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***CURRICULUM*: "CONTROL WITH MINIMUM ENVIRONMENTAL IMPACT"**

STRATEGIES OF BIOLOGICAL CONTROL OF BACTERIAL PATHOGENS OF KIWIFRUIT

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A mio padre e a mia madre

“The end of one journey always leads to the beginning of another”

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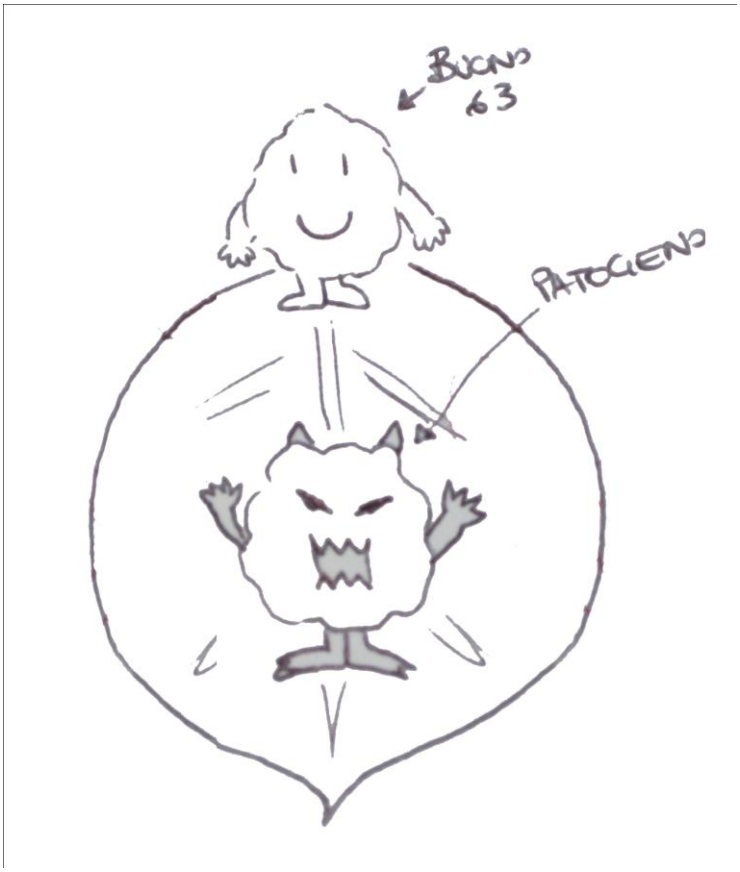

Abstract

The importance of kiwifruit cultivation is increasing all over the world and Italy plays a key role being one of its larger producer. Bacterial diseases are the most dangerous pathogens of kiwifruit, in particular bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae*.

Control through antibiotics and heavy metals points out environmental and safety issues, stimulating the search for alternative disease control measures. Aim of this Thesis was to investigate natural extracts and natural antagonists able to control kiwifruit bacterial diseases.

Some natural extracts showed antimicrobial activity, in particular garlic, pomegranate and lavender extracts were effective in the control of bacterial pathogens of kiwifruit as well as a biocontrol agent, strain hr63 belonging to *Pseudomonas fluorescens* species, which was able to control bacterial kiwifruit pathogens both, in *in vitro* and *in vivo* tests.

Keywords: Kiwifruit, Bacterial diseases, *Pseudomonas* spp., Biological control, Natural antagonists, Natural extracts.

Riassunto

La coltivazione dell'actinidia sta acquisendo sempre maggiore importanza nel mondo, e l'Italia è tra i primi paesi produttori di kiwi. Recentemente gravi danni sono stati registrati su actinidia a causa di tre differenti malattie batteriche, in particolare il cancro batterico causato dal batterio *Pseudomonas syringae* pv. *actinidiae.*

Il controllo attraverso l'utilizzo di antibiotici e metalli pesanti pone dei problemi di tipo ambientale, stimolando la ricerca di misure di controllo alternative. Lo scopo di questo lavoro era quello di investigare sostanze naturali e antagonisti naturali per il controllo biologico delle batteriosi dell'actinidia.

Alcuni estratti naturali mostravano capacità di controllare le malattie batteriche dell'actinidia, in particolare gli estratti di aglio, melograno e lavanda, così come un antagonista naturale appartenente alla specie *Pseudomonas fluorescens* (isolato hr63) che riusciva a controllare gli agenti di queste temibili malattie sia *in vitro* che *in vivo*.

SUMMARY

CHAPTER 1

INTRODUCTION

1.1. Kiwifruit: General description

Kiwifruit (*Actinidia deliciosa* [A. Chev.] C.F. Liang et A.R. Ferguson, Actinidiaceae family) is one of the most recently domesticated among all fruit plants. The genus *Actinidia* Lindl. contains approximately 60 species and 100 taxa. Actinidiaceae are perennial, climbing or scrambling plants and native to the mountainous southern and central China. All *Actinidia* species appear to be dioecious: the flowers on male vines produce viable pollen but lack a properly developed ovary; the flowers of female vines release shrivelled and non-viable pollen. From a botanical point of view, the fruits of the various *Actinidia* species are defined berries; they are fleshy, with many seeds, embedded in the flesh, and they do not split open at maturity. Horticulturally they display great diversity in size, shape, hairiness, and external colour. They can also vary in flesh colour, juiciness, texture, and composition. The flavour of fruits of almost all *Actinidia* species is considered edible, whereas in some species fruits are basically inedible or unpalatable (Ferguson, 1990; Zuccherelli, 1994; Testolin, 1998; Ferguson, 1999; Valli 2001; AA.VV., 2008).

Nearly all the kiwifruit cultivars grown in commercial orchards outside of China descend from few plants deriving from a single introduction of seed from China to New Zealand in 1904. In spite of such limited sampling of the gene pool, the first kiwifruit plantings in New Zealand contained considerable variation in the fruit carried by individual plants. From the mid 1960 kiwifruit also developed an economic relevance, and in the early 1970 few cultivars selected in New Zealand were introduced into Europe (Ferguson & Bollard, 1990; Testolin, 1998; Ferguson, 1999).

The species *A. deliciosa, A. chinensis* and *A. arguta* are those cropped outside

of China for commercial aims. *A. deliciosa* accounts for 95% of kiwifruit marketed throughout the world, of which "Hayward" is the leading commercial cultivar (fruit exceptionally large, broad-oval, with slightly flattened sides; skin light greenish-brown with dense, fine, silky hairs and light green flesh; superior flavour and good quality keeping; fruits ripening in early autumn; moderately vigorous vine, blooms occur late and it is medium in production). Other important cultivars are "Abbott" (oblong fruit of medium size, with vigorous, precocious, and productive vine); "Allison"(oblong fruit, slightly broader than "Abbott". Very vigorous and productive vine, blooming later than "Abbott"); "Bruno" (large fruit, elongated cylindrical, broadest at apex. Vigorous and productive vine, blooms occur slightly after "Allison"); "Monty" (oblong fruit, rather angular, widest at apex; of medium size. Highly vigorous and productive vine, sometimes excessively so); "Summer" (very precocious selection, fruits different from "Hayward", 80-90 grams); "Earligreen" (Natural mutation of "Hayward", very precocious, harvesting in early September, fruit similar to "Hayward"); "Green Light" (Natural mutation of "Hayward" with similar and precocious fruits). Male plants commonly used for pollination, on the other hand, are "Matua" and "Tomuri" (Morton, 1987; Ferguson, 2003; Bucci & Costa, 2006; Testolin & Ferguson, 2009).

Actinidia chinensis, also known as yellow kiwifruit, it's more recently cultivated and its importance is rapidly increasing. The fruits are smooth skinned, almost hairless with golden-yellow flesh and having a sweeter, aromatic flavour. The most widely planted cultivars are "Hort16A" (released in 2000 in New Zealand, is a vigorous vine which flowers about a month before "Hayward". It is very productive and can carry far bigger crop loads than "Hayward". The fruits are distinctive in appearance, elongated oval with a pronounced pointed stylar end or "beak". The skin can vary from light to dark brown and is covered by fine, downy hairs, similar to the fuzz on a peach. The fruit flesh loses its green colour during maturation and take a beautiful, clear, golden-yellow colour. Fruits are usually harvested three weeks before of "Hayward", therefore "Hort16A" requires an even longer frost free time of growth. When ripening, the fruits have a fine flavour, less acid but sweeter and more aromatic than 'Hayward' fruit); "Jintao" (Chinese cultivar released in Europe in 2001, is less vigorous but more productive than

"Hayward". It flowers about a week earlier than "Hayward" and the fruits are harvested earlier. The fruits are elongated, round in cross-section, and regular in size and shape when pollination is correct; their skin is smooth and brown and essentially hairless, the flesh of a brilliant golden yellow, and the core small and round in cross-section. The fruits are small but have a good, sweet flavour and store well); "Soreli" (The fruits are large and good looking, more than 100 g, their flesh is bright yellow, the flavour is good, while the storage life is short. They are harvested one month earlier than "Hayward")(Ferguson, 2003; Bucci & Costa, 2006; Testolin & Ferguson, 2009).

A. arguta, popularly known as baby kiwi or kiwiberry, it is a quite interesting crop especially for the niche market. Different cultivars are under trials, generally fruits are very small, without hairs and flavour is good and sweet. The uneven and protracted fruit maturation, the cost of harvesting and the short storage life delayed the diffusion of this species (Bucci & Costa, 2006; Testolin & Ferguson, 2009).

1.2. Marketing

Kiwifruit is still a minor crop, considering its amount of 0.2% respect to the total world production of fruits. However in few countries such as China, Italy and New Zealand kiwifruit is a rather important crop. The total area throughout the word planted in kiwifruit is about 120,000 ha. China has 65,000 ha, Italy 25,000, New Zealand 12,000 ha and Chile 10,000 ha. Considering only cultivated kiwifruit cvs., total world production is 1,650,000 t: China produces 500,000 t, about the same as Italy, New Zealand 350,000 t and Chile 160,000 t, so together, these four countries account for over 80% of the world commercial kiwifruit production. Approximately 7.5% of current commercial production would be of cultivars of *A. chinensis* and the rest of *A. deliciosa*, of which about 80% would be of Hayward. This cultivar still dominates international trade but cultivars of *A. chinensis* especially "Hort16A" are rapidly becoming more important. *A. arguta* remains potentially interesting, but at present the world plantings amount is irrelevant

(Ferguson & Testolin, 2009; World Kiwifruit Review, 2009; Carbone & Henke, 2010).

New Zealand and Italy with 300,000 t and Chile with 150,000 t are the larger kiwifruit exporters. EU countries are the most important destination for Italian kiwifruit export, whereas the demand of kiwifruit in China is so high that no exportation has been recorded rather than its production (World Kiwifruit Review, 2009; Carbone & Henke, 2010).

Italian production is concentrated in few regions: Lazio is the leading region for Italian kiwifruit, with 8,550 ha and production of 160,000 t, while Piemonte has 4,500 ha and 85,000 t, Veneto 3,200 ha and 75,000 t, Emilia-Romagna 3,800 ha and 75,000 t. Together, these four regions accounted for over 80% of Italian kiwifruit production (ISTAT, 2010).

1.3. Main diseases and pests of kiwifruit

Similar types of pests occur on kiwifruit plants in the countries in which they are cultivated and all tend to affect a broad range of plants. Armoured scales (e.g. *Hemiberlesia* spp., *Aspidiotus nerii*) are often the most serious but although the species involved are cosmopolitan, the abundance of a particular specie varies depending on the country in which it is situated. The other main group of pests, the leaf roller (e.g. *Platynota stultana, Cnephasia jactatana*), tend to be specific to each country and hazardous for the fruit. In a similar way, nematodes (e.g. *Meloidogyne* spp.) can be a problem in some countries (Steven, 1990; Blank *et al.,* 1999; AA.VV., 2008).

Kiwifruit are also susceptible to bacterial and fungal diseases. *Pseudomonas* species cause bacterial canker, bacterial necrosis and bacterial blossom blight. Some kiwifruit disorder, called “leader die-back”, “wood decay” and “elephantiasis” are caused by fungi typically responsible for complex diseases of woody tissue (e.g. *Chondrostereum purpureum, Cryptosporiopsis actinidiae, Phaeoacremonium inflatipes, P. aleophilum, P. rubrigenum, Phaeomoniella chlamydospora, Phialophora* spp., *Fomitiporia mediterranea, Fusarium* spp., *Cylindrocarpon* spp., *Phomopsis*

spp.). Symptoms are brown areas of hard necrotic tissue on internal wood followed by a spongy and friable discolored zone, deterioration of the wood structure with swelling and bark cracking, leaves and fruits deformed (Di Marco *et al.,* 2000; Riccioni *et al.,* 2007; Prodi *et al.,* 2008). Crown gall caused by *Agrobacterium tumefaciens* is a problem especially within the process of planting kiwifruit on soil already infested. The roots may be attacked by *Phytophthora cactorum* and *P. cinnamomi*, and also by oak root fungus (*Armillaria mellea*) which is fatal. *Sclerotinia* can also affect fruit on the wine but the other serious fungal diseases develop while or after the fruit storage (e.g. *Botrytis cinerea*, *Botryospheria dothidea, Alternaria alternata*) (Morton, 1987; Brook 1990; Zuccherelli 1994; AA.VV., 2008).

1.4. Bacterial diseases of kiwifruit

Pseudomonas syringae pv. *actinidiae* Takikawa *et al., Pseudomonas syringae* pv. *syringae* van Hall and *Pseudomonas viridiflava* (Burkholder) Dowson are the most dangerous bacterial pathogens of kiwifruit plants worldwide (Takikawa *et al.,* 1989; Balestra, 2004; Balestra *et al.,* 2008 and 2009).

1.4.1. Bacterial canker

Caused by *P. s.* pv. *actinidiae* (Psa), it is the most dangerous kiwifruit pathogen. It has been first recorded in Japan in 1984 (Serizawa *et al.,* 1989) and subsequently detected as cause of bacterial canker disease of kiwifruit in South Korea (Koh *et al.,* 1994) and Italy (Scortichini, 1994). Bacterial canker symptoms have also been observed on kiwifruit in China (Chang *et al.,* 1995). In both Korea and Japan, bacterial canker is considered as a limiting factor for the production of kiwifruit with significant losses in fruit production and plant mortality. Recently Psa was reported in Portugal (Balestra *et al.,* 2010), France (EPPO 2010/188) and New Zealand (Biosecurity New Zealand, 2010). Although Psa was originally described in Japan, its area of origin has not been ascertained. For example, comparison studies between Korean and Japanese strains showed that they have

different phylogenic origins (Lee *et al.,* 2005).

Psa is a vascular pathogen, whose most conspicuous symptom is the red-rusty exudation which covers bark tissues on trunks and twigs. Removal of the bark usually reveals a brown discoloration of the vascular tissues and reddening of the tissues beneath lenticels. Other symptoms are dark brown spots surrounded by yellow haloes appearing on leaves, brown discolouration of buds, fruit collapse, wilting and eventually plant mortality (**Fig. 1**) (Scortichini, 1994; Balestra & Varvaro, 1999; Janse, 2005). Its epidemiology is not clear: it has been observed that the pathogen is active between 10 to 20 °C and is limited by temperatures above 25°C. Inoculation studies showed that the bacterium can infect the plant through natural apertures (stomata, lenticels) and wounds. Symptoms are usually expressed during spring and autumn when climatic conditions are favourable to the spread of the disease (cool temperatures, persistent rains, high humidity). It is suspected that the bacterium is spread by heavy rain falls, strong winds, animals and humans. Over long distances, trade of infected planting material can spread the disease (Balestra *et al.,* 2009; Renzi *et al.,* 2009).

