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ASSESMENT OF BACTERIA TO CONTROL THE TWO-SPOTTED SPIDER MITE *Tetranychus urticae* (ACARI: TETRANYCHIDAE*)*

JORGE FRANCO MARINGOLI CARDOSO

Dissertation presented to obtain the title of Master in Plant and Animal Health, Food and Environmental Safety in Agribusiness. Area of concentration: Advancement of Knowledge for Sustainability in the Agricultural Production Process.

SÃO PAULO
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Adviser: Dr. Luís Garrigós Leite

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DEDICATION

To my father that, with a lot of patience, teaches me the value of discipline, hard work, and an open, clear and positive mind. Thanks dad.

To my mother, who teaches me how to be kind, persistent, and that never gave up on me.

To my adviser, Leite, that gave me space to grow and work on my own, while always being near when I needed help.

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ABSTRACT

CARDOSO, J. F. M. **Assessment of bacteria to control the Two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae).** 2022. Dissertation. (Master in Plant and Animal Health, Food and Environmental Safety in Agribusiness) – Biological Institute, State of São Paulo Agency for Agribusiness Technology, Department of Agriculture and Supply of the State of São Paulo, São Paulo, 2022.

The two-spotted spider mite *Tetranychus urticae* is an agricultural pest of worldwide relevance. Being a polyphagous, cosmopolitan organism with high reproductive rates, it is capable of affecting 1100 plant species and about 150 crops. Their infestations reduce the productivity of the field and can cause the death of plants. The main technique used for its control is the application of chemical pesticides that in many cases are not efficient and lead to the selection of resistant populations. As an alternative to the use of pesticides, biological control has been highlighted, where species of bacteria are capable of producing secondary metabolites with insecticidal action, showing potential as biological control agents. Thus, this work aimed to assess bacterial strains for the control of *T. urticae*. A screening was performed with previously selected 104 strains from the Collection of Microorganisms of the Laboratory of Biological Control, in the Advanced Centre of Research on Plant Protection and Animal Health (CAPSA). After the screening, the three strains that caused the highest mortality percentages were selected for a multiple rates test by dilution of the bacterial cultures to 2%, 5%, 10%, 20% and 40%. The three most effective strains (365, 268 and 321b) were molecularly identified as *Serratia* sp 365, *Bacillus* sp 268 and *Heyndrickxia* sp 321b. The bacteria with the highest performance, *Serratia* sp 365, was selected for a test on macaúba (*Acromia aculeata*) seedlings production. The experiment consisted of 6 treatments represented by the bacterial culture diluted at rates of 5%, 10%, 20% and 40%, and by the two controls water and liquid culture. For the rate test, the three best strains *Serratia* sp 365, *Bacillus* sp 268 and *Heyndrickxia* 321b showed LC_{50} of 38.67%; 57.32% and 150.65%, respectively. For the test with *Serratia* sp 365 on macaúba, the highest rates (40% and 20%) suppressed the mite population and the egg density after a single application of the bacterium. The two lowest rates (5% and 10%) suppressed the population and eggs only after two applications.

Keywords: *Biological Control. Screening. Acromia aculeata. Serratia. Bacillus. Heyndrickxia.*

RESUMO

CARDOSO, J. F. M. **Avaliação de bactérias para o controle do Ácaro-rajado *Tetranychus urticae* (Acari: Tetranychidae).** 2022. Dissertação. (Mestrado em Sanidade, Segurança Alimentar e Ambiental no Agronegócio) – Instituto Biológico, Agência Paulista de Tecnologia dos Agronegócios, Secretaria de Agricultura e Abastecimento do Estado de São Paulo, São Paulo, 2022.

O ácaro-rajado *Tetranychus urticae* é uma praga agrícola de relevância mundial. Por ser um organismo polífago, cosmopolita e com altas taxas reprodutivas, é capaz de afetar 1100 espécies de plantas e cerca de 150 culturas. Suas infestações reduzem a produtividade do campo e podem acarretar na morte das plantas. A principal técnica utilizada para seu controle é a aplicação de defensivos químicos que em muitos casos não são eficientes e levam à seleção de populações resistentes. Como alternativa ao uso de agrotóxicos, o controle biológico tem se destacado, onde espécies de bactérias são capazes de produzir metabólitos secundários com ação inseticida, mostrando potencial como agentes de controle biológico. Desta maneira, este trabalho teve como objetivo avaliar cepas bacterianas para o controle de *T. urticae*. Para tal, foi realizada uma triagem com 104 cepas da Coleção de Microrganismos do Laboratório de Controle Biológico, do Centro Avançado de Pesquisa em Proteção Vegetal e Saúde Animal (CAPSA). Após a triagem, as três cepas que causaram maior porcentagem de mortalidade foram selecionadas para um teste de diluição do caldo bacteriano nas concentrações de 2%, 5%, 10%, 20% e 40%. As três linhagens mais eficientes (365, 268 e 321b) foram identificadas molecularmente como *Serratia* sp 365, *Bacillus* sp 268 e *Heyndrickxia* sp 321b. A bactéria com melhor desempenho, *Serratia* sp 365, foi selecionada para um teste em produção de mudas de macaúba (*Acromia aculeata*). O experimento foi composto por 6 tratamentos representados pela cultura bacteriana diluída nas taxas de 5%, 10%, 20% e 40%, e por dois tratamentos controle, água e meio de cultura NB. Para o teste de diluição, as três melhores cepas *Serratia* sp 365, *Bacillus* sp 268 e *Heyndrickxia* 321b apresentaram CL50 de 38,67%; 57,32% e 150,65%, respectivamente. Para o teste com *Serratia* sp 365 em produção de macaúba, as menores diluições (40% e 20%) suprimiram a população de ácaros e a densidade de ovos após uma única aplicação da bactéria. As duas diluições mais altas (5% e 10%) suprimiram a população e os ovos somente após duas aplicações.

Keywords: *Controle Biológico. Screening. Acromia aculeata. Serratia. Bacillus. Heyndrickxia.*

SUMMARY

1 INTRODUCTION

Present-day agriculture has the incidence of pests as one of its main concerns. Their occurrence involves aspects directly related to food production and economy, being responsible for reductions in harvest volumes, decreasing product quality and even causing the death of crops, affecting the field's productive capabilities (MIRANDA, 2018).

The two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) is one of the most devastating pests of the current agricultural and ornamental production scenario (ATTIA et al., 2013; AL-AZZAZY; ALSOHIM; YODER, 2020). Due to its phytophagous and polyphagous feeding habit, it is capable of causing both physical and economical damage to more than 1,100 species of plants and approximately 150 types of crops (VAN LEEUWEN et al., 2010). Its ubiquitous nature renders a geographical distribution across many areas of the globe, occupying most zones of temperate subtropical climate (INCEDAYI et al., 2021), with more than 100 countries reporting infestations of *T. urticae* (CABI, 2021).

The damage inflicted upon plants by *T. urticae* occur during its feeding, which consists of disrupting epidermal cells of the host plant and sucking the contents of their mesophile. These injuries alter the plant's physiological processes and reduce its areas of photosynthetic activities (FLECHTMANN, 1985; EROGLU et al., 2019; INCEDAYI et al., 2021). The accumulation of these injuries develops into chlorotic spots, that rise along with increasing populations of *T. urticae*, resulting in premature defoliation, the cessation of plant growth and its eventual death (BERNARDI et al. 2010).

Currently, the main control technique used to manage populations of this mite is the chemical control through synthetic acaricides (ÇAĞATAY et al. 2018; SATO et al. 2007; WATANABE et al. 1994). However, due to the high reproduction rates of this species and its short life cycle, this practice stimulates the development of acaricide resistant populations (WYMAN; OATMAN; VOTH, 1979).

Since its first report as a pest, *T. urticae* has traced a history of developing resistances towards pesticides (GOODWIN et al., 1995), requiring higher rates and frequency of applications that lead to increasing health risks to producers and consumers, besides generating environmental impacts (EDGE; JAMES, 1982; SATO et al. 1994; ATTIA et al. 2013). ATTIA et

al. 2013). In addition, the excessive use of chemicals may promote environmental imbalances that favours *T. urticae*'s population growth, such as the elimination of natural predators that more are sensitive to chemicals (PONTES, 2006; KUMARI et al., 2017).

In Brazil, more than ten registered Active Ingredients are used to control *T. urticae*, with varied toxicological classifications (AGROFIT, 2019) and an array of scientific works have highlighted populations that are resistant to the main products on the market (KUMARI et al., 2017).

The high impact caused by infestations of this pest and the problems involved in its current control techniques emphasize the need for new technologies and management strategies, such as plant-based insecticides and agents of biological control (NICETIC et al., 2001; PONTES, 2006; POTENZA et al., 2006). Among the agents, different types of organisms are promising, such as predatory mites, entomopathogenic fungi and bacteria.

Bacteria are highly varied organisms used on different fronts of pest management (PALMA et al., 2014; SANTOS-MATOS et al., 2017; FIRA et al., 2018). They are able to inhabit many different aquatic and terrestrial environments, plant rhizospheres and even internally colonize plants. Certain genera have a considerable part of their genome dedicated to the production of secondary metabolites, which makes them capable of synthesizing multiple antagonistic substances with distinct chemical structures (PALMA et al., 2014; EHLING-SCHULZ; LERECLUS; KOEHLER, 2019).

There are still few studies that explored bacteria for the management of *T. urticae*, although their importance has shown considerable growth in recent years. Studies that explore species of *Pseudomonas*, *Actinobacter*, *Bacillus* and symbiotic bacteria of entomopathogenic nematodes attested to the effectiveness of these microorganisms to manage *T. urticae* (QESSAOUI et al., 2017; EROGLU et al., 2019; AL-AZZAZY; ALSOHIM; YODER, 2020).

Despite these advances, few bacterial-based products are used commercially. To date, the main product provided by bacteria on the market is abamectin, originally produced by the bacterium *Streptomyces avermitilis*, although there are already reports of low efficacy due to the development of acaricide resistance in *T. urticae* populations (HERRON et al., 2021). Thus, it is important to prospect new species of bacteria to control *T. urticae*, increasing the number of

strains with potential to effectively control this mite, and providing environmentally and human safe products (LACEY et al., 2015).

