing control measures will be required. For example, *Bb* is compatible with abamectin (de Oliveira & Neves, 2004), the two agents can be applied together and this may reduce the probability of the pest in developing resistance to the acaricide. It is also imperative to continue screening for more *Bb* strains or other with higher ovicidal effect and virulence to mite juveniles.

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