Psa is a gram-negative, aerobic, non-sporing, levan-positive, oxidase-negative, potato soft rot-negative and arginine dehydrolase-negative bacteria, and induces the hypersensitive response on tobacco leaves (group Ia of LOPAT test). In addition, Psa doesn't produce fluorescent pigment on medium B of King et al. (1954) (KB) and is arbutin and tyrosine-negative (Takikawa *et al.,* 1989; Balestra *et al.,* 2009).

In Italy a severe outbreak has been registered since 2008; in some areas Psa disease incidence in the field was very high (in Lazio region it ranged from 50% to 80%), in some cases, due to the extremely high percentage of disease, eradication of the whole orchard was required. From Lazio region the disease is rapidly spreading across the production areas of kiwifruit. The highest disease incidence was associated with the *A. chinensis* cultivars, especially cv. Hort 16 A, but also *A. deliciosa* plants showed symptoms (Balestra *et al.,* 2009; Renzi *et al.,* 2009).

Figure 1 – Leaves spot (left) and red-rusty exudation from twig canker (right) on *Actinidia* chinensis cv. Hort16A caused by *Pseudomonas syringae* pv. *actinidiae*.

1.4.2. Floral buds necrosis

Caused by *P. s.* pv. *syringae* (Pss), it is diffuse almost in every place where kiwifruit is cultivated. It has been reported in Italy for the first time in 1997 (Balestra & Varvaro, 1997).

Pss symptoms are canker on twigs, necrotic spots on leaves, browning and rots on flowers and buds (Gaignard & Luisetti, 1992; Balestra & Varvaro, 1997).

The pathogen is able to survive on kiwifruit organs as a typical epiphytic bacterium, but the diseases incidence depends on area, cultivars and weather conditions. Rainfall and high temperature during springtime favour the developing of the disease (Leben, 1965; Brook, 1990; Balestra, 1997; Balestra & Bovo, 2003; Gonzàlez *et al.,* 2003; Balestra, 2004; Janse, 2005; Calzolari *et al.,* 2006). Moreover Pss is characterised by an ice nucleation activity (INA) and by epiphytic survival (Rossetti & Balestra, 2008).

Ice nucleation activity. Plant tissues are injured by cold because water has the capacity to supercool with temperature below 0°C. The lower is the temperature, the higher is the probability of ice nucleation. In the absence of sites capable of ice nucleation, the water in plant tissues can supercool; freezing will not occur until the temperature becomes low enough that the most active ice nucleus associated with the plant is able to catalyze crystallization of supercooled water (Lindow *et al.,* 1982; Young, 1987). INA is the capability of some bacteria species

such as *Pseudomonas* spp. and *Erwinia herbicola* to catalyse ice formation by giving the water a binding site which allows water molecules to assume a reticular structure. Damages to "frost-sensitive" plants under natural conditions in presence of bacteria INA^+ usually occurs between -2 and -5°C. At these temperatures, ice forms from supercooled water in such plants, propagates throughout the plants, and frost damage occurs. (Lindow *et al.,* 1982; Young, 1987; Varvaro & Fabi, 1992; Testolin & Costa, 1995; Young *et al.,* 1997; Balestra, 2004). Ice damaging incidence on sensitive plants is proportional to the amount of bacteria INA+ present during the freezing (Mittlestadt, 1997).

Epiphytic survival. Various bacteria can colonize aerial plant leaves. Although the majority of them live as commensals, a few can alter the plant's health, by inducing disease symptoms or frost injury under favorable conditions. Epiphytic bacteria have been defined as bacteria that are capable of lives and multiply themselves on plant surfaces continuously (Leben, 1965; Beattie & Lindow, 1999; Lindow & Brandl, 2003).

Bacteria may arrive on leaf surfaces in several ways. Many phytopathogenic or saprophytic bacteria may be seed borne, growing and colonizing rapidly on pre-emergent seedlings and leaves. Leaf habitats are unusually open systems: surrounding vegetation and exposure to the atmosphere provides ample opportunity for aerial dissemination of phyllosphere bacteria. Immigration and emigration of phyllosphere bacteria also occur during rain. The air-borne dissemination of foliar bacterial pathogens is that they are carried by the wind in aerosols or ballistic particles generated when raindrops strike diseased plant parts or plants supporting surface bacterial populations. Also Insects may play a role in dissemination of phyllosphere bacteria, especially when leaves are wet (Beattie & Lindow, 1999; Lindow & Brandl, 2003; Pietrarelli *et al.,* 2006).

The leaf surface has long been considered an extreme habitat for microbial colonists because of continuously fluctuating physical and nutritional factors. It is subject to rapid changing in physical situation such as changes in water and nutrient availability, temperature, and intensity of UV light and ionizing and nonionizing radiation. Nutrients are made available to microbes either

endogenously from plant exudates or exogenously from compounds found in materials on plant surface. While inorganic nutrients are leached to the surface, organic substances which are mostly simple sugars like glucose, fructose, and sucrose, account for the majority of nutrients exuded from leaf tissues. Surface plant leaves offer a variety of habitats for microorganisms, including protected niches on the surface. Epiphytic sites include sites on the waxy cuticle that covers the epidermal, trichome, and guard cells, while endophytic sites include the substomatal cavities, the intercellular spaces, and the mesophyll cell surfaces. (Lindow *et al.,* 1993; Beattie & Lindow, 1999; Lindow & Brandl, 2003; Nix-Stohr *et al.,* 2008).

Leaf imprint studies demonstrate that bacteria do not occur in a uniform pattern across leaf surfaces, but are localized in particular sites such us at the base of the trichomes, stomata, and at the epidermal cell wall junctions, especially in the grooves along the veins. Bacteria were also found in depressions of the cuticle, beneath the cuticle and near hydathodes. In general, larger numbers of bacteria were found on lower more than on upper leaf surfaces, possibly due to the lower surface having a higher density of stomata or trichomes or a thinner cuticular layer (Varvaro *et al.,* 1993; Beattie & Lindow, 1999; Lindow & Brandl, 2003; Pietrarelli *et al.,* 2006).

The relationship between epiphytic bacterial population sizes on asymptomatic leaves of phytopatogenic bacteria and the incidence of the subsequent disease symptoms is generally accepted. Lindemann *et al.* (1984) found that frequencies with which epiphytic population sizes of *P. s.* pv. *syringae* were equal to or greater than approximately 10^4 CFU per leaflet on asymptomatic individual bean leaflets were predictive of disease. By those finding the disease it is determined by the development of large population sizes of the bacteria in association with leaves; in other words, phytopathogenic bacteria epiphytic population sizes can be good predictors of foliar disease (Hirano & Upper, 1993 and 2000; Beattie & Lindow, 1999).

1.4.3. Bacterial blight

Caused by *P. viridiflava* (Pv), it can infect leaves from late spring onwards, causing dark angular lesions surrounded by yellow haloes. Lesions may become extensive, and the necrotic tissue can eventually disintegrate. On the blossom early symptoms are brown, sunken areas of sepals of unopened buds which soon shed. It was first reported in Italy in 1990 (Brook, 1990; Varvaro *et al.,* 1990; Balestra & Varvaro, 1998; Calzolari *et al.,* 2006). Pv shows the same Pss characteristics as diffusion, epiphytic ability, causing damages with favourable weather condition and INA (Varvaro & Fabi, 1992**;** Balestra & Varvaro, 1998).

1.5. Control of bacterial diseases

To date only a few measures for the control of the bacterial diseases, in particular in organic agriculture, are available. Those options are:

1) Antibiotics (e.g. streptomycin) show good control effects. However their use is forbidden in many countries (as in EU) for the control of plant diseases because of risks of development of cross-resistance in phytobacteria and in human pathogens (Ghosh *et al.,* 2008).

2) Heavy metals, as copper compounds are the most effective way to control phitopathogenic bacteria, but they represent a problem due to their phytotoxicity, their accumulation in soil, the establishment of copper-resistant pathogen strains and the necessity of frequent applications. Moreover, according to the recent European restriction (CE Reg. 473/2002), the use of cupric salts will be limited (Balestra & Varvaro 1999; Varvaro *et al.,* 2001).

3) Elicitors are compounds stimulating any type of plant defence mechanisms. There are examples in which they provide very high levels of disease control, but many more examples of induced resistance providing lower levels or absent levels of disease control. Furthermore, too much elicitor can induce the vine to shut down resulting in decreased plant growth (Walters *et al.,* 2005).

4) Biological control agents and products (natural antagonist; natural extracts). Biological control agents have the possibility of multiplying on the plant preventing the establishment of the pathogen. The main criticism made of microbiological control is the lack of consistency. Also plant extracts showed good effect but like all chemicals will be washed off with time. However, biological control agents role -like that of antibiotics and heavy metals- is mainly to prevent new infections (Alabouvette *et al.*, 2006; Slusarenko *et al.*, 2008).

The main problems in bacterial disease control are the non-availability of suitable commercial antibacterial compounds. In general control would have to be integrated, with different components during the seasons acted upon to decrease the inoculum pressure and equip the plants with a level of natural resistance to infection. Moreover appropriate agronomic practices such as seed certification, appropriate irrigation and fertilization techniques are needed. Since environmental factors and variable colonization strategies play an important role in phytobacteria spreading, it is nevertheless difficult to reduce their damages without having recourse to effective preventive measures (Beattie & Lindow, 1999; Pietrarelli *et al.,* 2006).

1.6. Biocontrol of bacterial diseases

Different approaches may be used to prevent, mitigate or control plant diseases, even so growers often rely heavily on chemical fertilizers and pesticides. However, the environmental pollution caused by excessive use of pesticides, has led to the introduction of strict regulations on chemical pesticide use and pressure to remove the most hazardous chemicals from the market. Consequently, some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. Among these alternatives are those referred to as biological controls (Pal & McSpadden Gardener, 2006).

In principle there are the following ways of biocontrolling bacterial diseases

of crop besides resistance breeding: the use of micro-organisms (for periodic applications) and the use of natural substances inducing resistance or acting as bactericides.

1.6.1. Natural antagonists

Biological control can be defined as "the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms'' (Cook & Baker, 1983). Bacterial pathogens biological control comprises the decrease of inoculum or of the disease producing activity of a pathogen through one or more mechanisms including avirulent or hypo-virulent individuals or populations within the pathogenic species, antagonistic microorganisms and manipulation of the host plant to resist the pathogen more effectively (Buchenauer, 2004; Alabouvette *et al.*, 2006).

The first commercial development of a biocontrol measure on plant pathogenic bacteria started in the 1980s with strain K-84 of *Agrobacterium radiobacter* against crown gall of stone fruits in Australia (Kerr & Htay, 1974). In Europe, despite the many research efforts dealing with biological control of plant diseases, application of microbiological control was less successfull. Today there are only six BCA strains (*Ampelomyces quisqualis, Bacillus subtilis, Coniothyrium minitans, Gliocladium catenulatum, Paecilomyces fumosoroseus, Pseudomonas chlororaphis*) on Annex I of EEC directive 91/414, that is the list of products authorised for use in plant protection in the European Union. However interest in biological control of plant pathogens has increasing considerably because it may provide control of diseases that cannot or only partly be managed by other control strategies. Recent advance in microbial and molecular techniques have significantly contributed to new insights in underlying mechanisms by which introduced bacteria function (Buchenauer, 2004; Alabouvette *et al.*, 2006; Pal & McSpadden Gardener, 2006).

Biocontrol agents provide protection against an array of foliar, soilborne and post-harvest pathogens when released into soil or irrigation water, or when plants are treated (Gullino, 2005). Biocontrol mechanisms by which bacteria may protect

plants against pathogens are different. The bacteria may compete for space and nutrients in the rhizosphere or phyllosphere, or produce antibiotic substances. For example fluorescent *Pseudomonas* are capable of enhancing plant growth and of controlling pathogens by producing siderophores under iron-limiting conditions. Siderophores represents low-molecular weight iron(III)-transport compounds, which selectively complex iron(III) with very high affinity. Their function is to supply iron to the producing organism (Whipps, 1997; Buchenauer, 2004).

Antagonistic bacteria can produce several other metabolites including antibiotics, enzymes and volatiles, which may play important roles in control of pathogens. Antibiotics produced by bacterial biocontrol agents comprise chemically heterogeneous groups of organic low molecular weight compounds. For example antibiotics produced by *Pseudomonas* species include: phenazines, phloroglucinols, oomycin A, pyoluteorin, pyrrolin, 2,3-de-epoxy-2,3-didehydro-rhizoxin, viscosinamide, butyrolactones and butylbenzene-sulphonamide (Weller, 1988; Whipps, 1997; Raajmakers *et al.,* 2002).

Bacteriocins production has been demostrated for phyllosphere bacteria. Bacteriocins may be defined as antibacterial substances that display a specific inhibitory effect against organisms closely related to the producers. The chemical nature of the bacteriocins constitutes of low or high molecular weight proteins or low molecular weight nucleotides (Buchenauer, 2004).

Bacteria also can be beneficial to the host plant directly through the production of metabolites that either stimulate root development and plant growth or trigger the induction of systemic acquired resistance (Van Loon *et al.,* 1998; Raaijmakers *et al.,* 2002)

The effectiveness of endophytes as biological control agents (BCAs) is dependent on many factors. These factors include: host specificity, the population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance (Melnick *et al.,* 2008).

Table 1 - Principal natural antagonists of bacterial diseases.

Organism	Product	Target	Hosts	Formulation
Agrobacterium radiobacter	Norbac 84-C, Nogall	Crown gall	Fruit and nut trees	Water
Bacillus subtilis (different strains)	Rhizo-Plus, Serenade	Potato scab *(S. scabies),* Fireblight *(E. amylovora)*	Potato, Stone fruits	Water, Wettable power
Pantoea agglomerans P10c	Blossom bless	Fireblight *(E. amylovora)*	Apples, Pears	Wettable power
Pseudomonas fluorescens A506	BlightBan A506	Fireblight *(E. amylovora),* Frost damage	Stone fruits , Apple, Pear	Wettable power

1.6.2. Plant extracts

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most are secondary metabolites, of which at least 12,000 have been isolated. In many cases, these substances serve as plant defence mechanisms against predation by microorganisms, insects, and herbivores (Cowan, 1999).

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. Thought to be responsible for phenolic toxicity to microorganisms, those mechanisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999).

Quinones are aromatic rings with two ketone substitutions. These compounds, being coloured, are responsible for the browning reaction in cut or injured fruits and vegetables. The potential range of quinone antimicrobial effects is great, probable targets in the microbial cell are surface-exposed adhesins, cell

wall polypeptides, and membrane-bound enzymes (Duke *et al.,* 1985).

Flavones are phenolic structures containing one carbonyl group (**fig. 2**). They are known to be synthesized by plants in response to microbial infection. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Dixon *et al.,* 1983; Tsuchiya *et al.,* 1996).