Bacteria are capable of producing a wide variety of substances with insecticidal activity, as in the case of *Streptomyces* and *Saccharopolyspora* species (RUIU, 2015). In addition, the techniques required to produce bacteria can be simple, facilitating their production, formulation and application with cost reductions (LACEY et al., 2015). Hence, a constant search for strains with potential use is necessary, in addition to field tests that validate the efficiency of those selected for the control of the *T. urticae*.

2 OBJECTIVES

2.1 Main objective

To evaluate bacterial strains for the control of *T. urticae*

2.2 Specific Objectives

- Prospect bacteria for the control of *T. urticae*;
- Compare the virulence of the selected bacteria at different rates;
- Evaluate the effectiveness of *Serratia* sp 365 in the control of *T. urticae* in greenhouse conditions.

3 LITERATURE REVIEW

3.1 *Tetranychus urticae*

3.1.1 Characteristics, morphology and life cycle

Mites are a group of arachnid arthropods with a great variety of species and feeding habits, ranging from parasitic to predatory (VAN LEEUWEN et al., 2010). Many of them are of great commercial relevance as veterinary, medical and agricultural pests, while also being used as model organisms for biological studies (SABELIS, et al. 2010; SOUSA et al. 2019).

Amidst the arachnids, the Acari own the only group of mites that feeds on plants (GOTO, 2016), with around 7000 identified species and among them, the Tetranychidae are the family of phytophagous mites of highest agricultural relevance. With 70 genera and more than 1300 identified species, approximately 10% of them have the potential to become agricultural pests (MIGEON, DORKELD, 2019). In Brazil, 128 species of Tetranychidae are recognized, belonging to 24 genera and some of them are considered pests of multiple crops (FLECHTMANN, 2020).

The Tetranychidae have members whose size ranges from 200 to 900 micrometres and are capable of producing webs with important roles. As the mites move, their webs can extent from the leaves to the stems of infested plants. Eggs are laid and young mites develop under the webs. They determine the limits of the colony, provide protection from adverse climate conditions and UV rays, as well as aiding in dispersion to other habitats and discouraging predators (TEHRI, 2014; AGUT et al., 2018; MIGEON, DORKELD, 2019).

The two-spotted spotted spider mite, *Tetranychus urticae*, initially described by Koch in 1836 (MIGEON, DORKELD, 2019), belongs to the Tetranychidae family. Like other members of the this family, it has a short life cycle and high reproductive rates which makes their control difficult, and stimulates the development of populations resistant towards chemicals (SATO, 2017). The two-spotted spider mite has a greenish-yellow colouring, and its back has two characteristic pairs of dark spots, besides a cover of long setae. The sexual dimorphism is

expressed in the male's inferior size, around 0.25 mm, before the female, who has around 0.46 mm (BERNARDI et al. 2010; SATO, 2017).

Many environmental aspects may influence the duration of the reproductive cycle of *T. urticae*. External factors such as temperature, humidity, the nutrition and species of the host plant, the type of the crop or orchard, and exposure to pesticides changes the time required for this cycle to be completed and shows considerable variations, already explored by many authors (LAING, 1969; SHIH; POE; CROMROY, 1976; MONDAL; ARA, 2006; NAJAFABADI et al., 2014). Reports point out that under ideal conditions, free from stresses as predation, acaricides and in temperatures above 30°C with relative humidity below 60%, the total developmental period of *T. urticae* can range from 5 to 6 days (BERNARDI et al. 2010; RINCÓN, RODRÍGUEZ, COY-BARRERA, 2019; SATO, 2016).

The developmental stages that form this life cycle are: egg, larva, protonymph deutonymph and adult (BERNARDI et al. 2010). The eggs are round, translucent, and yellow or orange. Hard to spot with the naked eye, their diameter is approximately 0.13 mm. Adult females may lay their eggs directly on the lower surface of leaves or in their webs, commonly next to the ribbing and veins of the host plant. Hatching may occur in about 5 days after laying, under ideal temperatures, which range from 25º-30 ºC and 45-55% relative humidity (BERNARDI, et al. 2010; TEHRI, 2014).

After hatching, larvae (Figure 1) have three pairs of legs and oval or spherical shape, measuring an average of 0.16 mm. Protonymphs on the other hand, are pale green coloured, have four pairs of legs and reach 0.2 mm size (Figure 1). Deutonymphs are yellowish or pale brown, their size is around 0.3 mm and, at this developmental stage, the notorious spots that grant *T. urticae* the name "two-spotted" appear on their backs. It is also at this stage where the distinction between males and females begins. Males then start to surround females in transition to the adult stage to copulate after their emergence (MEENA et al., 2013; RINCÓN; RODRÍGUEZ; COY-BARRERA, 2019).

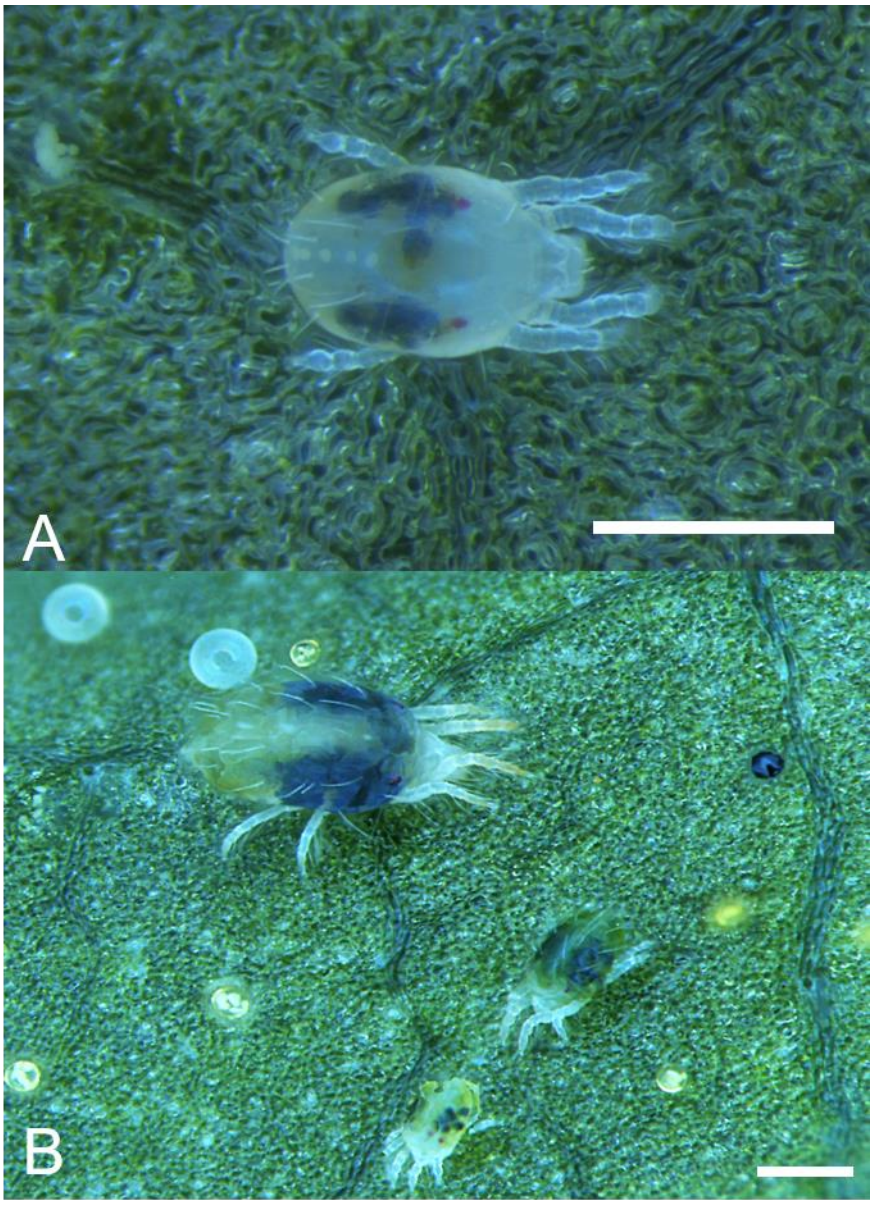

Figure 1: (A) Larva of *Tetranychus urticae*, characterized by having only three pairs of legs and pale colouring. (B) Size differences between developmental stages of *Tetranychus urticae*. Centralized, an adult female, accompanied below by a protonymph and then a larva. Scale: 0.2 mm. Photograph: (COSTA, 2021).

Adults carry setae at their backs. Males are slightly smaller and have slender bodies, measuring an average of 0.25mm (MEENA et al., 2013). Female adults (Figure 2) with a mean size of 0.472 mm, live around 10 to 30 days, and may lay eggs one or two days after reaching full maturity, producing up to 100 eggs in this period, with unfertilized eggs developing into males (SUEKANE et al., 2012; TEHRI, 2014; AGUT et al., 2018).

Figure 2: An adult female of *Tetranychus urticae*, characterized by its four pairs of legs and the body covered with setae. Scale: 0.2mm. Photograph: (COSTA, 2021).

Breeding may occur throughout the whole year, however, under adverse climatic or nutritional conditions, such as rigorous winters, eggs and females in reproductive stages may enter diapause, a state of lower metabolic activity, allowing them to endure such stresses. Upon entering diapause, females cease feeding, and some may acquire a reddish colouration. It is common that populations that infest greenhouses or areas of temperate climate lose their ability to enter diapause, reproducing even during winters (HOY, 2016).

3.1.2 *Tetranychus urticae* as a pest

Damages caused by *T. urticae* to host plants occur during its feeding. All developmental stages have mouthparts that bear stylets adapted to penetrate and, via the rostrum, ingest cell contents. The degree of these injuries is directly related to the stylet length and thickness. Larvae have stylets with lengths close to 100 micrometres, reaching 150 in adults stages (BENSOUSSAN et al. 2016; TEHRI, 2014; WAKIL, BRUST, PERRING, 2018).

Hence, specimens of *T. urticae* tear and suck the contents of mesophyll cells, causing them to collapse and degrading their chloroplasts. The remaining cellular matter is

coagulated into a necrotic mass. The resultant lesions reduce the amount of areas of the plant that are capable of performing photosynthesis, changing its physiological processes and resulting in lower concentrations of nitrogen, phosphorus and proteins inside other cells, which leads to a reduction on the plant's productivity (AGUT et al. 2018; BENSUSSAN et al. 2016; SUEKNE et al. 2012; WAKI, BRUST, PERRING, 2018).