Catechins, the most reduced form of the C3 unit in flavonoid compounds, deserve special mention (**Fig. 2**). These flavonoids have been extensively researched due to their occurrence in green teas. It was noticed some time ago that teas exerted antimicrobial activity and that they contain a mixture of catechin compounds (Toda *et al.,* 1989).

Tannins, is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatine from solution, a property known as astringency. They are divided into two groups, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units. Their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes and cell envelope, to bind proline rich proteins and to interfere with the protein synthesis (Scalbert *et al.,* 1991).

Terpenoids are secondary metabolites that are highly enriched in compounds based on an isoprene structure. Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Terpenoids are active against bacteria and their mechanism of action is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Scortichini & Rossi, 1991; Amaral *et al.,* 1998).

Coumarins are phenolic substances made offused benzene and a-pyrone

rings. This substance have antimicrobial properties against gram positive bacteria (Shihabudeen *et al.,* 2010).

Saponin**,** Steroids and Alkaloids have also been found to exert antimicrobial properties (Cowan, 1999; Shihabudeen *et al.,* 2010).

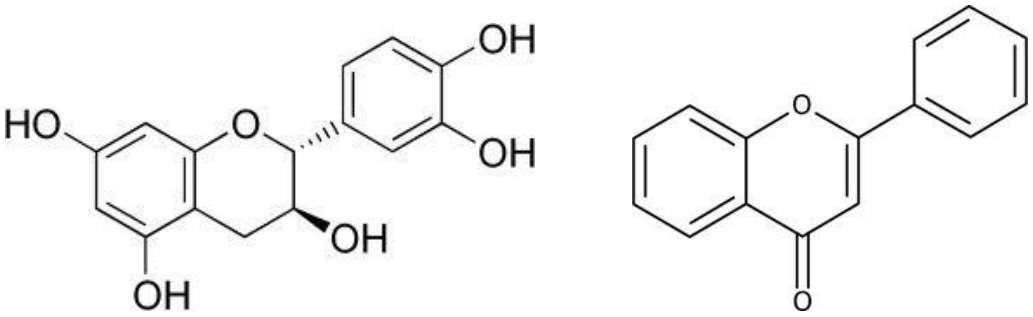

Figure 2 – Chemical structures of Catechins (left) and Flavones (right).

Extraction Methods. Initial screenings of plants for possible antimicrobial activities typically begin by using crude aqueous or alcohol extractions and can be followed by various organic extraction methods. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction.

For alcoholic extractions, plant parts are dried, ground to a fine texture, and then soaked in methanol or ethanol for extended periods. The slurry is then filtered and washed, and then it may be dried under reduced pressure and redissolved in the alcohol to a determined concentration. When water is used for extractions, plants are generally soaked in distilled water, blotted dry, made into a slurry through blending, and then strained or filtered. The filtrate can be centrifuged (approximately 20,000 *g*, for 30 min) multiple times for clarification (Cichewicz *et al.,* 1996; Taylor *et al.,* 1996). Crude products can then be used in disc diffusion and broth dilution assays to test for antibacterial properties and in a variety of assays to screen for antiviral activity (Cowan, 1999).

1.6.3. Essential oils

Essential oils (EOs) are aromatic oily liquids obtained from plant material. They can be obtained by different methods but steam distillation is most commonly used for commercial production of EOs. They are mostly used in food (as flavourings), perfumes (fragrances and aftershaves) and pharmaceuticals (for their functional properties) (Burt, 2004).

Some EOs show antimicrobial properties, moreover their components have been shown to exhibit antiviral, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties (Carson *et al*., 1995; Karpouhtsis *et al*., 1998; Mourey *et al*., 2002; Mari *et al.,* 2003; Burt, 2004).

Composition of the EOs is variable. They can comprise more than sixty individual components. Major components can constitute up to 85% of the EO, whereas other components are present only as a trace. A detailed compositional analysis is achieved by gas chromatography and mass spectrometry of the EO (Senatore, 1996; Russo *et al.,* 1998; Delaquis *et al.,* 2002). The phenolic components are chiefly responsible for the antibacterial properties of EOs (Burt, 2004).

The composition of EOs from a particular species of plant can differ depending on harvesting seasons and geographical sources: generally, EOs produced from herbs harvested during or immediately after flowering possess the strongest antimicrobial activity (Cosentino *et al.,* 1999; Marino *et al.,* 2001). EOs are volatile and therefore need to be stored in airtight containers in the dark in order to prevent compositional changes.

1.7. References

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CHAPTER 2

THESIS AIMS

The aim of this study was to find biological strategies to control bacterial diseases of kiwifruit by use of natural antagonists and natural extracts.

In the third Chapter a screening of a large number of bacteria naturally occurring on kiwifruit's phyllosphere for their antagonists' activity against *P. syringae* pv. *actinidiae, P. s.* pv. *syringae* and *P. viridiflava* was carried out. The strains which showed considerable antagonistic activity were successively submitted to further characterization analysis. The best potential biocontrol agents was selected and submitted to *in vivo* test to determine the potential antagonistic activity against kiwifruit bacterial pathogens.

The fourth Chapter objective was to perform a screening *in vitro* of antibacterial activity of several plant extracts from different countries, including some plant extracts from Italy, some essential oils and some native plants from New Zealand for their antibacterial property against *P. s.* pv. *actinidiae, P. s.* pv. *syringae* and *P. viridiflava*. The more effective crude extracts were characterized and used on planta against kiwifruit bacterial pathogens. Finally a formulation to improve and extend efficacy of active fractions with antimicrobial activity by use of microencapsulation technique was tested *in vivo*.

Apart from the period spent abroad, 8 months in New Zealand, at the Plant and food Research center of Ruakura (Hamilton) supervised by Dr. Joel Vanneste working on natural antagonists, all the *in vitro* researches was conducted at the Laboratory of Phytobacteriology, Department of Plant Protection, Viterbo (Italy) while *in vivo* tests in a greenhouse conducted by Department of Plant Protection sited at the farm of Tuscia University. In field tests were conducted in a kiwifruit orchard located in Latina, Latium region (Italy).

CHAPTER 3

BIOLOGICAL CONTROL OF KIWIFRUIT BACTERIAL PATHOGENS BY APPLICATION OF NATURAL, EPIPHYTIC LIVING NON-PATHOGENIC ANTAGONISTS TO THE PLANT PHYLLOSPHERE

3.1. Introduction

In recent years there has been an increasing interest in biological control of bacterial plant diseases using naturally-occurring saprophytic bacteria (Backman *et al.,* 1997; Wilson & Backman, 1999; Moss *et al.,* 2007). While bacterial biological control agents (BCAs) have been commercially developed for crown gall, caused by *Agrobacterium tumefaciens*; fire blight, caused by *Erwinia amylovora*; and frost injury, caused by ice-nucleation active (INA^+) strains of *Pseudomonas syringae* (Lindow, 1987; Stockwell *et al.,* 1993; Vanneste *et al.,* 2002), to date, no effective bacterial biological control agent of bacterial diseases of kiwifruit is available. This is in spite of numerous studies on biological control of *Pseudomonas* pathogens including *P. s*. pv. *glycinea* (Volksch *et al.*, 2001; May *et al.,* 2008), *P. s.* pv. *syringae* (Leonardi *et al.,* 1989; Braun-Kiewnick *et al.,* 2000), *P. s.* pv. *tomato* (Balestra *et al.*, 2001; Wilson *et al.,* 2002), *P. avellanae* and *P s.* pv. *coryli* (Gentili *et al.,* 2008).

The objectives of the current study was to screen naturally occurring saprophytic bacteria of the kiwifruit phyllosphere for their potential as biocontrol agents of kiwifruit pathogens *Pseudomonas syringae* pv. *actinidiae* (Psa), *P. s.* pv. *syringae* (Pss) and *P. viridiflava* (Pv). The BCAs which showed the best results were tested *in planta*. Furthermore, the effectiveness of several formulations was evaluated for long term preservation of these bacterial antagonists.

3.2. Material and Methods

3.2.1. Isolation of potential BCAs

Leaves were collected during February-March 2009 (which are summer months in New Zealand) from an experimental organic kiwifruit orchard sited at Ruakura Research Center in Hamilton (New Zealand). The orchard is composed by three different species of kiwifruit: *A deliciosa* (cv. Hayward), *A. chinensis* (cv. Hort16A®) and *A. arguta* (different cvs.).

Each sampling, 10 undamaged leaves of medium size and 6 fruits were picked randomly from three plants. The leaves, handled only by the petiole, and the fruits, were placed in paper bags and taken to the laboratory for immediate processing (Korsten *et al.,* 1995). A total of 100 leaves and 60 fruits were collected, one-third of each group from *A deliciosa,* one-third from *A. chinensis* and one-third from *A. arguta*.

Leaves were washed separately in plastic bag containing 50 ml of $MgSO_4$ buffer (see appendix) for 2 hours. Fruits' top (stem end) and bottom (calyx end) were cut and washed separately in plastic container containing 10 ml of 10 mM $MgSO_4$ buffer for 2 hours. A dilution series was made of each sample solution and 100 µl each of the 10^3, 10^4 and 10^5 dilutions were spread onto LB (Luria Bertani) agar plates. After 48 h incubation at 27°C the total colonies growth on plates were counted. Representatives of each group with similar morphological characteristic were reisolated for further tests. All isolated bacteria were maintained on NG (Nutrient glycerol) agar slants and kept at 5°C.

3.2.2. Screening of potential antagonists strains

All the strains isolated from kiwifruit phyllosphere were tested *in vitro* for their antagonistic activity against several strains of Psa[1], Pss and Pv (**Tab. 1**) obtained from the bacterial collection of Tuscia University.

The screening test was carried out preparing MM (Minimal media) agar plates with 5 ml overlays of MM soft-agar seeded with one of six strains of Pss and one of six strains of Pv at a final concentration of $1x10^6$ CFU/ml. After 24 hours of incubation at 28°C the plates were checked and the eventual inhibition zone around the spots was measured.

The bacteria able to inhibit all 12 strains of Pss and Pv were tested in the following experiment: in the centre of a MM-agar plate, the strain to be tested was grown in a 10 mm diameter area, by depositing a suspension of ca $1x10^6$ CFU/ml. After 48 h of incubation, suspensions 10^6 CFU/ml of 10 strains of Pss and 10 strains of Pv (**Tab. 1**) were streaked from the edge of the dish to the edge of the potential BCAs. The dishes were incubated at 27°C for 24 hours. The inhibition areas were then measured from the edge of the BCAs colony (Vanneste *et al.,* 1992). Both tests were repeated twice.

[1] Since part of this work was carried out in Plant & Food Research at Ruakura Research Centre (Hamilton, New Zealand) during 2009 when Psa was not present in New Zealand, *in vitro* experiments concerned only Pss and Pv, as the laboratory did not have the authorization from MAF Biosecurity New Zealand to work with Psa which is considered in New Zealand an unwanted organism.

Table 1- Bacterial strains used in this study.

Species	Strain name	Host	Place of origin
P. s. pv. *actinidiae*	LT1	*Actinidia chinensis*	Latium (Italy)
P. s. pv. *actinidiae*	LT3	*Actinidia deliciosa*	Latium (Italy)
P. s. pv. *syringae*	VT2	*Actinidia deliciosa*	Latium (Italy)
P. s. pv. *syringae*	VT5	*Actinidia deliciosa*	Latium (Italy)
P. s. pv. *syringae*	VT8	*Actinidia deliciosa*	Latium (Italy)
P. s. pv. *syringae*	VT12	*Actinidia deliciosa*	Latium (Italy)
P. s. pv. *syringae*	VT16	*Actinidia deliciosa*	Latium (Italy)
P. s. pv. *syringae*	VT19	*Actinidia deliciosa*	Latium (Italy)
P. s. pv. *syringae*	VR22	*Actinidia deliciosa*	Veneto (Italy)
P. s. pv. *syringae*	VR25	*Actinidia deliciosa*	Veneto (Italy)
P. s. pv. *syringae*	VR26	*Actinidia deliciosa*	Veneto (Italy)
P. s. pv. *syringae*	NZ3	*Actinidia deliciosa*	New Zealand
P. viridiflava	VT3	*Actinidia deliciosa*	Latium (Italy)
P. viridiflava	VT6	*Actinidia deliciosa*	Latium (Italy)
P. viridiflava	VT14	*Actinidia deliciosa*	Latium (Italy)
P. viridiflava	VT17	*Actinidia deliciosa*	Latium (Italy)
P. viridiflava	VR21	*Actinidia deliciosa*	Veneto (Italy)
P. viridiflava	VR23	*Actinidia deliciosa*	Veneto (Italy)
P. viridiflava	VR24	*Actinidia deliciosa*	Veneto (Italy)
P. viridiflava	VR29	*Actinidia deliciosa*	Veneto (Italy)
P. viridiflava	VR32	*Actinidia deliciosa*	Veneto (Italy)
P. viridiflava	NZ2	*Actinidia deliciosa*	New Zealand

3.2.3. Characterization and identification of BCAs

The potential BCAs selected were submitted to morphologically and biochemically (e.g. fluorescence production test, levan production test, oxidase test) (Klement *et al.,* 1990; Goszczynska *et al.,* 2000) and analyzed for hypersensitive reaction on tobacco and ice nucleation activity tests (Goszczynska *et al.,* 2000). Moreover strains hr45, hr49, hr51, hr54, hr58, hr61 and hr63 were identified by analysis of DNA sequence of the 16S rDNA (Palacio-Bielsa *et al.,* 2009).

Hypersensitive reaction on tobacco assays. BCAs strains were streaked onto LB-agar plates and incubated at 28°C for 48 h. Cells were suspended in 10 ml of sterile distillate water (SDW) and the density of the suspension was adjusted to

1 x 10^8 CFU/ml using a spectrophotometer at 600 nm. BCAs bacterial suspensions were infiltrated in replicates into the intercellular spaces of tobacco leaves (cv "Burley") (abaxial surface) at the 8 to 10 leaf stage (Klement *et al.,* 1990). Other controls consisted of suspensions of Hr^+ pytopathogenic bacteria (Pss VT2) and Hr^- antagonist bacteria (*Pantoea agglomerans* P10c). After 24 hours presence of dry and necrotic tissue in correspondence of infiltration was recorded as positive reaction (Goszczynska *et al.,* 2000).

Ice nucleation assays. BCAs bacterial suspensions 1 x 10^8 CFU/ml were prepared. Suspensions of cell material were placed in 10 µl droplets on the surface of a paraffin-coated aluminium weighing pan and cooled on the surface of ethylene glycol-water (1:1 v/v) in the refrigerating circulator bath. As the temperature was slowly lowered to -2, -5 and -7°C the number of drops frozen was scored. BCAs were considered ice nucleation active at a particular temperature if the suspensions froze within 10 min. Four drops per strain were tested. Other controls consisted of suspensions of INA bacteria (Pss VT2) and non-INA bacteria (*P. agglomerans* P10c) (Kieft, 1988; Balestra & Varvaro, 1998).