As a population of *T. urticae* develops on the host pant, the damages caused by its feeding accumulates and usually manifests in stages. The first one is the emergence of chlorotic spots on infested leaves, leading to premature yellowing and eventual defoliation (BERNARDI et al. 2010; RINCÓN, RODRÍGUEZ, COY-BARRERA, 2019). Along with the growing infestation, other leaves are targeted, and then the stems, stalks, and fruits. The webs begin to be noticeable and plant growth is reduced. In severe infestations, the plant dies and the colony migrates to neighbouring plants (KUMRAL et al. 2019; LAING, 1969).

Records point out that *T. urticae* was first considered an agricultural pest of high relevance around 1962, when its resistance to pesticides used at the time was confirmed (GOODWIN et al., 1995). Previously, Tetranychidae were considered sporadic pests. However, after the conclusion of the second world war, the extensive use of synthetic pesticides such as DDT (Dichloro-Diphenyl-Trichloroethane) turned them into significant pests, particularly in greenhouses, through the selection of resistant populations. The efficacy of a pesticide commonly lasted for a year after its introduction (HOY, 2016). Since then, a history of resistance development can be traced around chemicals that seek to control populations of *T. urticae* (GOODWIN et al., 1995).

Currently, the geographical distribution of *T. urticae* reaches worldwide status, since it can be found in regions such as Afrotropical, Australasian, Nearctic, Neotropical, Oriental and Palearctic, in a total of 110 countries that have reported infestations (Figure 3). Some of the crops that are threatened by its damage are: cotton, beans, soy, strawberries, papaya, tomatoes, peaches and ornamental plants like roses (GALLO et al. 2002), with estimates suggesting that more than 80% of worldwide investments on acaricides are dedicated to attempts of managing this pest (VAN LEEUWEN et al., 2010).

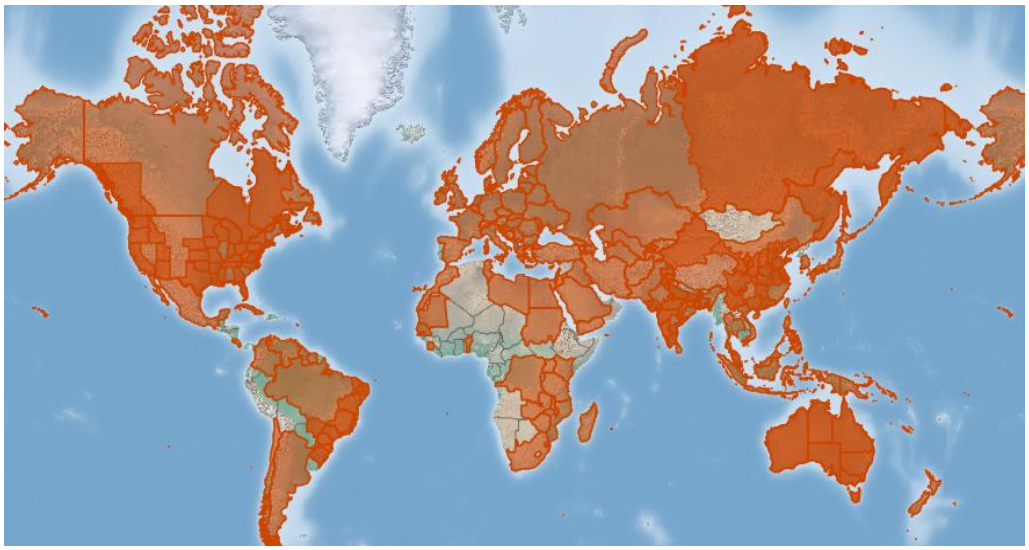

Figure 3: Geographical distribution of *Tetranychus urticae*. Countries highlighted in orange have records of the two-spotted spider mite as a pest organism. Source: Invasive Species Compendium. Wallingford.

In Brazil, around 42 crop productions hold *T. urticae* established as a relevant pest. Strawberries, papaya, cotton, beans, tomatoes and ornamental plants are a few examples (SATO, 2017), and some of them raise an important aspect of such infestations: crops have different sensitivities to damages caused by *T. urticae*. Certain plants are capable of bearing small populations without suffering great damage. However, species of pears, as an example, start to defoliate even with a very low number of injuries (HOY, 2016). Sensitivity is also a particularly severe problem when the visual aspect of the harvest is relevant, as is the case with fruits and ornamental plants, in which injuries on leaves, flower or fruits cause the market price of produces to considerably decrease and even impede their sales (PASCUAL-RUIZ et al., 2014).

3.2 Pest management and biological control with bacteria

Since the start of their usage, after 1945, chemical pesticides have been playing an important part on the development of agriculture, reducing product losses and improving harvests. Associated to the growing world population and the necessity for agricultural growth, production grew around 11% per year in the period of 1950 to 2000. Three billion tons of pesticides are used worldwide every year, but only 1% of them is used in an efficient way to manage pest organisms of target plants (HAYES; HANSEN, 2017; TUDI et al., 2021). The excessive use of these synthetics then started to cause serious problems, affecting non-target organisms, contaminating terrestrial and aquatic ecosystems, carrying risks to humans and selecting resistance populations of pest organisms (CARVALHO, 2017; TUDI et al., 2021).

Given this scenario, alternatives to the chemical management of pests started to be explored, and one of the most promising options resides in biological control. This refers to cases where a biological agent impedes the natural tendency of a population to grow exponentially and to the mechanisms through which an agent provides this population control. This type of relationship occurs naturally in all ecosystems. The biological control technique has been used for 2000 years, but it is only at the end of the 19th century that the modern and industrialized use started to be explored (VAN DEN BOSCH; MESSENGER; GUTIERREZ, 1982; VAN LENTEREN et al., 2017).

Biological control in agriculture has four main techniques to stimulate the interaction between natural enemies and pest organisms, defined by Eilenberg, Hajek and Lomer, (2001) as: Classical biological control, Inoculation biological control, Inundation biological control and Conservation biological control. One of these techniques, inundation, makes use of the properties that some organisms have to provide population control, selecting those that present potential, producing them in high rates and applying them seasonally in crops. It is then possible to prevent pest organisms from multiplying themselves to the point of causing damages to planting and harvesting, even in periods where the agent’s population would naturally decrease, due to lack of resources or adverse conditions (RIDGWAY, R. L., VINSON, 1977; VAN LENTEREN et al., 2017).

The technique of seasonal application of natural enemies includes different classes of organisms, such as nematodes, gastropods, parasitoids, predators, vertebrates and microorganisms. Following predators and parasitoids, microbiological agents are the most commonly used organisms. These can be classified as fungi, yeasts, viruses or bacteria (RIDGWAY, VINSON, 1977).

The European continent is the largest consumer of bacteria as applied biological control agents, reaching this level through to political actions, Integrated Pest Management programs and high consumer demands. Europe is followed by North America, and Latin America has considerably grown as a market. In the last few years, biological control programs using invertebrates and microbial agents reached 30 million acres worldwide (VAN LENTEREN et al., 2017).

The pesticide market was worth 58.46 billion dollars in 2015, while the whole value of biological control reached only 1.7 billion. But, albeit considerably smaller than consumption of chemical pesticides, the market for bioproducts holds a bigger growth, that since 2015 is higher than 15% a year, while the development of chemicals grows by 6% a year (VAN LENTEREN et al., 2017).

Due to it being an emerging market, the majority of biocontrol producers is composed of medium to small sized businesses, and details about the production and registry of species used to control pests is sparse and at times contradictory. A single strain can be registered under different names, with a lack of information about certain strains and with countries that do not have reports about producers of biological products. Although it is difficult to quantify how many strains and species are under use in the world, it is estimated that at least 209 strains and 94 species of microorganisms are registered, produced and used (VAN LENTEREN et al., 2017).

Products composed of bacterial agents can contain living cells or just the metabolites of interest, that are produced by these bacteria and later separated from the cells. Their action upon the pest organism may occur in different ways, such as the induction of

resistance of the plant to the pest, and the antibiosis promoted by the metabolites of the control agent (KÖHL; KOLNAAR; RAVENSBERG, 2019).

One of the strategies with greatest agricultural potential is the induction of plant resistance to pathogens. Beneficial microorganisms can act on the plant to stimulate its immune response in a local manner, where injuries and necrosis were caused by a pathogen, in the entire plant, or even in neighbouring plants. Such resistance can be active only though the presence of a pathogen, or induced permanently, resulting in a more effective and faster immune response upon infestations (CONRATH et al., 2015; MAUCH-MANI et al., 2017).

The antibiosis promoted by metabolites occurs through antagonistic organisms, such as biological control agents, whose growth may depend on the direct consumption of nutrients by the pest organism, or whose secretion occurs naturally. Many species with this property have a small range of organisms that can be targeted, and killed, which can prevent adverse effects in the field by attacking non-target organisms (KÖHL et al., 2011). However, this leads to the necessity of more abundant screenings that can acquire new lines of agents, and learn their effects and modes of action (KÖHL; KOLNAAR; RAVENSBERG, 2019). Köhl et al.(2011) state that the presence of degrading enzymes that act on pathogens are present in many ecosystems, and because they undergo rapid degradation, they show very low toxicological and eco-toxicological risks.

The genus *Bacillus* gained prominence in recent years, with growing numbers of species with biotechnological potential. Belonging to the phylum *Firmicutes*, bacteria of this group have cylindrical shapes and produce endospores with great longevity, which makes bacteria with this characteristic to have longer viability in the field, possibly reducing the need of multiple applications (LIMA, 2010).

Gram positive and aerobic or facultative-anaerobes, the number of described species in the genus *Bacillus* constantly grows, with more than 140 identified species. They are cosmopolitan organisms than can colonize soils, water, air, surfaces of plants and their rhizospheres, gastrointestinal tracts of animals and extreme environments, being related to a great

diversity of habitats to be explored in species surveys and isolations. Certain species even have a considerable amount of their genome dedicated to the production of secondary metabolites (FIRA et al., 2018).