Molecular identification (16S rDNA). The genomic DNAs of each bacterial strain were extracted from single colonies grown for 48h at 28°C on KB-agar (King B) plates. Approximately 2x10^9 cells were harvested and their DNA was extracted with the PureLink™ Genomic DNA kit (Invitrogen, USA) according to manufacturer's instructions for Gram negative bacteria. The DNA was then eluted with TE-buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0).

The 16S rDNA gene was amplified by Polymerase Chain Reaction (PCR) using 25 to 35 ng of genomic DNA in a final volume of 25 µl 50 pmol of each forward and reverse primer, 2X reaction buffer (including 2mM $MgCl_2$), 1 unit of Taq DNA polymerase (Invitrogen), 1nM of dNTPs. Reaction mixtures were incubated in a Mastercycler Gradient thermal cycler (Eppendorf) at 94°C for 12 min (for initial denaturation and activation of Taq polymerase); followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 1.5 min; followed by a final extension period of 12 min at 72°C.

The 16S rDNAs were amplified with universal reverse oligonucleotide primer 27F (specific for Bacteria) (59-AG AGTTTGATCCTGGCTCAG-39) and 1492R (59-GGTTACCTTGTTACGACTT-39).

For sequencing DNA concentration and quality were determined by gel electrophoresis on agarose gel (10 g l^{-1}) and its concentration was fluorimetrically estimated, adjusted to a final concentration of 25 ng/µl.

DNA sequences were resolved using a 3130XL Genetic Analyzer System fitted with 50 cm capillary arrays (Applied Biosystems) loaded with POP-7 polyacrylamide matrix (Applied Biosystems). DNA templates were prepared using Big Dye v3.1 terminator chemistry (Applied Biosystems). DNA sequences were compared to those in GenBank using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.,* 1997) network service from the National Center for Biotechnology Information to determine their approximate phylogenetic affiliations (NCBI) (Weisburg *et al.,* 1992; Doika *et al.,* 2000; Palacio-Bielsa *et al.,* 2009).

3.2.4. Mode of action

Several tests were carried out to understand the characteristics and the mode of action of the bests potential BCAs after *in vitro* tests.

Antibiotic production assay. The production of antimicrobial compounds by hr61, hr62 and hr63 strains was tested both in liquid minimal medium and in agar (overlay technique).

In liquid minimal medium assay, the lack of multiplication of Pss and Pv in the cell-free culture filtrates of the BCAs strains revealed the antagonist activity of the potential BCAs against kiwifruit bacteria pathogens. The lack of multiplication was checked by plate counting.

Suspensions of BCA strains hr45, hr49, hr50, hr52, hr53, hr54, hr58, hr61, hr62, hr63 and hr64 were added into glass tubes containing 10 ml of MM-broth. The tubes were incubated at 27°C for 48 hours to allow bacterial growth to mid-log phase. Cells were removed by centrifugation (15,000 *g* x 2') and filtration (filter 0.22 mm diameter, Fisher Scientific). Five ml of supernatant fluid and 100 µl of 2

strain each of Pss and Pv (**Tab. 1**) ($1x10^6$ CFU/ml) suspension were added in new glass tubes. After 24 hours of incubation at 27°C, several ten-fold dilutions of the broth were spread on MM-agar plates. The colonies growth were counted after 48 h of incubation. As control uninoculated MM-broth was used (Ishimaru *et al.,* 1988; Vanneste *et al*., 1992).

For the overlay technique, a single centre spot (1 cm in diameter) of the potential BCAs to be tested for antibiotic production hr49, hr51, hr61, hr62 hr63 and hr64 were incubated for 48 h at 27°C on MM-agar plates. Cells of the resulting producer colony were removed and killed by exposure to $CHCl_3$ vapor (Chloroform) and overlaid with 5 ml of warm MM soft agar seeded with about $1x10^6$ CFU/ml of Psa strain LT1, Pss VT2 and Pv VT3 (**Tab. 1**). Antibacterial activity was recorded as formation of clear zone of inhibition after further incubation for 48 h at 27°C (Ishimaru *et al.,* 1988; Vanneste *et al*., 1992; Galasso *et al.,* 2002; Biondi, 2008).

Incubation Temperature. The influence of temperature on the antimicrobial metabolites production has been assessed in the following experiment: glass tubes containing 5 ml MM-broth inoculated with hr63 were incubated with agitation at different temperature (22°C, 27°C, 32°C and 37°C) for 48 hours. Uninoculated MM-broth was used as control. After filter-sterilization, the cell-free fractions were added with 100 µl of Pss VT2 and Pv VT3 (**Tab. 1**) (concentration of 1 x 10^6 CFU/ml) and incubated at 27°C for 24 h. Bacterial growth was monitored picking up and spreading on MM-agar plates 100 µl of broth serial dilutions of each tubes. After a 48 h incubation at 27°C the colonies growth on plates were counted and estimated the CFU/ml (Fu *et al.,* 2010).

Temperature effect. The temperature effects on antimicrobial metabolites activity were checked by the potential BCA strain hr63 growing at 27°C for 48 hours in a glass tube containing 50 ml MM-broth. After filter-sterilization, the suspension was divided into several glass tubes which were kept 3 minutes at -18°C, 4°C, 27°C, 42°C, 60°C and 100°C. Each tubes was then added with 100 µl of Pss VT2 and Pv VT3 (**Tab. 1**) (concentration of 1 x 10^6 CFU/ml) and incubated at

27°C for 24 hours. Bacterial growth was determined picking up and spreading on MM-agar plates 100 µl of broth serial dilutions of each tubes. After a 48 h incubation at 27°C the colonies growth on plates were counted and estimated the CFU/ml (Vanneste *et al*., 1992).

Inactivation of the antibiotic activity by aminoacids. A single spot of potential BCA strains hr61, hr62 and hr63 was grown for 48 h in the centre of a MM-agar plate, removed, and then killed with chloroform vapours as described in the antibiotic production assay paragraph (overlay technique). Holes were punched into the agar around the spot where BCAs strains had grown. One of the following solutions was then added to each hole: 5 µl of a 4 mg/ml solution of the following filter sterilized amino acids: L-alanine; L-arginine; L(+) asparagine; L-aspartic acid; L-cystein; L-glutamine; L-glutamic acid; glycine; L-histidine; L-isoleucine; L-leucine; L-lysine; L-methionine; L-phenylalanine; L-proline; L-serine; L-threonine; DL-thryptophan; L-thyrosine and L-valine (Merck). 5 µl of sterile distilled water were used as negative control. The plates were then incubated for 1h and afterwards overlaid with 5 ml of warm MM soft agar seeded with Pss VT2 and Pv VT3 (100 µl at $1x10^6$ CFU/ml). Inactivation of the antibiotic activity by these different aminoacids was determined by visual examination after an additional 24 h at 27°C (Vanneste *et al.*, 1992).

3.2.5. Epiphytic survival

In *in vivo* tests, potential BCAs antibiotic resistant mutants were used.

Antibiotic resistant mutants. Antibiotic resistant derivatives (ant^r) were selected as single colonies arising, after a suspension (0.1 ml at 10^9 CFU/ml) of each BCAs strain was spread onto LB-agar supplemented with streptomycin (100 µg/ml; Sigma) plates and incubated at 27°C for 72 hours. The colonies morphology, the antimicrobial properties and the growth rate of the derivatives were compared with that of the wild type strains (hr61, hr62 and hr63). Stock cultures of ant^r

strains were cryopreserved at -80°C in LB-broth containing 15% glycerol (Lottmann *et al.*, 2000; Bazzi *et al.,* 2006; Jahanifar *et al.,* 2008).

For growth rate test, MM-broth was dispensed into sterile sealed glass containers (10 ml final volume). The glass containers were then inoculated with 100 µl of an overnight culture of hr61, hr62, hr63, hr61 ant^r, hr62 ant^r, hr63 ant^r and shaken. Bacterial growth was monitored by removing 100 µl of broth after 3, 6, 12 and 24 hours incubation at 27°C and spreading on LB-agar plates serial dilution of broth of each tube. After 48 h incubation at 27°C the colonies growth on plates were counted and the initial concentration in CFU/ml was estimated (Tassou *et al.,* 2000). Broth test were repeated twice with two replicates each.

To evaluate the differences in antagonistic activity between BCAs strains and BCAs strains ant^r the overlay technique, as previously described, was used. Control strain was the Pss VT2 strain. The differences in antagonist activity between BCA strains and BCAs ant^r strains was revealed by the presence of inhibition zones surrounding the area of growth (Vanneste *et al.*, 1992; Galasso *et al.,* 2002; Biondi, 2008).

Ability to colonise kiwifruit leaves. Potential BCAs strains hr61, hr62 and hr63 were assessed for their ability of colonizing kiwifruit leaves. 2 years-old, Potted kiwifruit cv. Hayward, were used. The plants were watered daily by drip-irrigation and a mineral solution was distributed weekly into the pots to maintain optimum nutritional conditions. The experiments were carried out in a greenhouse where temperature was maintained at 25±2 °C during the day and 15±2 °C during the night; the relative humidity was maintained between 70 and 80%, during the whole experiment, by using automatic cooling. Heating and drip-irrigation data were recorded by logger at 60-min intervals.

For inoculation, BCAs strains hr61 ant^r, hr62 ant^r and hr63 ant^r were, streaked onto LB-agar plates and incubated at 28°C for 48 h. Cells were suspended in 100 ml of sterile distillate water (SDW) and the density of the suspension was adjusted to 1 x 10^8 CFU/ml using a spectrophotometer at 600 nm. Kiwifruit plants were sprayed with each bacterial suspension until leaves got homogeneously wet by spraying plants with a CO^2-pressurized handheld sprayer equipped with a large

orifice (diameter 1.4 mm, Tee-Jet 8004) nozzle operating at a pressure of 28 g cm^2 to produce fine spray droplets. Inside the greenhouse, 2 h before and 2 h after bacterial inoculation, the relative humidity was maintained at 90% to favour stomata opening.

Each BCA strain was sprayed on 5 kiwifruit plants. For estimation of bacterial population sizes, 1 hour, 2, 4, 6, 8 days after spraying, 5 leaves were harvested from each treatment (replicates) of plants treated with different BCAs. Leaves were immersed separately in 20 ml of washing buffer ($MgSO_4$) in plastic bag and agitated using Stomacher equipment (Seward, model 400 Circulator) for 5 minutes. Serial dilutions of leaf washings were plated onto LB-agar plates amended with 100 µg of streptomycin per ml using spiral plate system (Don Whitley Scientific, mod. WA01GG). Plates were incubated 48 h at 27°C and the single colonies developed were counted to obtain the CFU/cm^2 of leaves.

The population level on the upper and lower leaf surface was monitored also by leaf imprints on plates of reisolation medium (LB-agar supplemented with streptomycin).

3.2.6. Greenhouse experiments

Hr63 was assessed for the ability to suppress bacterial population of kiwifruit pathogens on kiwifruit plants in greenhouse. 2 years-old potted kiwifruit cv. Hayward, were used for the test. Experiment conditions were the same as 3.2.5. paragraph.

Briefly, an overnight culture of bacteria hr63 ant^r grown in LB-broth was resuspended in $MgSO_4$ buffer (final concentration $1x10^8$ CFU/ml). 15 plants were sprayed with hr63 suspension. The following day 1 x 10^8 CFU/ml bacterial suspension of Psa LT1, Pss VT2 and Pv VT3 were sprayed each on 5 plants treated with hr63 and 5 not treated. After 1, 7 and 14 days 5 leaves per plot were sampled. In lab, leaves were immersed separately in 20 ml of washing buffer ($MgSO_4$) in plastic bag and agitated using Stomacher equipment for 5 minutes. Serial ten-fold dilutions was prepared by transferring 1 ml each plastic bag onto 9 ml of sterile water. From any dilution 100 µl was plated on LB-agar and LB-agar supplemented

with streptomycin plates using spiral plate system. Plates were incubated 48 h at 27°C and the single colonies developed were counted to obtain the CFU/cm^2 of leaves (Bazzi *et al.,* 2006). The plant studies were repeated twice. The BCAs were applied 1 day prior the pathogens inoculation for their acknowledged preventive way of action (Braun-Kiewnick *et al.*, 2000; Khan *et al.*, 2010).

On the plates with streptomycin were able to growth only hr63 colonies, while on the plates of LB-agar both the pathogen and the antagonists colonies. The population size of hr63, Psa, Pss and Pv was defined:

Total colonies hr63 = mean colonies growth on LB-agar supplemented with streptomycin.

Total colonies Psa or Pss or Pv = mean colonies on LB-agar minus mean number of colonies growth on LB-agar supplemented with streptomycin.

3.2.7. Formulation

The effectiveness of several formulations was evaluated for long-term preservation of the potential BCA strain hr63. The viability of hr63 in NB-broth and LB-broth was tested by addition of trehalose (10 mM) or polyvinylpyrrolidone (PVP) (2%) or glycerol (10 mM) in 1 L of broth. 100 µl of 1 x 10^8 CFU/ml culture of hr63 was inoculate individually onto each broth and incubated at 25 ± 2 °C. The broth cultures were analyzed for viable cell population at different intervals up to decline phase. A serial ten-fold dilutions was prepared by transferring 1 ml each of inoculum onto 9 ml sterile water. From any dilution 100 µl was plated on LB-agar plates using spiral plate system (Don Whitley Scientific, mod. WA01GG). The plates were incubated at 27 ± 2 °C and individual colonies were counted. In total, three replications were used for each dilution (Lee *et al.,* 2006; Manikandan *et al.,* 2010).

3.2.8. Statistical analysis

In *in vitro* tests the mean and standard error were calculated.

In *in vivo* tests bacterial population dynamics were evaluated using the log-

transformed population sizes. The population sizes were expressed per square centimetre of leaf, being estimated using "APS assess 2.0" software to determine leaf areas. Data were statistically analysed using GraphPad Prism 4 software by analysis of variance (ANOVA) and the significance of the treatments were determined by using Tukey's HSD test ($p > 0.05$) (Steel *et al.,* 1997).

3.3. Results

3.3.1. Determination of total bacterial population

Bacterial population size on kiwifruit leaves was $1x10^4$ CFU/ml. No differences were observed between *A. deliciosa*, *A. chinensis* and *A. arguta* leaves. However some differences were observed in fruits: the highest population was recorded on *A. deliciosa*'s fruits with a mean of 1.4×10^6 CFU/cm^2. *A. chinensis* and *A. arguta* showed lower populations (1.4×10^4 and 5.8×10^3 CFU/cm^2) (**Fig. 1**).