Within this genus, a bacterium that is particularly studied and produced is the species *Bacillus thuringiensis* (Bt). It was initially isolated in 1901 by the biologist S. Ishiwata in Japan, and later at, 1911, isolated and described by Berliner, that named it according to taxonomic aspects. It is a species that belongs to a complex called *Bacillus cereus*, which also has other species promoting biological control (POLANCZYK; ALVES, 2003). Its utility lies in controlling insect vectors of important diseases in the fields of human health, in bioremediation and biosynthesis (VALTIERRA-DE-LUIS et al., 2020).

In the field of agriculture, Bt is an entomopathogenic bacterium that produces protein crystals that are toxic to many insects like lepidopterans, coleopterans and dipterans (VALTIERRA-DE-LUIS et al., 2020), as well as to certain bacteria and nematodes, and has been used in the formulation of pesticides. The genes encoding the toxic crystals have been used in the production of transgenic plants, focused on resisting pest damages (JOUZANI; VALIJANIAN; SHARAFI, 2017).

These bacteria have two growth stages: a vegetative phase, and a sporulation phase, when the bacteria differentiate into spores capable of surviving adverse conditions and germinate when exposed to the necessary nutrients (LIMA, 2010). As one of the main organisms used in biological control, many studies have explored the potential of Bt (MELO; SOCCOL; SOCCOL, 2016).

Still in the genus *Bacillus*, the species *B. subtilis* has also been recognized as a bacterium with medical and industrial relevance (KARPOV et al., 2020). It is a gram-positive bacterium that mainly colonizes soils and plant roots and is characterised by forming biofilms that allow bacteria inside to resist injuries. This species is used as model organism in studies that seek to understand the function, potential and the structure of these biofilms and understanding the principles of spore formation (MIELICH-SÜSS; LOPEZ, 2015).

When applied in agriculture, the *Bacillus* group is composed of bacteria capable of secreting antibiotics and hydrolytic enzymes, altering the environment so that it becomes beneficial to their growth. *B. subtilis* is a source of compounds of biotechnological interest for producing a great diversity of secondary metabolites with distinct chemical structures while also being able to colonize interiors of plants, thus receiving the name endophytic (FIRA et al., 2018).

Their mode of action over plants exhibits direct and indirect mechanisms of biocontrol, acting directly on pathogenic organisms with their secondary metabolites and degradative enzymes (LU; GUO; LIU, 2018), or indirectly, with induced plant growth and the development of systemic resistance, and increased tolerance to biotic stresses (HASHEM; TABASSUM; ABD_ALLAH, 2019). They are applied to control fungal pests of fruits and legume crops (RYU et al., 2014). In this sense, it may be possible to discover bacteria that unite the ability to produce toxic substances to *T. urticae* while also controlling other sicknesses and inducing greater plant growth, among other benefits.

Another group of bacteria that have emerged as agents for biological control are the symbiotic bacteria of entomopathogenic nematodes (EPNs). Although about 40 families of nematodes are associated to insects, only two of them, *Steinernematidae* and *Heterorhabditidae* are capable of causing the death of the insect host, by carrying and regurgitating their symbiotic bacteria in the body cavities of the host, and are available for use in biological controls (DILLMAN, STERNBERG, 2012).

The families *Steinernematidae* and *Heterorhabditidae* relate exclusively to two genera of bacteria: *Xenorhabdus* (Thomas & Poinar) and *Photorhabdus* (Boemae, Luis & Kuhl), respectively (BRIDA et al., 2017). These are small, cylindrical worms that inhabit soils and infect host insects through their natural orifices. Upon reaching the hemocoel, they regurgitate their symbiotic bacteria, carried in their digestive tract, which then starts to feed on the tissues of the host, causing its death within 24 to 72 hours (GAUGLER; KAYA, 2018).

The mode of action of EPNs is widely studied in the area of biological control (MACHADO et al., 2005; SHAPIRO-ILAN, D., DOLINSKI, 2015; SANDA et al., 2018), as

well as novel and optimized production techniques that allow for their large scale use (SHAPIRO-ILAN, D., DOLINSKI, 2015). However, since EPNs are adapted to survive in soil, their action upon pests that damage aerial parts of plants is reduced (KUNG; GAUGLER; KAYA, 1990).

Due to the diverse biological activities of the secondary metabolites produced by their symbiotic bacteria (ANTONELLO et al., 2017), certain studies seek to isolate symbiotic bacteria from nematodes, multiply them in a culture medium and test the toxicity of their secondary metabolites. Analysis can consist of applying them directly on pest organisms or in the form of filtrates, as in a study carried out by Eroglu et al. (2019) involving the performance of symbiotic bacteria on a population of *T. urticae,* demonstrating that even when separated from nematodes they are capable of producing efficient toxins in insect control (FFRENCH-CONSTANT; BOWEN, 2000; SHAPIRO-ILAN; HAZIR; GLAZER, 2017; EROGLU et al., 2019).

Finally, it remains to highlight the potential of the *Serratia* genus. Petersen and Tisa (2013) describe that the species *Serratia* can also be found in a myriad of environments, ranging from soils and aquatic environments to digestive tracts, with many species capable of producing extracellular enzymes and secondary metabolites. Species of this group range from opportunistic pathogens to humans, plants, insects and nematodes, to plant growth promoters and, although rare, certain species associate with nematodes in a similar way to symbiotic bacteria of the genera *Heterorhabditis* and *Steinernema*.

One of the first descriptions of this genus acting as a biological control promoter comes from Mercer, Greenwood e Grant (1992), who highlighted a strain of *Serratia marcescens* as a control agent for phytoparasitic nematodes. The bacteria promoted control through the production of chitinases, that act on the eggs and cause premature death of newly hatched nematodes. Wong-Villarreal et al. (2021) studied a strain of *Serratia* sp affiliated with the *S. ureylitica*, which, in addition to affecting population control of phytoparasitic nematodes by acting on the eggs, has endophytic properties. Méndez-Santiago et al. (2021) reported the management of the fungus *Rhizoctonia solani* promoted by a strain of *Serratia marcescens*, in

addition to the stimulation of plant growth by phosphate solubilization in the first 48 hours after application and by the production of auxin, highlighting another possible use for this genus in programs of pest management.

3.3 Control techniques of *T. urticae*

3.3.1 Chemical Control

Synthetic acaricides used in the management of *T. urticae* have a large range of mode of actions and chemical structures (DEKEYSER, 2005). However, although the quantity of compounds meant for this control seems vast, the quantity of products registered and utilized for specific crops may be small (VAN LEEUWEN et al., 2010).

Chemical pesticides are easy to use, many have long shelf lives and cause great populational reduction on *T. urticae*. By having such a simple use process, their popularity is very high. In the European Union alone 1 billion dollars has been spent per year to buy pesticides destined to control *T. urticae* (ATTIA et al., 2013). Data provided by the Brazilian Institute of Geography and Statistics (IBGE, 2015) points out that the use of chemical pesticides in Brazil increased around 700% in the last 40 years, albeit the size of areas used for agricultural production only increased around 98%.

This group of qualities is, however, what drives the issues associated with the use of chemicals, that are often bound to intensive and constant applications. Hence comes the difficulties in managing *T. urticae* populations: the recurrent need of alternate products, since the excessive use accelerates the resistance development, the elimination of natural predators such as predatory mites that aid in populational control, and risks to the wellbeing of producers and consumers (ATTIA et al., 2013; VAN LENTEREN et al., 2017).

Currently, the chemical management of *T. urticae* in Brazil counts with more than ten registered Active Ingredients of acaricidal activity, such as abamectin, azadiractin, bifentrin and diafentiurom. The toxicological classification of these products varies from “low toxicity” to “extremely toxic” and their Environmental Classification points out that many of them are registered as “Dangerous to the environment” (AGROFIT, 2019).

Beside the possible risk that pesticides pose to humans and the ecosystem, there is an extensive documentation regarding the development of resistant populations of *T. urticae* to

chemical products, including some of the main acaricides registered in Brazil, with the resistance intensity reaching eight thousand times in cases of repeated use of these products, such is the case of fenpiroximate and etoxazole (KUMARI et al. 2017; SATO, 2016). In addition, another issue in managing *T. urticae* via the use of chemicals occurs in crops where the consumption of produces after the harvest is immediate and with small preparations, can lead to toxins being retained in produces, possibly leading to consumer exposure to the chemicals (GHOSH, 2013).

3.3.2 Biological Control

Regarding the natural enemies of phytophagous mites, the group that receives most attention are the predatory mites. Belonging to the *Phytoseiidae* family, at the end of 1950, they started to be considered efficient in managing multiple such mites (WATANABE et al., 1994). Currently, they are the main agents of biological control of *T. urticae* (SATO, 2017). Species like *Phytoseiulus persimilis* (Athias-Henriot, 1957) and *Neoseiulus californicus* (Mcgregor, 1954) are of great relevance in Europe and the United States (FERLA; MARCHETTI; GONÇALVES, 2007).

Presently in Brazil, besides *Neoseiulus californicus* (AGROFIT, 2019), another species has grown in use, *Phytoseiulus macropilis* (BANKS, 1904), an omnivorous predatory mite that can occur naturally in plantations. However, natural populations of this species occur in fewer numbers, at times as a consequence of the use of chemical pesticides, limiting the natural biological control of *T. urticae*. Therefore, the availability of commercial products that allow for its synergistic use with other antagonistic organisms is relevant, so that the biological control is well established (BERNARDI et al. 2010)

Predatory mites are used in the field by the distribution of traps that house them and are then set up in the field to the initial focus of the infestation. With this technique, the potential to reduce the pest population can reach 90% in one week, offering high efficiency and the ability to be included in pest management programs (BERNARDI et al. , 2010; VACACELA AJILA et al. 2019). Many studies have sought to measure the compatibility between predatory mites and chemical pesticides, so that they may be applied together (REIS et al., 1998; DUSO; VAN LEEUWEN; POZZEBON, 2020).

Another control technique that has risen in popularity is the use of natural products from plants, such as neem oil. Even at small doses, it provides a populational reduction of *T. urticae* and affects the fecundity of adult females without being harmful to populations of predatory mites (BRITO et al., 2006; VERONEZ; SATO; NICASTRO, 2012).

Lastly, a relevant area that has grown in recent years is the use of entomopathogenic fungi, particularly from the species *Beauveria bassiana*. Studies that expose populations of *T. urticae* to conidia from *B. bassiana* attest to its efficiency, and pair this fungi with adjuvants such as neem oil to allow their persistence in the field even under adverse conditions such as dry and warm environments (SÁENZ-DE-CABEZÓN IRIGARAY; MARCO-MANCEBÓN; PÉREZ-MORENO, 2003; GATARAYIHA; LAING; MILLER, 2010).

4 MATERIAL AND METHODS

4.1 Bacterial strains

The bacterial strains were obtained from the Collection of Microorganisms of the Laboratory of Biological Control, in the Advanced Centre of Research on Plant Protection and Animal Health (CAPSA) in Campinas-SP, maintained by cryopreservation in glycerol at -80ºC. The 104 bacterial strains initially assessed were chosen based on previous bioassays conducted in lab condition to control insect pests.

To activate the strains, each one was grown in 100 ml Schott Flasks containing 60 ml of liquid culture medium Nutrient Broth (NB) (Beef extract – 1 g/L, yeast extract – 2 g/L, peptone – 5 g/L, sodium chloride – 5 g/L), shaken constantly at 150 rpm under 25ºC for 120 hours.

4.2 Rearing of *T. urticae*

The adult female of *T. urticae* used in the experiments were obtained from rearing on plants of *Canavalia ensiformis*, Fabaceae, in the Laboratory of Acarology (LA/IB) in the CAPSA in Campinas-SP.

Seeds of *Canavalia ensiformis* were soaked in water for twelve hours, dried and transferred to 500 ml plastic pots containing the substrate VIVATTO PRO 20 – TECHNES™, type F, followed by their incubation in the dark for the plant emergence. The pots were then transferred to an acclimatized chamber and kept under constant relative air humidity of 70±10%, temperature of 25±1ºC and photoperiod of 14 twelve hours for 30 days.

A population of mites provided by the Laboratory of Acarology was then reproduced on developed plants of *C. ensiformis*. The infested plants were kept under constant temperature of 25±1ºC, relative air humidity of 70±10% and photoperiod of 14 hour, as described by Sato, et al. (2007).

4.3 Arenas for bacterial essays against *T. urticae*

For the essays with bacterial strains, the mites were gathered in arenas, each one consisting of 15-diameter petri dish and containing a layer of moisturized hydrophilic cotton on the bottom, besides a leaf of *C. ensiformis* with its axial surface facing the cotton. The stems of the leaves were covered by moisturized hydrophilic cotton to keep it turgid, and the border of the leaves were also covered to prevent the mites from escaping (Figure 4).

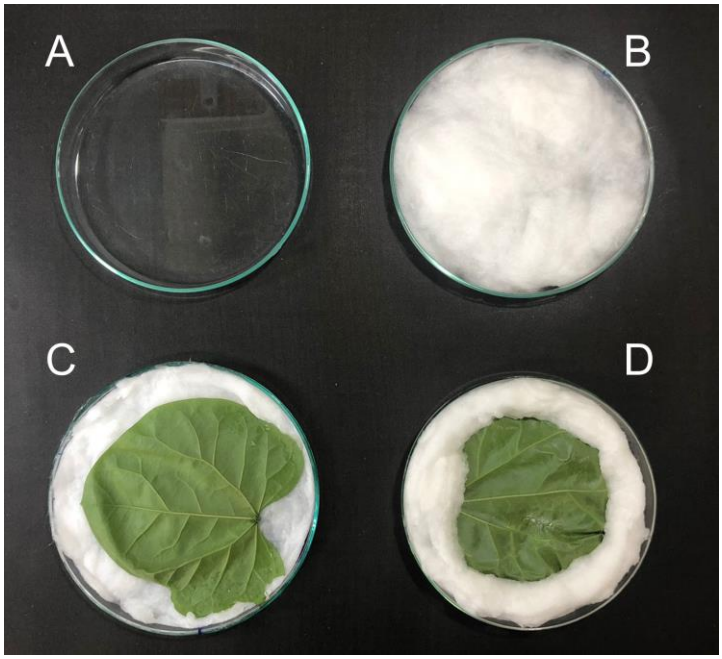

Figure 4: Arena preparation steps: (1) petri dish, (2) petri dish with a layer of hydrophilic cotton soaked in water, (3) petri dish with a leaf of *C. ensiformis*, (4) stem and borders of the leaf covered in cotton to prevent mites from escaping. Photo: (CARDOSO, 2021).

By using a paint brush (nº 0) with soft bristles, the adult females of *T. urticae* were collected from the infested plants and transferred to the arenas. After two hours, females that died or were hurt by the transference process were removed and replaced to keep the number standardized at 20 mites for the screening test, and 60 for the rate test.

4.4 Screening of bacteria for the control of *T. urticae*

This study assessed the performance of 104 bacterial strains for the mortality of *T. urticae*. Three replications were established for each bacterium, all represented by an arena containing 20 adult females. Two control treatments were also established, one sprayed with water and another with the liquid culture medium NB.

The bacterial strains were applied over the mites with a Potter spray tower, using 7 lb/psi pressure, by spraying 2 ml of the bacterial culture over the arenas with the mites. The arenas were then incubated under constant temperature of 25±1ºC and relative air humidity of 60±10%.

Evaluations were carried out 24, 48 and 72 hours after the spraying and consisted of counting and removing dead mites. Dead females were visually identified by their atypical yellow colouring, by their flaccid or deteriorated bodies, and by touching their bodies with a nº 0 paint brush to ensure their lack of movement. Bacterial strains that caused more than 75% mortality were selected for the following test and for molecular identification.

4.8 Molecular Identification

To make the molecular identification of the bacterial strains, the gene encoding the 16S ribosomal RNA of each strain was amplified by the Polymerase Chain Reaction (PCR) and sequenced.

The strains were obtained from the Collection of Microorganisms and activated in NB liquid culture medium according to the methodology described before. They were then individually inoculated into petri dishes containing Nutrient Agar (NA) solid culture medium (Peptone – 5g/L; HM Peptone B# - 1.50 g/L; Yeast extract – 1.50 g/L; Sodium chloride – 5 g/L; Agar – 15 g/L) and subjected to 24 hours of growth time at 35ºC. The PCR process was performed from a bacterial colony of each bacterial strain, collected from their respective petri dishes by means of a sterile bacterial inoculation loop.

For each one of the strains, the end of the loop containing bacteria was inserted into a micro tube containing the PCR reagents, and shaken. The PCR solution was composed of 10 µL of 5X PCR buffer solution; 1 µl fD1 primer (10 µM); 1 µl rP1 primer (10 µM); 1 µl of dNTPs (10 mM); 0.2 µl GoTaq DNA polymerase (5 U/µl Promega) and 36.8 µl autoclaved MilliQ H2O.

The fD1 (5' – AGAGTTTGATCCTGGCTCAG – 3') and rP1 (5' – ACGGTTACCTTGTTACGACTT – 3') primers used were described by Weisburg et al., 1991. The amplification of the genes of each strain was performed using a thermocycler (model T100, BioRad) programmed for: initial denaturation at 94°C/4 min, 40 cycles of 94°C/30 s – 60°C/30 s – 72°C/90 s and final extension at 72°C/4 min.

The amplifications were verified through electrophoresis in a 0.8% agarose gel, plus ethidium bromide (100 ng/mL), and recorded in a photo documenter coupled to a UV transilluminator. The amplified products were purified by precipitation with polyethylene glycol (SCHMITZ; RIESNER, 2006) and then subjected to sequencing reaction by the chain termination method (SANGER; NICKLEN; COULSON, 1977).

The chain termination reaction consisted of 5 µL of PCR product, 1 µL of Big Dye 3.1 reagent (Applied Biosystems), 1.5 µL of dilution buffer, 0.3 µL of fD1 or rP1 primer (10 mM) and 2.2 µl of sterile MilliQ H2O. The reaction was carried out in a thermocycler (model T100, BioRad) programmed for: initial denaturation at 95°C/1 min followed by 25 cycles of 95°C/5 s – 60°C/4 min. The sequencing reaction products were precipitated by adding 40 µL of 75% isopropanol, centrifuged at 12,000 g/10 and then 100 µL of 75% isopropanol was added and the products again centrifuged at 12,000 g/5 min.

After discarding the supernatants, the pellets were dried, resuspended in 10 µL of formamide and denatured at 95°C/2 min. A 3500XL Genetic Analyzer capillary sequencer (Applied Biosystems) was used to perform the sequencing. The sequences obtained were compared to those deposited in GenBank and in the Ribosomal Database Project, release 11 (http://rdp.cme.msu.edu) (COLE et al., 2014). The phylogenetic trees were constructed using the

MEGA 6 program (TAMURA et al., 2013) using the Neighbor Joining method with 1000 replications.

4.5 Rate test

This study assessed the three bacterial strains selected previously in respect to their performance against *T. urticae* at four different rates.

The experiment consisted of five treatments, each represented by the bacterial culture diluted to 2%, 5%, 10%, 20% and 40%, besides a control treatment represented by the culture medium NB diluted to 40%. Each treatment consisted of three replications, each one represented by an arena containing 60 adult females of *T. urticae*.

The selected strains were grown in 100 ml-Schott flasks containing 60 ml of liquid culture medium (NB), and incubated under 25ºC on a shaker at 150 rpm for 120 hours. After growing the strains, a sample of the bacterial culture was collected and analysed regarding the rate of cells/ml through the use of a Neubauer haemocytometer (Table 1).

Table 1: Rates in cells/ml of the three bacterial strains selected for the rate assessment test.

Strain	Density of the undiluted bacterial cultures (cells/ml)
365	$2{,}25 \times 10^8$
268	1.5×10^8
321b	1.25×10^7

The bacterial cultures were diluted with sterilized water and NB culture medium, in order to keep the total proportion of NB in all dilutions equal to 40%. The diluted cultures

were applied over the mites with a Potter spray tower, using 7 lb/psi pressure, by spraying 2 ml of the bacterial culture over the arenas with the mites. The arenas were then incubated under constant temperature of 25±1ºC and relative air humidity of 60±10%.

Evaluations were carried out 24, 48, 72, 96 and 120 hours after spraying and consisted of counting and removing the dead mites, identified visually by their atypical yellow colouring, by their flaccid or deteriorated bodies, and by touching their bodies with a nº 0 paintbrush to check their lack of movement. The entire experiment was conducted three times and the bacterium that caused the highest mortality rates was selected to the following test.