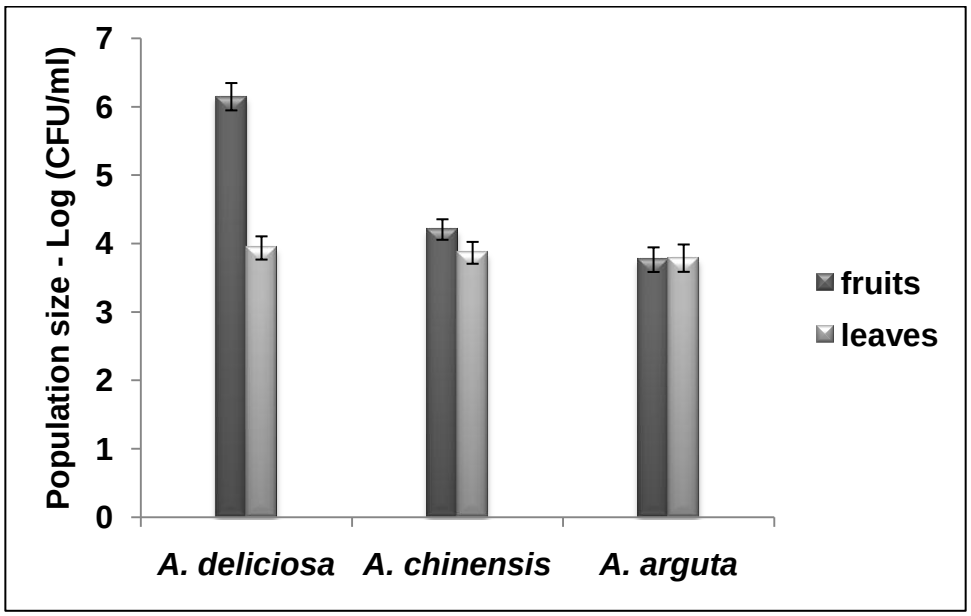

Figure 1 - Total bacterial population on fruits and leaves of three different species of *Actinidia*. Errors bars indicate the standard error.

3.3.2. Screenings of potential antagonists strains

After leaves and fruits washing, 1968 strains were collected and analysed: 1416 from fruits and 552 from leaves. At the end of the first antagonist test, 20 strains were selected for their antagonist activity against all 12 strains of Pss and Pv (**Fig. 2**). Potential BCAs strains were named from hr45 to hr64 (**Tab. 2**). 19 strains on 20 were coming from fruits. Strains hr53, hr 56, hr58, hr 61 and hr64 showed larger inhibition areas.

In the second antagonist test 11 of 20 potential antagonists strains tested confirmed antagonist activity on all strains of Pss and Pv, in particular hr61, hr62, hr63 and hr64 showed the best effectiveness (**Fig. 2**). Some strains, as hr46, hr50, hr52, hr53 and hr55, showed small or none antagonist activity (**Tab. 2**).

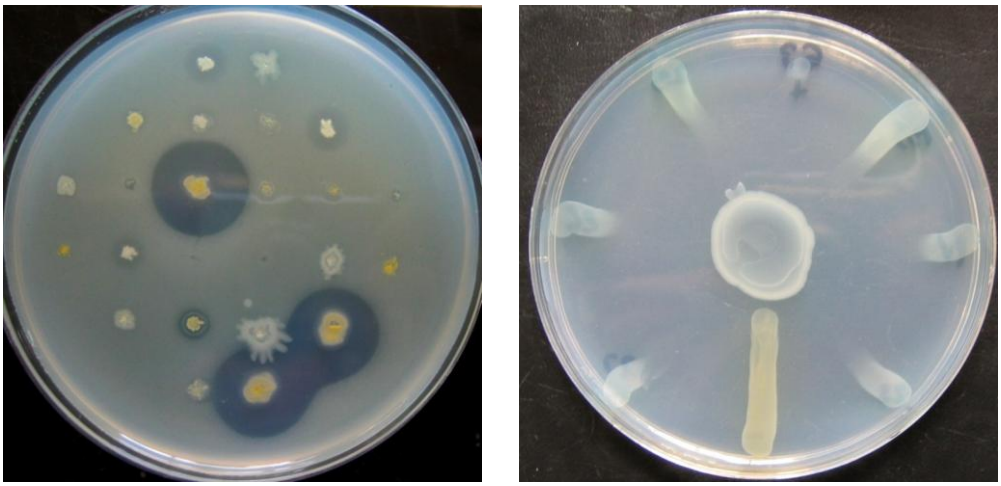

Figure 2 – Growth inhibition in *in vitro* inhibition assays. On the left, zones of growth inhibition of *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* can be detected around BCAs spot placed on the plate. On the right, zones of growth inhibition can be detected around the potential BCAs strains spot in the center.

Table 2 - *in vitro* inhibition assays results. Potential BCAs strains were named from hr45 to hr64.

BCAs strains	TEST A		TEST B	
	Number of Pss and Pv strains tested/inhibited	Inhibition zone (mm) ±SD	Number of Pss and Pv strains tested/inhibited	Inhibition zone (mm) ±SD
hr45	12/12	2.4 ± 0.7	20/20	6.2 ± 1.6
hr46	12/12	2.8 ± 1.6	0/20	0
hr47	12/12	2.5 ± 0.9	20/20	6.3 ± 1.8
hr48	12/12	2.9 ± 1.4	15/20	2.5 ± 0.2
hr49	12/12	1.8 ± 0.6	20/20	7.7 ± 2.2
hr50	12/12	1.2 ± 0.4	0/20	0
hr51	12/12	2.8 ± 1.5	20/20	6.0 ± 2.3
hr52	12/12	1.8 ± 1.0	0/20	0
hr53	12/12	3.8 ± 1.4	0/20	0
hr54	12/12	2.2 ± 0.8	20/20	3.1 ± 1.1
hr55	12/12	2.0 ± 0.9	20/20	6.7 ± 1.5
hr56	12/12	3.0 ± 2.0	0/20	0
hr57	12/12	2.3 ± 1.5	8/20	2.9 ± 0.3
hr58	12/12	3.5 ± 1.9	20/20	6.2 ± 2.1
hr59	12/12	2.7 ± 1.8	10/20	2.5 ± 0.1
hr60	12/12	2.2 ± 1.2	15/20	3.2 ± 0.3
hr61	12/12	4.9 ± 2.6	20/20	23.1 ± 5.1
hr62	12/12	1.9 ± 1.1	20/20	13.6 ± 3.8
hr63	12/12	2.4 ± 1.5	20/20	18.3 ± 2.5
hr64	12/12	3.3 ± 1.8	20/20	10.4 ± 2.3

3.3.3. Characterization and identification of BCAs.

Fluorescence production test, levan production test and oxidase test results are detailed in **table 3.**

Hypersensitive reaction on tobacco and ice nucleation activity test. None of the potential antagonist strains collected from kiwifruit induced an HR on tobacco leaves or induced ice nucleation within -7°C (**Tab. 3**). These tests allowed to confirm that the potential BCAs are neither plant pathogens nor a risks as frost injury.

Molecular identification (16S rDNA). Strain hr45 showed highest similarity to *Pseudomonas fluorescens* species (97%), strain hr49 to *Pseudomonas fluorescens* (97%), strain hr51 to *Pantoea agglomerans* (99%), strain hr54 to *Pseudomonas fluorescens* (98%), strain hr58 to *Pantoea agglomerans* (98%), strain hr61 to *Pseudomonas fluorescens* (95%) and strain hr63 to *Pseudomonas fluorescens* (99%).

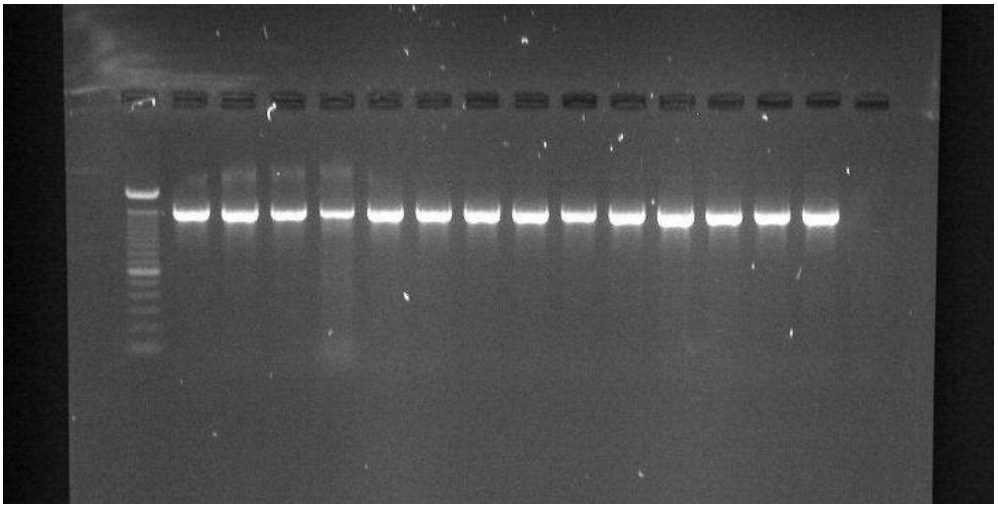

Figure 3: Amplification of 16S fragment. In the left column the 100 bp DNA ladder; in the right column was placed water as control.

>gb|GU198119.1| Pseudomonas fluorescens strain LMG 14571 16S ribosomal RNA gene, partial sequence
Length=1500