4.6 Field test

This study assessed *Serratia* sp 365 for its efficiency against *Tetranychus* sp on macaúba (*Acromia aculeata*) seedling production, infested naturally with the mite (Figure 5). The experiment was conducted from March 22th to April 19th in 2022, in a farm located at Serra Negra County (-22.566436° S -46.667846° w).

The bacterium was grown for five days in 50L bioreactors with automatic agitation and aeration, containing 25 L of NB liquid culture medium. After growing the bacterium, a sample of the culture was collected and analysed regarding the rate of cells/ml through the use of a Neubauer haemocytometer.

The experiments consisted of four treatments, each one represented by the bacterial culture (16.75x10^8 cells/ml.) diluted to 5%, 10%, 20% and 40%, besides two control treatments, one consisting of spraying water and another spraying NB medium diluted to 40%. The bacterial cultures were diluted with sterilized water and NB culture medium, in order to keep the total proportion of NB for all dilutions equal to 40%.

Figure 5: Colony of *Tetranychus* sp with web formation in a macaúba seedling (*Acromia aculeata*). Photograph: (CARDOSO, 2022).

Each treatment consisted of a plot containing 1400 macaúba seedlings (Figure 6), all being four months old, planted individually in 50cm² paper pots, grouped in plastic boxes and aligned in two columns of 25 boxes each, in total an area of 12m² per treatment. The treatment plots were spaced apart by 1 meter and raised 0, 5m aboveground on a platform. The plots were irrigated daily by sprinkling, from three days post application.

Figure 6: Plots utilized in the bioassay on macaúba, composed of 1400 seedlings each. Photo: (CARDOSO, 2022).

The bacterial culture at different rates were applied twice in an interval of seven days, over the macaúba seedlings, at the rate of 8.000 L/ha, using a backpack pump sprayer. Evaluations were conducted prior to the first application as well as two and six days after each application, consisting of randomly collecting 10 leaves for each treatment, each leaf representing a replication, and counting the number of dead mites, living mites, and mite eggs in four random 1cm² spots for each leaf. The experiment was conducted twice in time.

4.7 Statistical analysis

All experiments were carried out in a completely randomized design. The data obtained in the screening of bacteria were normalized by the formula of Abbott (1922) ([number of individuals killed in the replication – number of individuals killed in the control] / [100 – individuals killed in the control] x 100) and submitted to analysis of variance (ANOVA), with the means compared and analysed by the Scott & Knott multiple comparison algorithm (SCOTT; KNOTT, 1974).

Mortality estimates for the rate assessment test was performed using Probit analysis (STAT PLUS). The field test had its data on mortality values (%) normalized using the arcsine square root transformation. The data on population (cm^2) and egg density (cm^2) were normalized by logarithmic regression. All data was then submitted to analysis of variance (ANOVA) and the means were compared and analysed by Tukey's test ($p < 0,05$) (RStudio, v 1.4.1717). The population reduction values (%) were determined based on the control treatments.

5 RESULTS

5.1 Screening of bacteria for the control of *T. urticae*

For the 104 strains tested as undiluted cultures, the mortality rates obtained 72 hours after application ranged from 0% to 100%. Using the Scott & Knott (1974) algorithm, the strains were separated into three groups with statistical differences (F= 4.838; P < 0.001) (Figure 7). Group C compromised 79 strains which caused mortalities ranging from 0% to 34.11%. Group B has 19 strains which caused mortalities ranging from 37.16% to 59.26%. Group A has five strains, with mortalities ranging from 64.42% to 100%.

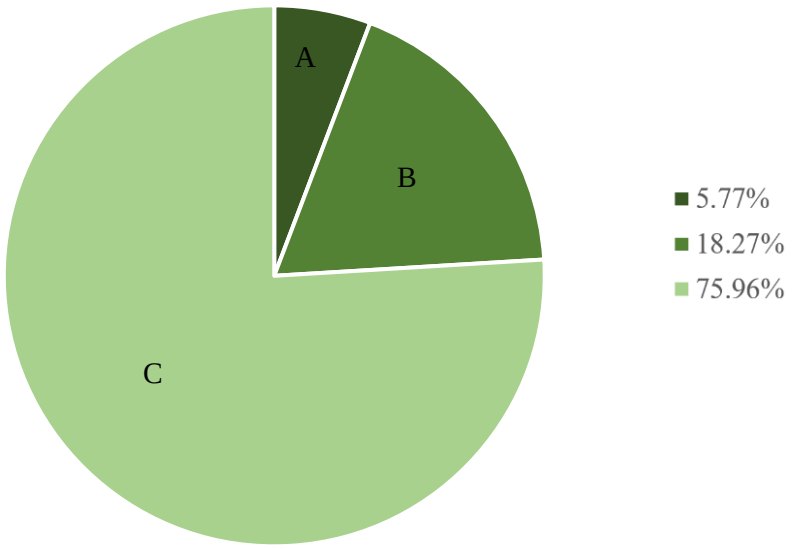

Figure 7: Proportion between the number of bacterial strains that form the three groups with statistical differences in mortality. Of the 104 strains tested, group C represents 75.96%, group B 18.27 and group A represents 5.77%.

In group A, only the strains 365, 268 and 321b caused mortalities above 75%, and were selected for the performance test with different rates of bacterial culture.

5.4 Molecular Identification

The 16S ribosomal gene sequence of strain 365 showed similarity between 99.3% and 99.6% to the species *Serratia marcescens* subsp *marcescens*, *Serratia surfactantfaciens* and

Serratia nematodiphila (Figure 8). The strain 268 showed 100% similarity to the species *Heyndrickxia* (ex-*Bacillus*) *sporothermodurans* and 99.7-99.8% with the species *Heyndrickxia* (ex-*Bacillus*) *vini*, from the *Bacillus subtilis* group (Figure 9). As for the strain 321b, it showed 100% similarity with the species *Bacillus proteolyticus* and *Bacillus sanguinis*, from the *Bacillus cereus* group (Figure 10).

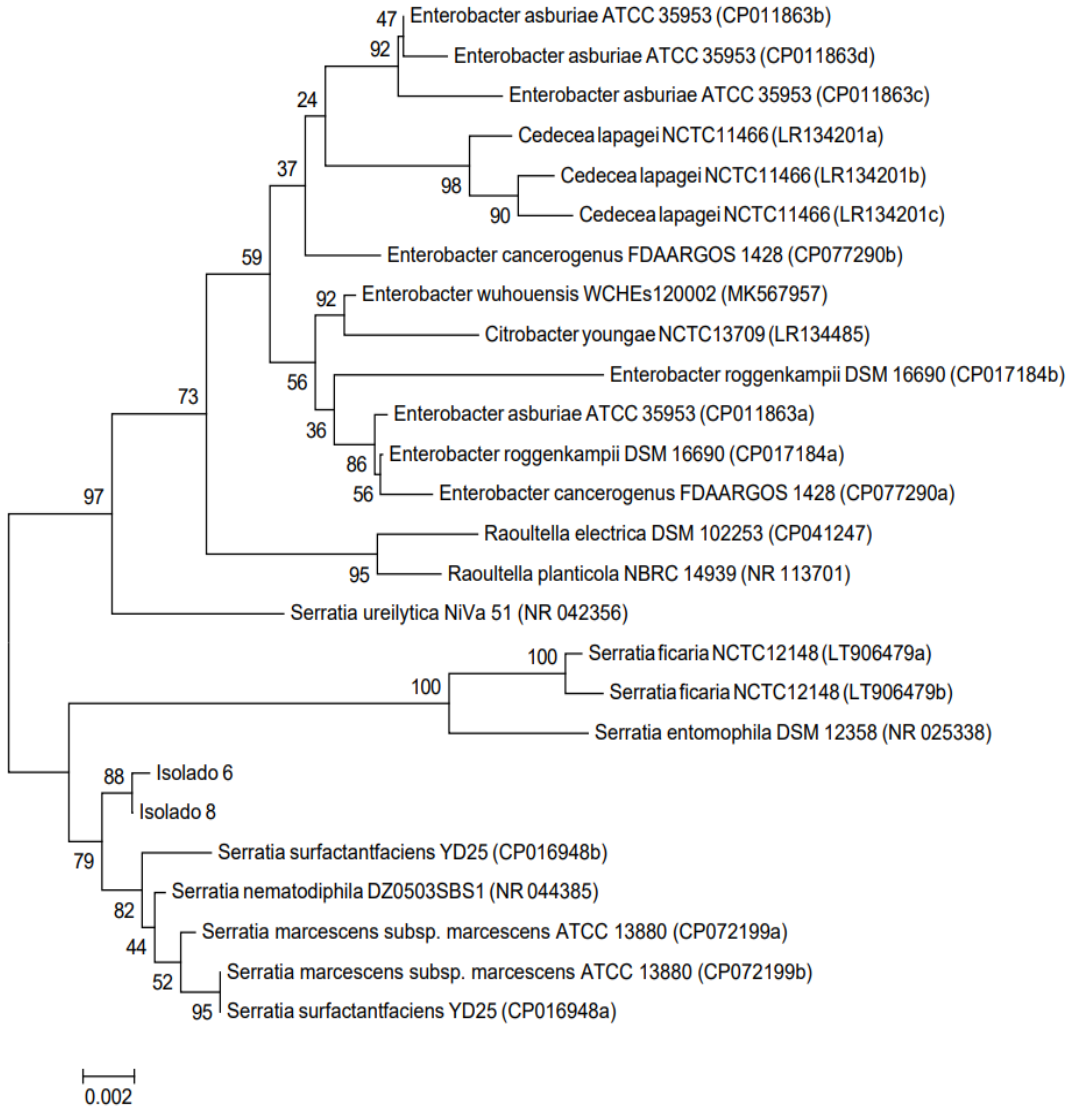

Figure 8: Maximum probability phylogenetic tree showing the phylogenetic positions of bacterial strain 365, based on sequencing the 16S ribosomal gene, with genetic similarity to the species *Serratia marcescens* subsp *marcescens*, *Serratia surfactantfaciens* and *Serratia nematodiphila.*

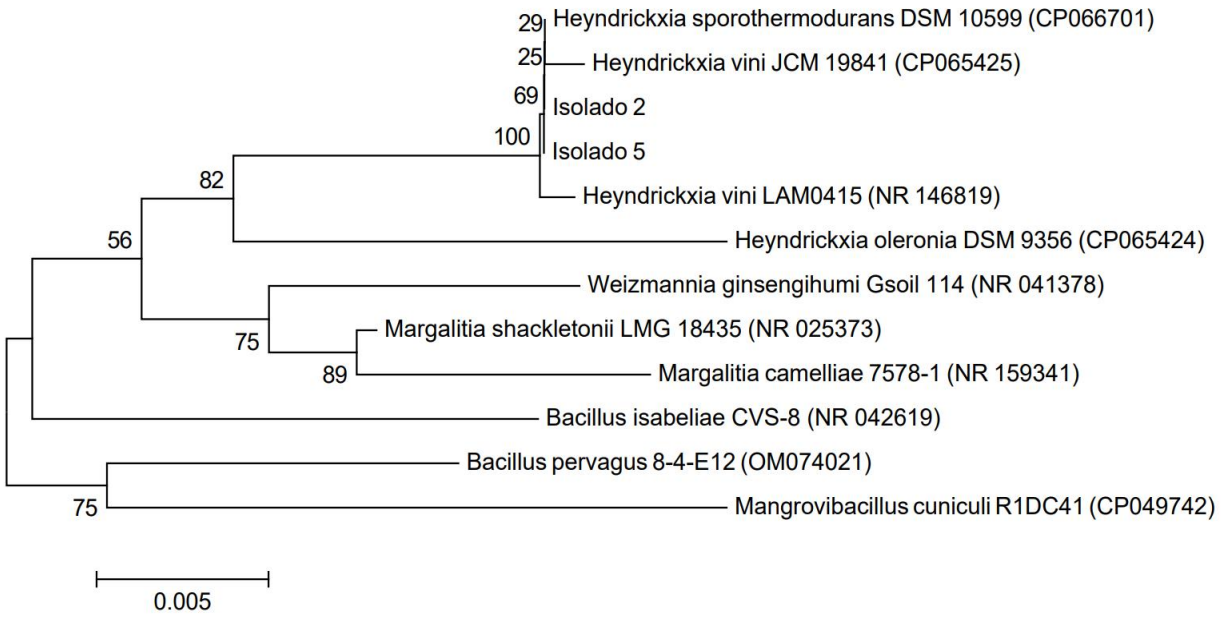

Figure 9: Maximum probability phylogenetic tree showing the phylogenetic positions of bacterial strain 268, based on sequencing the 16S ribosomal gene, with 100% similarity for *Heyndrickxia* (ex-*Bacillus*) *sporothermodurans* species and 99.7-99.8% similarity with the species *Heyndrickxia* (formerly *Bacillus*) vini, from the group of *Bacillus subtilis*.

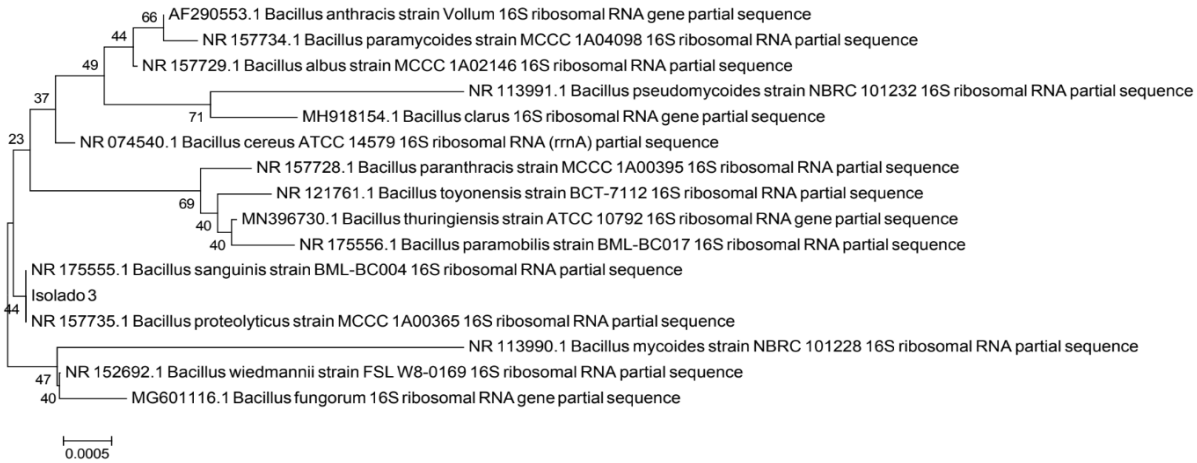

Figure 10: Maximum probability phylogenetic tree showing the phylogenetic positions of the bacterial strain 321b, which showed 100% similarity with the species *Bacillus proteolyticus* and *Bacillus sanguinis*, of the *Bacillus cereus* group.

Currently, the correct taxonomic positioning of several bacterial species, such as those obtained in the present study, requires multilocus sequencing or even complete genomes (LIU et al., 2017; CHO et al., 2020; GUPTA et al., 2020).

5.2 Rate test

The bacterium *Serratia* sp 365 was the most virulent to *T. urticae*, with LC_{50} at the rate of 38%, differing numerically from the other strains with LC_{50} at rates of 57.35% and 150.65%, respectively (Table 2).

For the bacterium *Serratia* sp 365, increasing the rate from 2% to 40% reduced the LT_{50} from 12 to 5 days, respectively, with similarities between the two highest rates, which differed significantly in relation to the lowest rates (Table 3).

Table 2. Mortality (%) and LC_{50} of mites after 120 hours of application of different rates of bacterial culture.

Bactéria	Rates						LC_{50} (range)		Slope	X^2
	0%	2%	5%	10%	20%	40%	%	cells/ml		
365	13.7±2,4	22.2±1.9	30.7±3.6	38.0±2.8	46.1±3.4	47.8±4.2	38.67 (29.16-56.79)	$8,70x10^7$ ($6,56x10^7$-$1,28x10^8$)	0.56	1.921
268	12.5±3.9	19.2±1.8	17.8±2.4	28.3±3.6	24.4±3.4	29.7±4.8	57.32 (22.68-100)	$8,60x10^7$ ($3,40x10^7$ – $1,50x10^8$)	0.73	9.274
321b	7±12.5	8.5±1.1	10.4±1.9	12.8±3.1	11.1±1.5	18.1±4.0	150.65 (8.15-2785.42)	$1,88x10^7$($1,02x10^6$ – $3,48x10^8$)	0.945	106.241

Table 3. LT_{50} of mites 120 hours after application of different rates of *Serratia* sp 365 bacterium culture.

Parâmetros	Doses				
	2%	5%	10%	20%	40%
LT_{50}	12.23 (10.0-16.3)	9.03 (7.8-11.0)	7.4 (6.5-8.8)	5.83 (5.3-6.5)	5.29 (4.9-5.9)
Slope	2.0	1.9	1.8	2.1	1.9
X^2	0.2	0.6	0.0	1.0	0.1

5.3 Field test

After the first and second application of *Serratia* sp 365, there was an increase in mite mortality (Figure 11), with significant differences when compared to the control treatments of water and NB medium (first application: F= 14.74; df= 5.114; P<0.001) (second application: F= 63.89; df=5.114 P<0.001) (Table 4). Consequently, the population of the mite and mite egg density were reduced. The highest rates (40% and 20%) suppressed the mite population and lowered the egg density after an application of the bacteria, significantly differing from the control treatments (population: F= 12.34; df= 5.114; P<0.001) (eggs: F= 8.20; df= 5.114; P<0.001). The two lowest rates (5% and 10%) suppressed the mite population and egg density only after two applications, starting to differ significantly from the control treatments after the second application (population: F= 204.71; df= 5.114; P<0.001) (Eggs; F= 83.00; df=5.114; P<0.001).

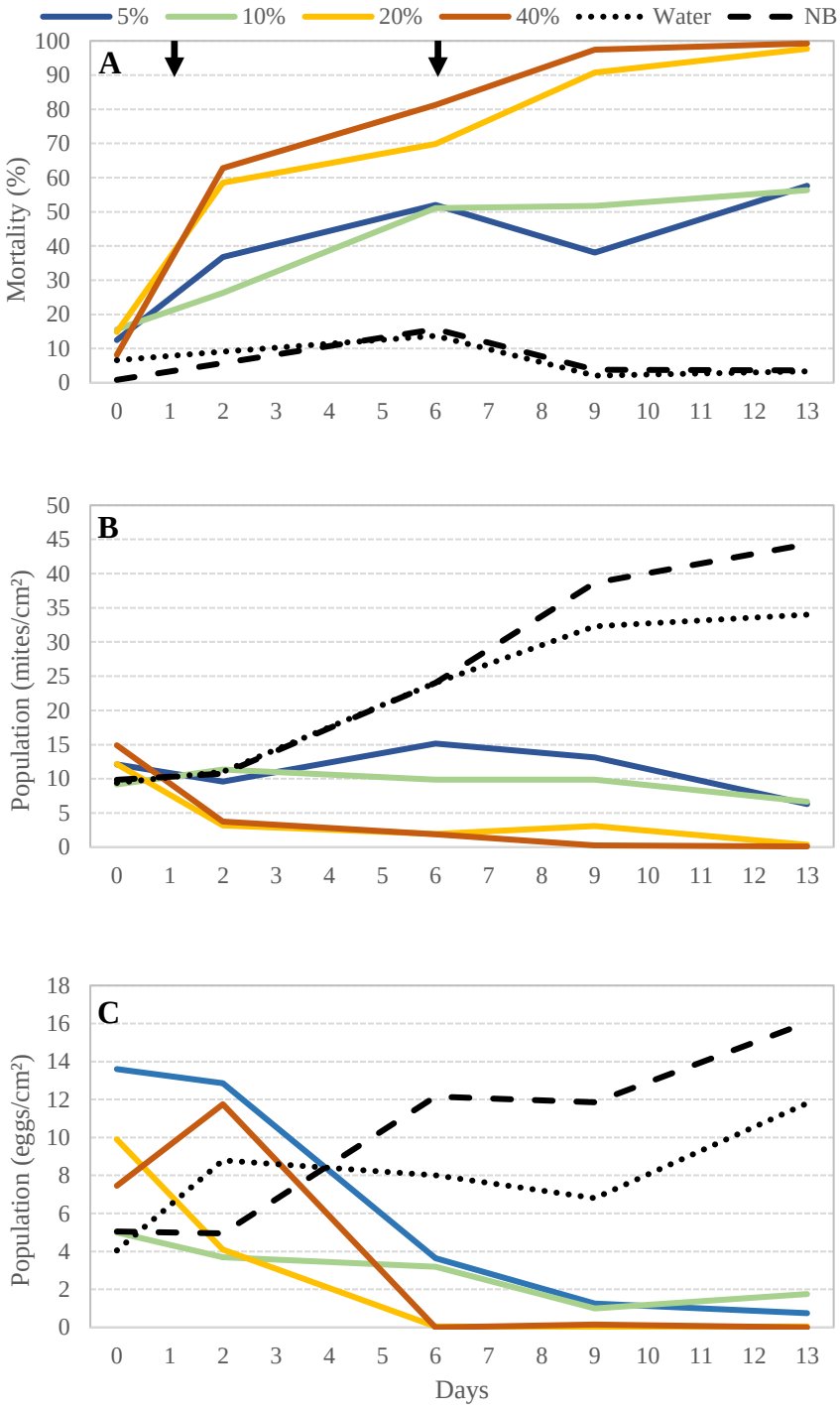

Figure 11: Mortality percentage (A), mite population (B) and mite egg density (C) *Tetranychus* sp after two applications of *Serratia* sp 365.