Score = 2586 bits (1400), Expect = 0.0
Identities = 1403/1404 (99%), Gaps = 1/1404 (0%)
Strand=Plus/Plus

```
Query  1    ATGC-AGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAA  59
            |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34   ATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAA  93

Query  60   TGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCAT  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94   TGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCAT  153

Query  120  ACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTC  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  154  ACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTC  213

Query  180  GGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGA  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  214  GGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGA  273

Query  240  GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  274  GGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG  333

Query  300  GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  334  GGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCT  393

Query  360  TCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTT  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394  TCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTT  453

Query  420  GACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAG  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  454  GACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAG  513
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Figure 4: BLAST alignments of hr63 16S sequences with the reference *Pseudomonas fluorescens* sequence (Genbank: GU198119.1).

Table 3 – Main characteristics of selected BCAs. Identification tests: HR (hypersensitive reaction on tobacco); OX (oxidase test); LE (levan production); FL (fluorescence pigment production on King B); INA (ice nucleation activity). **-** = negative, **+** = positive.

BCA strains name	Collected from:		Identification tests					Belonging to:
	part	host	HR	OX	LE	FL	INA	
hr45	Fruit	*A. deliciosa*	-	+	+	+	-	*Pseudomonas fluorescens*
hr46	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr47	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr48	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr49	Fruit	*A. deliciosa*	-	+	+	+	-	*Pseudomonas fluorescens*
hr50	Fruit	*A. deliciosa*	-	-	-	+	-	*Pseudomonas spp.*
hr51	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr52	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr53	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr54	Fruit	*A. deliciosa*	-	-	-	-	-	*Pseudomonas fluorescens*
hr55	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr56	Fruit	*A. deliciosa*	-	-	-	-	-	*Pantoea agglomerans*
hr57	Fruit	*A. chinensis*	-	-	-	-	-	*Pantoea agglomerans*
hr58	Fruit	*A. chinensis*	-	-	-	-	-	*Pantoea agglomerans*
hr59	Fruit	*A. chinensis*	-	-	-	-	-	*Pantoea agglomerans*
hr60	Fruit	*A. chinensis*	-	-	-	-	-	*Pantoea agglomerans*
hr61	Leaf	*A. chinensis*	-	+	+	+	-	*Pseudomonas fluorescens*
hr62	Fruit	*A. arguta*	-	+	+	+	-	*Pseudomonas fluorescens*
hr63	Fruit	*A. chinensis*	-	+	+	+	-	*Pseudomonas fluorescens*
hr64	Fruit	*A. chinensis*	-	-	-	-	-	*Pantoea agglomerans*

3.3.4. Mode of action

Antibiotic production assay. In Liquid minimal medium assay was detected antibacterial activity against Pss and Pv in cells-free filtrates of hr49, hr61, hr62 and hr63 strains. For those BCAs, Pss and Pv populations, from $1x10^5$ CFU/ml, remained stable or slightly decreased within 12 hours (**Fig. 5**). Hr62 and hr63 extracts were more effective than hr49 and hr61. Hr45, hr50, hr52, hr53, hr54, hr58, hr64 did not produce detectable antibacterial substances in cell-free filtrates (**Fig. 5**).

In the overlay technique assay, zones of inhibition around the spot of BCAs were consistently observed with hr61, hr62 and hr63, while less consistent inhibition was observed with hr49, hr51 and hr64 (**Tab. 4**). This result confirm those obtained with liquid minimal medium assay.

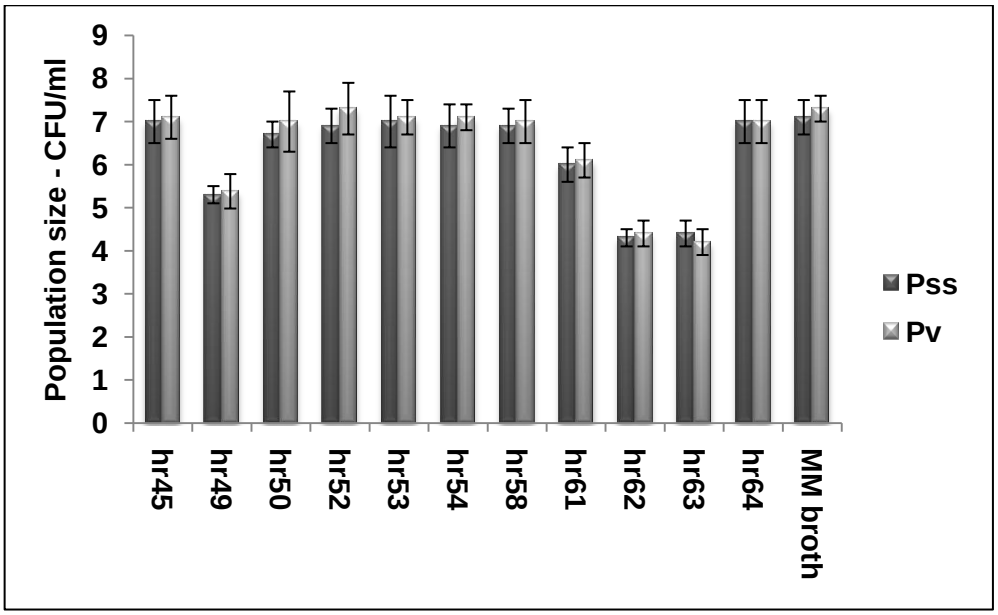

Figure 5: Growth inhibition in liquid media tests of *Pseudomonas syringae* pv. *syringae* (Pss) strain VT2 and *P. viridiflava* (Pv) strain VT3 by BCAs cell-free supernatant. Each point represents the mean bacterial population size determined from three replicate experiments. The vertical bars represent the standard error of the mean log-transformed population size at the sampling time.

Table 4 – BCA strains hr49, hr51, hr61, hr62, hr63 and hr64 antibiotic inhibition areas (mm) on *Pseudomonas syringae* pv. *actinidiae* (Psa) LT1, *P. s.* pv. *syringae* (Pss) VT2 and *P. viridiflava* VT3.

	Hr49	Hr51	Hr61	Hr62	Hr63	Hr64
Psa	5.3 ± 0.4	3.3 ± 0.2	35.7 ± 1.5	25.2 ± 0.5	25.7 ± 2.2	9.4 ± 0.7
Pss	6.7 ± 0.5	4.7 ± 0.3	28.5 ± 1.7	22.0 ± 1.4	25.0 ± 2.3	8.8 ± 0.7
Pv	8.5 ± 0.7	4.4 ± 0.4	33.0 ± 1.4	19.2 ± 1.0	26.2 ± 1.3	8.5 ± 0.6

All parameters are expressed as mean±SD.

Incubation Temperature. Antimicrobial metabolites production of strain hr63 is highest at an incubation temperature of 27°C. There is a significant reduction of antimicrobial metabolites production at 22°C and 32°C while no production was observed at 37°C compared to bacteria growth on MM broth at 27°C (**Fig. 6**).

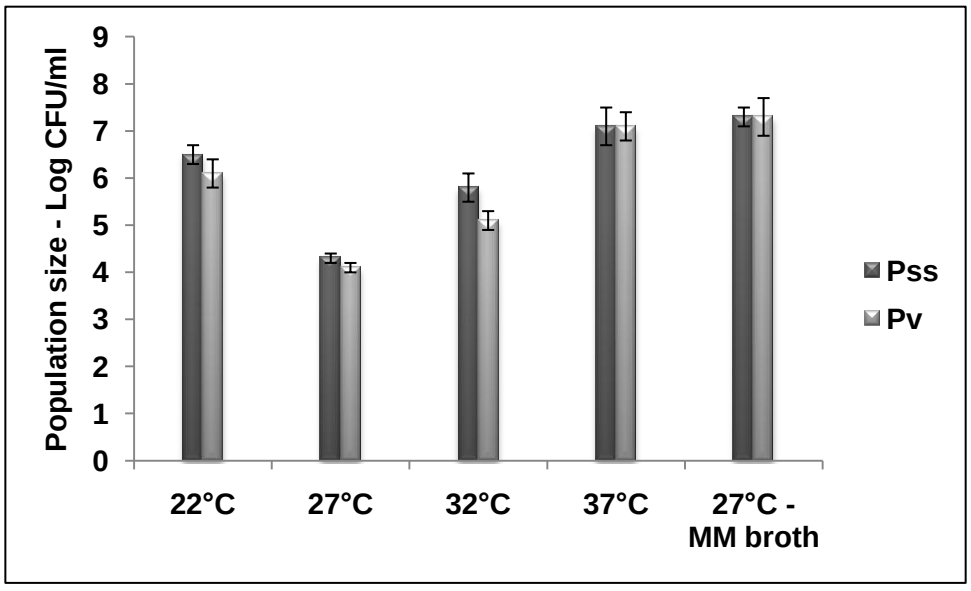

Figure 6: *Pseudomonas syringae* pv. *syringae* (Pss) VT2 and *P. viridiflava* (Pv) VT3 Log_{10} of CFU/ml growth after 12 hours of incubation at 27°C on cell-free supernatant of hr63. On abscissa the growth temperatures of hr63 in MM-broth. Each point represents the mean bacterial population size determined from three replicate experiments. The vertical bars represent the standard error of the mean log-transformed population size at the sampling time.

Temperature effect on antibiotic activity. Extreme temperature (-18°C and 100°C) for few minutes inactive antibacterial metabolites produced by hr63. At 60°C metabolites are partially inactive and between 4°C and 42°C no effects were observed on metabolites activity (**Fig. 7**).

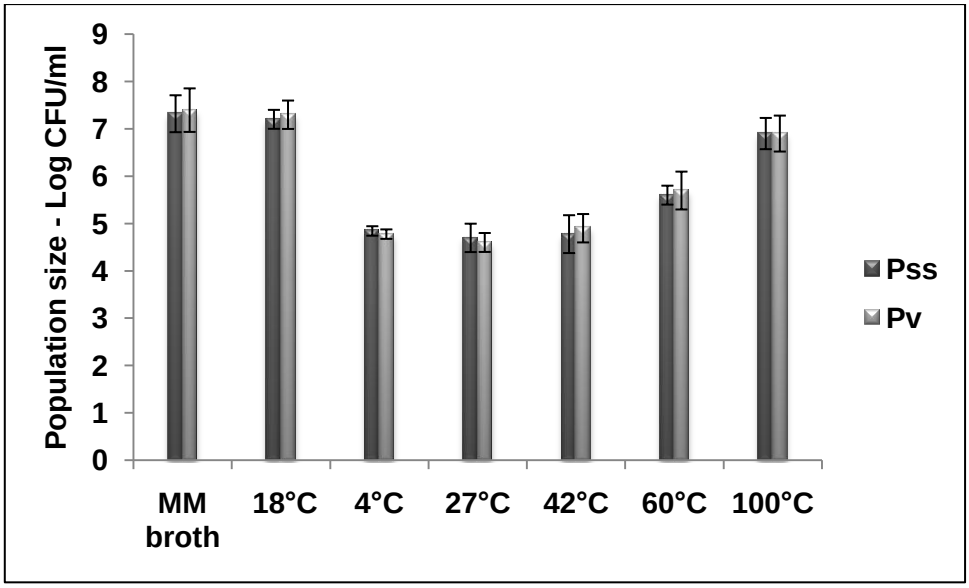

Figure 7: *Pseudomonas syringae* pv. *syringae* (Pss) VT2 and *P. viridiflava* (Pv) VT3 Log_{10} of CFU/ml growth after 12 hours of incubation at 27°C on cell-free supernatant of hr63 kept for 3 minutes at the temperatures on the abscissa. Each point represents the mean bacterial population size determined from three replicate experiments. The vertical bars represent the standard error of the mean log-transformed population size at the sampling time.

Inactivation of the antibiotic activity by aminoacids. The antimicrobial activity of hr63 against Pss and Pv was inactivated by addition of leucine. All the others aminoacids did not produce modification on hr61, hr62 and hr63 antagonistic activity (**Fig. 8**).

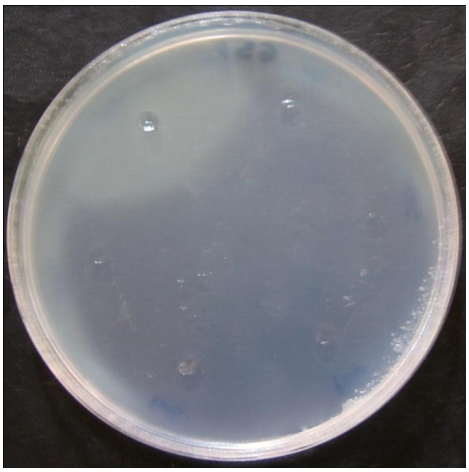

Figure 8 - Antagonist activity of strain hr63 on *Pseudomonas syringae* pv. *syringae* VT2. On top-left, adding aminoacid Leucin, hr63 lose antagonistic activity.

3.3.5. Epiphytic survival

Antibiotic resistant mutants. One colony each of hr61, hr62 and hr63 growth on LB-agar supplemented with streptomycin (100 µg/ml; Sigma) plates, morphologically similar to the wild-types, were selected and named hr61 ant^r, hr62 ant^r and hr63 ant^r.

Antibiotic resistant mutants hr61 ant^r, hr62 ant^r and hr63 ant^r demonstrated to have growth rates similar to those of the corresponding parental strains on MM-broth (**Fig. 9**) and to maintained the same *in vitro* antagonistic activity against Psa, Pss and Pv (**Tab. 5**).

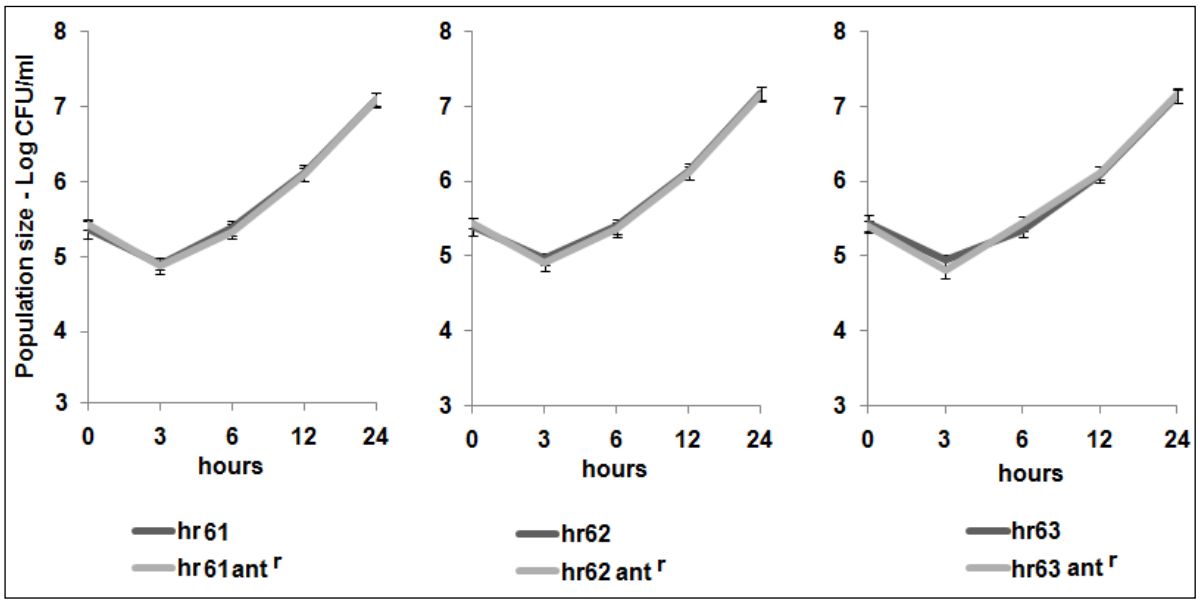

Figure 9 – Population dynamics on MM-broth of hr61, hr62 and hr63 and the correspondent antibiotic resistant strain.

Table 5 – Inhibition areas in millimeter by overlay technique test of BCA strains hr61, hr62 e hr63 and their correspondent antibiotic resistant hr61, hr62 and hr63 ant^r on *Pseudomonas syringae* pv. *actinidiae* (Psa) LT1, *P. s.* pv. *syringae* (Pss) VT2 and *P. viridiflava* (Pv) VT3.

	hr61	hr61 ant^r	hr62	hr62 ant^r	hr63	hr63 ant^r
Psa	35.7 ± 1.5^a	33.5 ± 2.4^a	25.2 ± 0.5^b	27.5 ± 1,9^b	25.7 ± 2.2^b	27.2 ± 2.2^b
Pss	28.5 ± 1.7^b	28.0 ± 2.4^b	22.0 ± 1.4^c	20.2 ± 1.7^c	25.0 ± 2.3^b	26.7 ± 2.4^b
Pv	33.0 ± 1.4^a	31.7 ± 1.9^a	19.2 ± 1.0^c	18.5 ± 2.0^c	26.2 ± 1.3^b	28.2 ± 2.4^b

All parameters are expressed as mean±SD. Values not marked with the same letter are significantly different ($p<0.05$).

Ability in colonisation on kiwifruit leaves. Strains hr61, hr62 and hr63 showed ability to colonize kiwifruit leaves during the experiment time. In particular they were able to increase populations of 2 unit log in 8 days. Hr63 showed the best characteristics reaching 5 x 10^6 CFU/cm^2 at the end of the experiment (**Fig. 10**).

Leaf imprinting experiment showed as hr63 was able to colonize equally the leaf surfaces (**Fig. 11**).

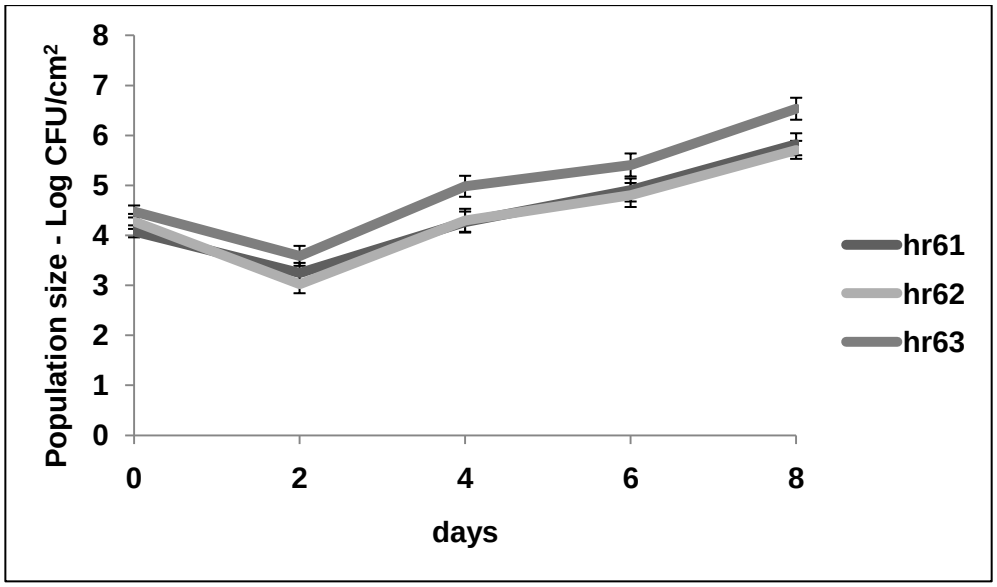

Figure 10 - Population dynamics of BCAs hr61, hr62 and hr63 on kiwifruit leaves, errors bars indicate the standard error.

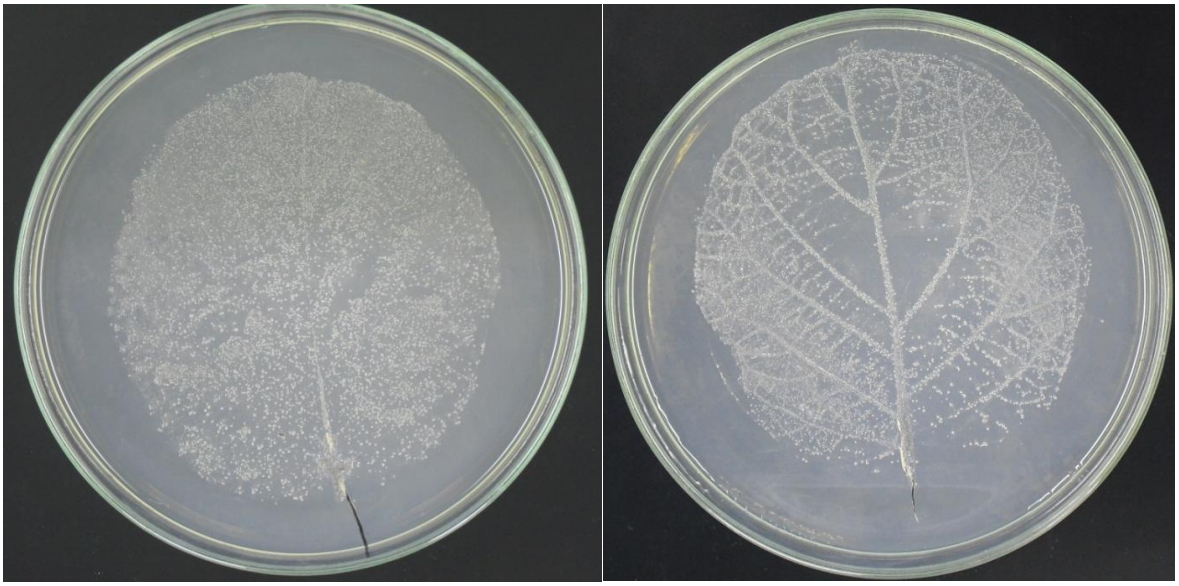

Figure 11 – Leaf imprinting of upper (left) and lower (right) kiwifruit leaf surfaces 2 days after hr63 ant^{r} spraying. Leaf was imprinted on LB-agar plates supplemented with streptomycin.

3.3.6. Greenhouse experiments

The BCA strain hr63 sprayed on plants of kiwifruit was able to maintain populations of Psa, Pss and Pv on leaves below $1x10^4$ CFU/cm^2 (**Fig. 12**). In particular, the strain hr63 was able to settle on leaves around $1x10^4$ CFU/cm^2 and during the experiment time grew of about 1.5 units log to $5x10^5$ CFU/cm^2. phytopathogenic strains, sprayed one day after the antagonist, had the same population of hr63 at the inoculation time (around $1x10^4$ CFU/cm^2) but afterwards they slightly decreased in Psa and Pv or maintained the same level in Pss case.

In the control plants, treated only with Psa, Pss and Pv, population of pathogens on leaves in absence of the antagonist increased to level higher than $4x10^5$ CFU/cm^2, growing of 1 log in 12 days (**Fig. 12**).

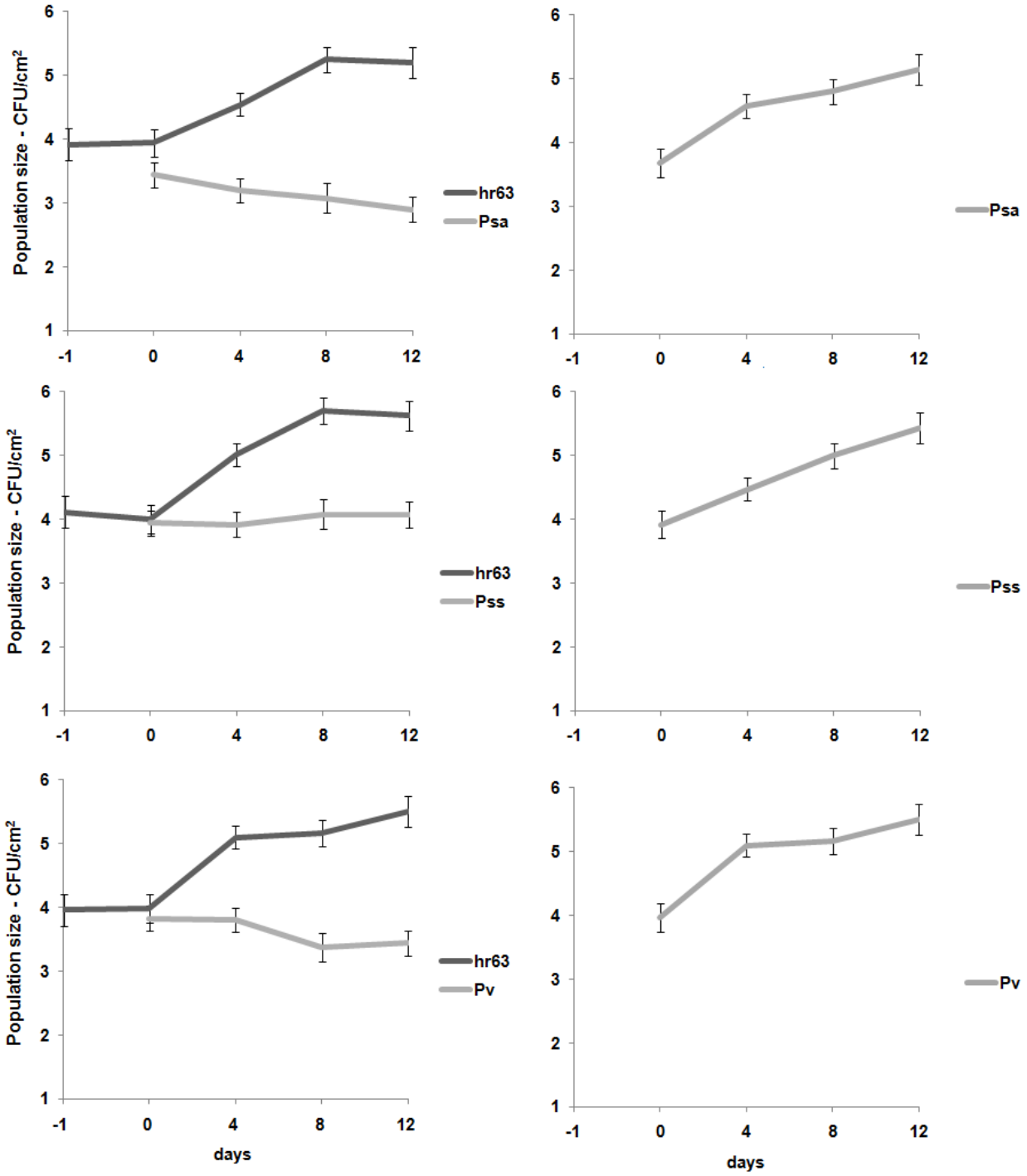

Figure 12 - Population dynamics on kiwifruit plants in greenhouse experiment. On the left side the BCA hr63 was sprayed 1 day before *Pseudomonas syringae* pv. *actinidiae* (Psa) LT1, *P. s.* pv. *syringae* (Pss) VT2 and *P. viridiflava* (Pv) VT3; on the right side the pathogens were sprayed alone. Errors bars indicate the standard error. The values are the means of five replicates.

3.3.7. Formulation

Among the different amendments tested in NB and LB, addition of glycerol and trehalose maintained the population level of 1 x 10^6 CFU/ml up to 120 days. However, addition of glycerol (10 mM) resulted in a greater level of hr63 cells throughout the period of observation, followed by trehalose (10 mM) (**Tab. 6**). In NB, the initial population of 10^5 CFU/ml increased to 10^8 CFU/ml up to 15 days and slightly decreased after 30 days after incubation, whereas in NB without chemical amendments, the population declined up to 10^3 CFU/ml at 60 days after incubation. Compared to LB, NB was more desirable for the development of liquid-based bioformulation of *P. fluorescens* hr63.

Table 6 – Viability of *Pseudomonas fluorescens* hr63 cultures of different ages in nutrient broth amended with different chemicals.

Days	Population (CFU/ml)					
	NB+ glycerol	NB + trehalose	NB Broth alone	LB+ glycerol	LB + trehalose	LB Broth alone
0	2.9 x 10^5	3.0 x 10^5	2.8 x 10^5	2.8 x 10^5	2.5 x 10^5	2.9 x 10^5
3	3.7 x 10^7	2.8 x 10^7	2.0 x 10^7	3.0 x 10^8	2.6 x 10^8	3.7 x10^8
6	2.2 x 10^8	2.3 x 10^8	3.7x 10^8	8.6 x 10^8	5.8 x 10^8	8.0 x 10^8
15	6.1 x 10^8	1.3 x 10^8	7.1x 10^7	9.8 x 10^8	1.7 x 10^8	5.8 x 10^7
30	6.0 x 10^7	3.6 x 10^7	4.2 x 10^7	6.0 x 10^7	3.1 x 10^7	5.1 x 10^7
60	5.3 x 10^6	3.2 x 10^5	2.4 x 10^4	4.4 x 10^6	2.6 x 10^5	3.7 x 10^4
120	1.1 x 10^6	2.2 x 10^4	3.2 x 10^3	5.6 x 10^5	1.7 x 10^4	2.0 x 10^3

3.4. Discussion

The search for natural antagonists is a very promising branch in control of plant disease, mainly because these organisms survive on the same host plants and help to control bacterial diseases reducing environmental risks.

No differences were observed on the amount of natural-occurring bacterial populations on leaves of *A. deliciosa*, *A. chinensis* and *A. arguta,* while on the surface on *A. deliciosa* fruits has been noticed a larger amount of bacteria respect to *A. chinensis* and *A. arguta* fruits. This could be explained because of *A. deliciosa* fruits hairy surface, which allow a more favourable conditions for bacteria surviving.

In vitro antagonistic tests allowed to select potential BCAs isolated from kiwifruit leaves and fruits with better antagonistic characteristics.

In the first antagonist test twenty strains showed remarkable antagonist activity against all phitopathogenic bacteria tested. These strains, named from hr45 to hr64, were almost all collected from fruits, which proved to be a more suitable niche for antagonistic bacteria. In this experiment potential BCAs showed different levels of antagonism, and in particular hr53, hr56, hr58, hr61 and hr64 showed larger inhibition zones.

In the second antagonistic test, where antagonist activity is determined by the release and diffusion on media of antimicrobial substances, some strains (e.g. hr61, hr62, hr63 and hr64) confirmed the antagonist activity, while others (e.g. hr46, hr50, hr52, hr53 and hr56) which were effective in the first test, did not show any antagonist activity, therefore they didn't release antimicrobial substances on media.

Characterization tests allowed to obtain information about the potential BCA strains. According to the HR, INA and analysis of DNA sequences of 16S rDNA, none of the potential BCAs gathered were nor pathogens to animal/plant or human beings. *Pseudomonas fluorescens* and *Pantoea agglomerans* were the two most prevalent species found as potential BCAs from kiwifruit in this study. Both species are already known as BCAs (Braun-Kiewnick *et al.*, 2000; Nunes *et al.*, 2002; Vanneste *et al.,* 2002; Pusey *et al.*, 2009).

Finding out BCAs' mode of action is very important to select the best ones;

the antibiotic production assay indicate that the strains hr49, hr61, hr62 and hr63 produced metabolites with antibacterial activities, whereas was not detected antibacterial metabolites production from the other strains, thus their antagonistic activity on plate is probably mainly due to a better ability to compete for space and nutrients. This is in accord with the second *in vitro* antagonist test.

Hr63 produce higher quantity of antimicrobial compound when it growth at 27°C. Moreover antimicrobial compounds produced by hr63 are inactivated with temperature lower than 4°C and higher than 60°C. Aminoacids test showed as the antimicrobial compound produced by strain hr63 interferes with the leucine biosynthetic pathway, the addition of leucine turns off that pathway and therefore removes the target of the antimicrobial compound from strain hr63.

At the end of the *in vitro* tests three potential BCAs, hr61, hr62 and hr63, all belonging to *P. fluorescens* species, emerged as best candidates for Psa, Pss and Pv control.

Establishment of BCAs on aerial plant surface is the main challenge in biocontrol. All potential BCAs selected hr61, hr62 and hr63 were able to colonize leaves and improve population size in greenhouse test on kiwifruit plants. In particular hr63 proved to be the most effective BCA to colonize plant surface and therefore it was selected for antagonist test *in vivo* against Psa, Pss and Pv.

It's not well known the relation between bacterial pathogens inoculation and the symptoms appearance on kiwifruit plants. Some preliminary tests confirmed the difficulty to obtain standardized symptoms. Consequently the *in vivo* antagonist activity of hr63 was evaluated using the phytopathogenic bacteria epiphytic population sizes, which according to Hirano & Upper (1983) is a good predictor of foliar disease. In greenhouse test, Psa, Pss and Pv were not able to increase population size on kiwifruit plants in presence of the BCA hr63 sprayed 1 day before. So we can assume that hr63 is effective in controlling bacterial diseases of kiwifruit.

The development of formulations which could increase the shelf life besides providing tolerance to adverse conditions and user friendly assumes greater importance for the success of bio-inoculant technology in sustainable crop protection**.** In this study, addition of glycerol to NB preserved the viability of hr63

cells in liquid formulation for the storage period of 6 months. This finding is commensurate with those of Manikandan *et al.* (2010) who developed the liquid formulation of *P. fluorescens* Pf1. The enhanced survival of hr63 cells in the liquid formulation might be attributed to the action of chemical amendments. The choice of glycerol and trehalose as chemical amendments in this study is justified that they hold a high amount of water and are capable of enhancing cell tolerance to desiccation, osmotic pressure and temperature stress (Fillinger *et al.,* 2001).

The efficacy of biological control agents is highly dependent upon their ability to adapt to local climates. Antagonists that suppress disease in laboratory or in greenhouse experiments may fail to establish threshold populations required for biological control in the field. Aerial surface of plants can be inhospitable to bacteria growth, because of UV light changing temperature, water and nutrient availability (Stockwell *et al.,* 1998). For that reasons further tests are needed.

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CHAPTER 4

USE OF NATURAL EXTRACTS AS ANTIMICROBIAL AGENTS OF KIWIFRUIT PHYTOPATOGENIC BACTERIA

4.1. Introduction

Several plant products are well-known for their antimicrobial activity against human-borne pathogens (Friedman *et al.,* 2002; Di Pasqua *et al.,* 2005); some studies also investigated the potential use of these extracts against phytopatogenic bacteria (Balestra *et al.,* 1998; Varvaro *et al.,* 2001; Pradhanang *et al.,* 2003; Slusarenko *et al.,* 2008; Dan *et al.,* 2010).

Some of the most successful examples include:

Garlic, it has been used as a medicine for millennia for its properties to inhibit different enzymes essential for microbial pathogen infections by its organosulfur compounds (Lawson, 1998; Ankri & Mirelman, 1999; Obagwu & Korsten, 2003). The antibacterial principle of garlic was identified as diallylthiosulphinate and named allicin (Cavallito *et al.,* 1944). Allicin is produced during the crushing of garlic cloves by the interaction between the aminoacid alliin and the enzyme alliinase; allicin is a precursor of a number of secondary products formed in crushed garlic preparations and possesses various biological activities (Stoll & Seebeck, 1951; Miron *et al.,* 2000; Harris *et al.,* 2001; Slusarenko *et al.,* 2008);

Fig, fruit extract is rich in phenols, essential oils and flavonoids, which are effective on bacteria through produced compounds such as resveratrol, psoralen and bergapten. These compounds have demonstrated antibacterial activity (Ogungbamila *et al.,* 1997; Ulate-Rodriguez *et al.,* 1997; Salameh *et al.,* 2004; Zao *et al.,* 2005).

Pomegranate, it's one of the oldest edible fruit and it has a long history as a medicinal fruit used extensively in the folk of many cultures. Fractions of ellagic

acid, gallagic acid, punicallins, and punicalagins extracted from pomegranate revealed antimicrobial activity when assayed against human bacterial pathogens (Prashanth *et al.,* 2001; Ghosh *et al.,* 2008). Pomegranate peel is rich in polyphenols including ellagitannins, gallotannins, ellagic acids, gallagic acids, catechins, anthocyanins, ferulic acids, and quercetins (Reddy *et al.,* 2007; Aguilera-Carbo *et al.,* 2008; Opara *et al.,* 2008);

Lavender, essential oil has been found to be active against many species of bacteria (Hammer *et al.,* 1999; Kokoskova & Pavela, 2005). Linalool, a major component of lavender oil show antibacterial activity (Pattnaik *et al.,* 1997). Unfortunately, very little is known of any synergistic relationships that occur between the constituent oil; moreover there are no correlations between the linalool or linalyl acetate content and the antibacterial activity and different oil components may be responsible for the activity against different microorganisms (Lis-Balchin & Hart, 1998; Cavanagh & Wilkinson, 2002);

Bay tree, is a culinary plant and it's traditionally used as an analgesic to treat a variety of complaints, neuralgia and intestinal cramps. Bay leaf oil tested for its bactericidal activity has shown to be active against *Salmonella enterica* and *Escherichia coli*. The essential oils extracted from bay leaf had antimicrobial activity (Raharivelomanana *et al*., 1989; Friedman *et al.,* 2002; Thakare, 2004; Erturk, 2006; Decorato *et al.,* 2007).

Antimicrobial activity of other plant extracts was several times reported, including kiwifruit (Motohashi *et al.,* 2001), olive (Markin *et al.,* 2003; Decorato *et al.,* 2007), grapevine (Jayaprakasha *et al.,* 2001 and 2003), argan (El Monfalouti *et al.,* 2010), citronella (Pradhanang *et al.,* 2003), geranium and mint (Hammer *et al*., 1999), manuka and tea tree (Perry *et al.,* 1996; Carr, 1998; Hammer *et al.,* 1999; Pradhanang *et al.,* 2002; Carson *et al*., 2006) and sage (Kokoskova & Pavela, 2005; Karata & Ertekin, 2010).

Also some Newzealander native plant extracts showed interesting biological characteristics (Bloor, 1995).

Among the proteins naturally present in egg or milk, lysozyme is the one that has recently attracted increasing attention, due to its potent antimicrobial activity against a wide range of microorganisms. Lysozyme is an enzyme that catalyses the

breakdown of peptidoglycan polymers of bacterial cell wall (Hughey & Johnson 1987; Benkerroum, 2008).

Furthermore, the isolation of the fractions with potential antimicrobial activity, has been targeted with the aim of improving their effectiveness. Between different compounds indicated as antimicrobial agents, following the case of gallic acid (GA) and ellagic acid (EA), phenolic compounds presents in different plant as pomegranate, berries, figs and grapes (Amakura, *et al.,* 2000; Vattem & Shetty, 2005; Naz *et al.,* 2007; Jurenka, 2008).

Gallic acid (**Fig. 1**) is an acid existing in plant material in the form of free acids, esters, catechin derivatives and hydrolysable tannins. This ubiquitous chemical is one of the most biologically-active phenolic compounds of plant origin. GA has been shown to possess antimicrobial activity against both human and plant pathogens (e.g. *Erwinia carotovora*) (Karamaæ *et al.,* 2006), moreover is the fraction with more antimicrobial activities in pomegranate fruit juice (Naz *et al.,* 2007).