Table 4. Mortality (%), population density and egg density of the mite *Tetranychus* sp after two applications of *Serratia* sp 365.

Rate	Mortality		Population		Eggs	
	1ª Application	2ª Application	1ª Application	2ª Application	1ª Application	2ª Application
40%	81.29±7.96 a	99.25±0.74 a	1.9±0.63 a	0.1±0.07 a	0.00 a	0.00 (a)
20%	69.86±11.56 ab	97.69±1.10 a	1.95±0.68 a	0.35±0.15 a	0.05±0.05 a	0.05±0.05 ab
10%	51.04±9.64 b	56.33±8.21 b	9.85±2.70 b	6.65±1.13 b	3.20±1.60 ab	1.75±0.68 b
5%	52.05±10.92 b	57.61±7.89 b	15.15±4.11 b	6.3±1.02 b	3.65±1.96 abc	0.75±0.25 ab
NB	15.69±7.56 c	3.59±1.38 c	24.05±4.99 b	44.3±2.56 c	12.15±4.21 bc	16.05±2.60 c
H_2O	13.73±7.67 c	3.37±1.04 c	24.05±5.24 b	34.00±2.52 c	8.00±2.71 c	11.80±1.28 c

Means followed by distinct letters in the lines differ significantly by Tukey's test, P≤5.

6 DISCUSSION

Of the 104 strains tested, only six stood out in terms of mortality for *T. urticae* (64.4 to 100%), and only three were selected with mortalities above 75%, which represents less than 3% of the total evaluated bacteria. Other studies have also found difficulties in selecting bacteria with potential to control *T. urticae* (IDRIS; LABUSCHAGNE; KORSTEN, 2007; LEMESSA; ZELLER, 2007; DHANASEKARAN; THANGARAJ, 2014; WANG et al., 2018; EROGLU et al., 2019), emphasizing the importance of initial screenings with the largest possible amount of strains, since few organisms have antagonistic potential.

This small percentage of selected strains may be related to the variety of factors that make an organism capable of acting as an antagonist, and determine its mode of action. When dealing with bacteria as antagonists of *T. urticae*, their main modes of action can be by the action of the cell or of metabolites, acting on the mite by ingestion of the bacteria or by contact with its body surface.

The feeding habit of *T. urticae*, which scrapes, breaks and sucks liquids from the host plant, allows the ingestion of the bacteria when sprayed on the plant, but the amount ingested may be too small to allow the effective action of the bacteria inside the mite. The mite's small body surface may restrict the amount of bacterial culture that reaches its body (BENSOUSSAN et al., 2016), and may require larger volumes of spray per area. In addition, the mite may present variations in its susceptibility depending on its life stage, with adult females being generally more susceptible than the larval stages (EROGLU et al., 2019).

On the other hand, bacteria can evade the immune system of *T. urticae*, reducing the chances to induce resistance on the mite when compared to chemicals (SANTOS-MATOS et al., 2017). In addition, bacteria can be endophytically absorbed by the plant and reach the mites orally, increasing the chances of success (KWON; PARK; LEE, 2013).

Another aspect that may favour the control of *T. urticae* with bacteria is the culture medium itself, which, in addition to allowing the optimization of the production of the bacteria and its metabolites, can also cause a direct adverse effect to the mite. In a similar study with

bacteria of the genus *Xenorhabdus* and *Photorhabdus*, Eroglu et al. (2019) used the NB culture medium for providing the lowest mortality in control treatments, allowing greater visibility of the effect caused by the bacteria on *T. urticae* during the evaluations. However, for commercial productions, another culture medium could be explored to allow higher yields of the bacteria and metabolites, regardless of their action on the mite.

The strain 365 was selected for presenting an LC_{50} of 38.6%, numerically lower than those determined for strains 268 (57.3%) and 321b (150.6%). The strain 365 may belong to the species *Serratia marcescens* subsp *marcescens*, *Serratia surfactantfaciens* or *Serratia nematodiphila*, while the strain 268 was identified as either *Heyndrickxia* (ex-*Bacillus*) *sporothermodurans* or *Heyndrickxia* (ex-*Bacillus*) *vini* from the group *Bacillus subtilis* (Figure 9), and the strain 321b as *Bacillus proteolyticus* or *Bacillus sanguinis*, from the group *Bacillus cereus*.

When studying the genus *Serratia* to control phytoparasitic nematodes, Méndez-Santiago et al. (2021) also found genetic similarities between the same species of strain 365. For the genus *Bacillus* and *Heyndrickxia*, Gupta et al. (2020) reported similarities between the same species identified for the strain 321b, stressing the need for multilocus or complete genome sequencing as a way to determine the species.

For the bacterium *Serratia* sp 365, increasing the rate from 2% to 40% reduced the LT_{50} from 12 to 5 days, respectively. Thus, higher rates certainly contribute to better control and greater reduction of mite damage in the plant, but may become economically unfeasible. The ideal rate may be the one that results in the best cost-benefit, which may cause a population reduction of the mite and/or reduce its reproduction rate, leading to a consequent suppression of its population. Even if the product does not provide high mortality rates of the mite, if it reduces the reproduction rate, that can reach 100 eggs in a 30-day life period (TEHRI, 2014), it can contribute to its control.

Based on the mortality obtained in the field test, there are 2 groups formed by the lowest rates of 5 and 10% (57.6% and 56.3%), and the highest, 20 and 40% (97.6% and 99.2%) (Figure 11, Table 4). However, even at the lowest rates, the bacterium suppressed the population

of mites and egg density after the second application, suggesting that mortalities of 57.6% and 56.3% already eliminate the pest if they remain cumulative.

The 5% rate killed 52% of the mites, even though it was almost 8 times lower than the LC_{50} obtained in the laboratory, of 38%. The lower mortalities obtained in the laboratory in treatments with lower dilutions may be due to three factors: the volume of spray per area; the rate of bacterium in the bacterial culture; and the daily irrigation.

The volume of bacterial culture used in the laboratory bioassays (1 ml/100cm²) was around 8 times smaller than that used in the field (8 ml/100 cm^2). The bacterial culture used in the field test had a rate (16.75 x 10^8 cells/mL) about 8 times higher than the culture used in the laboratory test (2.25 x 10^8 cells/mL). For mite control with Vertimec™ abamectin, flow rates up to 2000 L/hectare are recommended (AGROFIT, 2019). The volume of spray used in the field test represents 8,000 litres per hectare, a very high flow rate for open fields, but not for protected crops. Daily irrigation may have favoured mite mortality by the bacterial culture, keeping it hydrated and active throughout the evaluation period. New studies should be carried out trying to assess lower flow rates in protected and field conditions.

This is the first study with a bacterium of the genus *Serratia* to control mites, and is also the first study to carry out tests from the initial screening to the test in commercial seedling production. Other studies reported the action of the genus *Bacillus* on *T. urticae* (LI et al., 2018), but this is the first to report the pathogenicity of the species *H. sporothermodurans* or *H. vini* and *B. proteolyticus* or *B. sanguinis* on the two-spotted spider mite.

The bacterium *Serratia* sp 365 was highly effective for the control of *Tetranychus* sp, providing population reductions that ranged from 37% to 92.1% after the first application, and from 80.4% to 99.7% after the second application. For the egg density, the results were even better, with reductions of 54.4% to 100% after the first application, and 85.2% to 100% after the second application. New studies should be carried out to assess the susceptibility of the different stages of the mite to the bacterial culture. Future studies should also be carried out in order to know the susceptibility of the different life stages of *T. urticae* to the bacterium, since eggs may be more resistant to pathogens (EROGLU et al., 2019).

The present study assessed more than 100 bacterial strains to control the mite *T. urticae*, compared different rates of the three most virulent and assessed the effectiveness of the most virulent in commercial macaúba seedling production. New studies should be carried out seeking to know which is the main active ingredient of the bacterial culture, whether the bacterial cells or the metabolites of the bacteria; how to optimize the production method and formulation of the agent considering that some *Serratia* species do not form spores and, consequently may generate formulations with shorter shelf-lives; to determine the volume of culture with the best cost benefit; to assess the persistence in the field; to assess the selectivity for natural enemies, since living predatory mites were found in the bacterial treatments of the field experiment. In addition, the two other bacteria that showed the second and third highest virulence also deserve to be assessed in the field and compared with the selected *Serratia* sp 365.

7 CONCLUSION

- The selected strains were restricted to less than 3% of the total strains assessed;
- *Serratia* sp 365, *Bacillus* sp 268 and *Heyndrickxia* sp 321b were selected with LC_{50} equal to 38.67; 57.32 and 150.65, respectively;
- For *Serratia* sp 365, the rates of 20% and 40% suppressed the mite population and the egg density after a single application of the bacterium, while the rate of 5% and 10% suppressed the mite population and egg density only after two applications.

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