Ellagic acid (**Fig. 1**) is a naturally occurring phenolic lactone compound found at high concentrations in pomegranate, strawberries, raspberries, cranberries. EA is present in plants in the form of hydrozable tannins called ellagitannins as the structural components of the plant cell wall and the cell membrane. Ellagitannins are esters of glucose with ellagic acid which, when hydrolyzed, yield ellagic acid (Vattem & Shetty, 2005). Studies have shown EA antibacterial activity towards human bacterial pathogen (*Helicobacter pylori*). EA kills bacteria by inhibiting arylamine Nacetyltransferase activity (Martini *et al.,* 2009). Based on different studies the ellagitannins are thought to be the primary constituent responsible for the antibacterial effects of Pomegranate extracts (Braga *et al*., 2005; Jurenka, 2008).

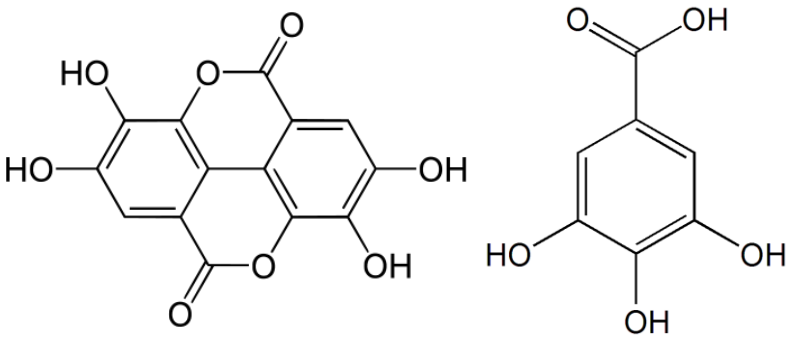

Figure 1 - Chemical structures of Ellagic acid (left) and Gallic acid (right) -Hydrobenzoic acids-.

Concerning the techniques of extraction of plant material, previous report (Adomi & Umukoro, 2010) and some preliminary tests proved that alcoholic extraction was the most effective way to extract antimicrobial components among others previously tested.

Initial screening of potential antibacterial compounds from plants is generally performed with crude extracts (Rojas *et al.,* 1992). The two most commonly used screens to determine antimicrobial susceptibility are the agar drop diffusion assay (Navarro *et al.,* 1996; Di Pasqua *et al.,* 2005) and the broth dilution assay (Hess *et al.,* 1995; Cowan, 1999).

The agar disc diffusion technique proved useful data as a preliminary screening test for a large number of natural extracts, indeed the hydrophobic nature of most essential oils and plant extracts prevents the uniform diffusion of these substances through the agar (Janssen *et al.,* 1987; Rios *et al.,* 1988). Further tests using broth dilution assays were made to ensure that the solubilising agent itself did not have an inhibitory effect, or if so, that its effect should be minimized (Hili, 1997).

Regarding natural extracts, a system to improve the effectiveness and to provide a sustained and targeted delivery that minimizes the amount of active ingredient used is essential. Microencapsulation is a specific procedure that encompasses a substance in microscopic capsules designed to release that same substance outwards. In the case of agrochemicals the microcapsules, generally

sized from 10 to 50 micron, are made up of nylon and include a porous membrane that allows a progressive and uniform release of the active principles (Jana *et al.,* 2001; Glenn *et al.,* 2010; Takei *et al.,* 2008).

The microspheres are composed by a polymer or lipid matrix in which the active principle is finely dissolved or dispersed. They can be employed in different ways in pharmaceutical fields, food industry and agriculture, where, they permit a prolonged release of pesticides and herbicides. Polymers must be chemically inert (inactive) and biocompatible, easily sterilizable, non-toxic and biodegradable and they should not leave impurities, additives or polymerization residuals (Cortesi *et al.,* 2002; Esposito *et al.,* 2002).

These polymers have the advantage of being able to alloy a considerable amount of active principles and excipients without altering their chemical and physical properties. We are dealing with a solid substance in the form of transparent granules and with a peculiar amine odour, soluble in organic solvents such as methanol, ethanol, acetone and methylene chloride, that also has the capacity for forming a swell pellicle in contact with water.

The objectives of the current study were mainly the following : *i*) screening *in vitro* of several natural crude extracts for their antimicrobial activity against Psa, Pss and Pv; *ii*) screening *in vitro* of several crude extracts of New Zealander native plant against for their antimicrobial activity against Pss and Pv; *iii*) study of effectiveness of plant crude extracts in greenhouse and field experiments to control kiwifruit bacterial pathogens; *iv*) formulation of active fractions with microencapsulation technique with the aim to improve the extracts' efficacy.

4.2. Materials and Methods

4.2.1. Preparation of natural extracts

During the spring-summer 2008, a total of 21 fresh plant materials were collected from organic fields and purchased from local organic markets and identified (Ceoloni *et al.,* 2006). These plants were distributed in 17 families. The scientific names, sources, and tested parts are detailed in **Tab. 1.** The New Zealander native plants, detailed in **Tab. 2**, were collected in Waikato region, New Zealand, during summer 2009 and identified by the "The Native Trees of New Zealand" handbook (Salmon, 1986).

Plants materials were dried at 45°C for 48 hours and ground in a grinding machine; exposure to sunlight was avoided to prevent the loss of active components. For the extraction, hundred millilitre of a 95% ethanol extraction fluid was mixed with 20 g of powdered plant material (w/v, 1/5). The mixtures were kept for 48 hours in agitation (50 oscillations per minute) at room temperature (22°C), protected from sunlight. This mixture was filtered through a sterile muslin cloth (Shan *et al.,* 2005; Erturk *et al.,* 2006).

The extracted liquid was subjected to rota-evaporatoration (Buchi RE-111 Rotavapor) to remove the ethanol. The water bath temperature was adjusted to 75°C. The semisolid extracts produced were redissolved in water to make a 100 mg/ml (1%) solution and then filtered. The pH was adjusted to 7. The crude extracts were stored at 4°C until tested (Cowan, 1999; Erturk *et al.,* 2006; Bouamama *et al.,* 2008; Li *et al.,* 2008).

The essential oils (EO) of lavander, mint, citronella, tea tree, geranium, manuka, sage, incense, argan and neem (**Tab. 1**) were purchased from Muller & Koster s.p.a. which provides the products with gas chromatography analysis. EO, before used, were diluited in sterile water added with Dimethyl sulfoxid (DMSO) at final concentration of 0.5% to enhance the oil solubility ensuring minimal effects on bacterial growth (Hili, 1997; Lo Cantore *et al.,* 2004; Iacobellis *et al.,* 2005; Sacchetti *et al.,* 2005; Prabuseenivasan *et al.,* 2006).

Lysozyme powder (Sigma), was diluted in water (10 g l^{-1}).

Table 1 – Natural extracts used in the experiments.

Scientific name	Family	Common name	Origin	Part tested
Allium sativum	*Alliaceae*	Garlic	Italy	Bulb
Ficus carica	*Moraceae*	Fig	Italy	Fruit
Actinidia deliciosa	*Actinidiaceae*	Kiwifruit	Italy	Fruit
Punica granatum	*Punicaceae*	Pomegranate	Italy	Fruit peel
Laurus nobilis	*Lauraceae*	Laurel	Italy	Leaves
Olea europea	*Oleaceae*	Olive	Italy	Leaves
Vitis vinifera	*Vitaceae*	Grapevine	Italy	Fruit peel
Lavandula spica	*Lamiaceae*	Lavander	France	Essential oil
Mentha piperita	*Lamiaceae*	Mint	America	Essential oil
Cymbopogon nardus	*Poaceae*	Citronella	Giava	Essential oil
Malaleuca alternifolia	*Myrtaceae*	Tea tree	Australia	Essential oil
Pelargonium zonale	*Geraniaceae*	Geranium	China	Essential oil
Leptospermum scoparium	*Myrtaceae*	Manuka	New Zealand	Essential oil
Salvia officinalis	*Lamiaceae*	Sage	Spain	Essential oil
Boswellia sacra	*Burseraceae*	Incense	Somalia	Essential oil
Argania Spinosa	*Sapotaceae*	Argan	Marocco	Essential oil
Azadirachta indica	*Meliaceae*	Neem	India	Essential oil

Table 2 – New Zealander plant extracts used in the experiments.

Scientific name	Family	Common name	Place	Part tested
Alectryon excelsus	*Sapindaceae*	Tītoki	New Zealand	Leaves
Aristotelia serrata	*Elaeaocarpaceae*	Makomako	New Zealand	Leaves
Arthropodium cirratum	*Laxmanniaceae*	Maikaika	New Zealand	Leaves
Cordyline australis	*Laxmanniaceae*	Cabbage tree	New Zealand	Leaves
Corynocarpus laevigatus	*Corynocarpaceae*	Karaka	New Zealand	Leaves
Griselinia littoralis	*Griseliniaceae*	Kapuka	New Zealand	Leaves
Hebe pauciramosa	*Plantaginaceae*	Hebe	New Zealand	Leaves
Olearia paniculata	*Asteraceae*	Akiraho	New Zealand	Leaves
Phormium tenax	*Hemerocallidaceae*	Harakeke	New Zealand	Leaves
Pittosporum crassifolium	*Pittosporaceae*	Karo	New Zealand	Leaves, seeds
Pittosporum eugenioides	*Pittosporaceae*	Tarata	New Zealand	Leaves, seeds
Podocarpus totara	*Podocarpaceae*	Totara	New Zealand	Leaves
Pseudopanax gilliesii	*Araliaceae*	-	New Zealand	Leaves, buds
Pseudopanax laetus	*Araliaceae*	-	New Zealand	Leaves
Pseudopanax lessonii	*Araliaceae*	Houpara	New Zealand	Leaves

4.2.2. Bacterial pathogens

Psa, Pss e Pv strains utilized in the experiments were isolated from diseased kiwifruit plants in central Italy. The strains were named Psa LT1, Pss VT2 and Pv VT3. They were cryoconserved at -80°C for long-term storage in the phytobacteriological collection of the Department of Plant Protection, Tuscia University of Viterbo, Italy. The subcultures of pathogens were obtained by growing bacteria for 48–72 hours at 27±2 C° on NA (Nutrient agar) agar plates.

4.2.3. Screening of antibacterial activity of natural extracts

The screenings to assess the antimicrobial activity of natural extracts were carried out by drop diffusion test (Klement *et al.,* 1990) and broth dilution test (Hammer *et al.,* 1999; Tassou *et al.,* 2000; Burt, 2004).

For paper disk diffusion technique strains Psa LT1, Pss VT2 and Pv VT3 were utilised. Natural extracts (100 mg/ml concentration) and essential oils (1% concentration) aliquot of 15 µl were transferred onto sterile filter paper (Whatman No.1) disks (6 mm in diameter) (Oxoid). Disks were placed onto NA-agar plates (three paper disks per plate) previously inoculated with 100 µl bacterial suspension (ca. 10^6 CFU/ml) and incubated at 27 ± 2°C for 48 h. As control sterile water and streptomycin (100 µl ml^{-1}) were used (Klement *et al.,* 1990; Iacobellis *et al.,* 2005).

After incubation at 27±2 °C for 48 h, inhibition zones were observed through a stereomicroscope and their diameter was measured in mm from each disc border (Klement *et al.,* 1990). The *in vitro* spot tests were repeated twice with three replicates each.

New Zealander native plant extracts experiment, carried out in Plant & Food Research at Ruakura Research Centre (Hamilton, New Zealand) during 2009 when Psa was not present in New Zealand, concerned only Pss and Pv, as the laboratory did not have the authorization from MAF Biosecurity New Zealand to work with Psa which is considered in New Zealand an unwanted organism.

For broth test, NB-broth (Nutrient Broth) was dispensed (10 ml final volume) into sterile sealed glass containers. The strains employed were Psa LT1, Pss VT2

and Pv VT3, while the extracts were garlic, fig, pomegranate, bay tree, Lysozyme, grapevine, lavander, mint, tea tree, sage, and the mixtures lavander-garlic and lavander-pomegranate, at the same concentration utilized in the spot test. The glass containers were then inoculated with 100 µl of an overnight culture of Psa LT1, Pss VT2 and Pv VT3 and natural extracts and later shaken. As control, sterile water and streptomycin (100 µl ml^{-1}) were used. Bacterial growth was monitored picking up 100 µl of broth after 3, 6, 12 and 24 hours incubation at 27°C and spreading on NA-agar plates serial dilution of broth of each tubes. After a 48 h incubation at 27°C the colonies growth on plates were counted and estimated the CFU/ml (Tassou *et al.,* 2000). Broth test were repeated twice with two replicates each.

4.2.4. Greenhouse tests with crude extracts

Potted 2 years-old kiwifruit plants cv. Hayward, were used for *in vivo* tests. The plants were watered daily by drip-irrigation and a mineral solution was distributed weekly into the pots to maintain optimum nutritional conditions. The experiments were carried out in a greenhouse where temperature was maintained at 25±2 °C during the day and 15±2 °C by night; the relative humidity was maintained between 70 and 80%, during the whole experiment, by using automatic cooling. Heating and drip-irrigation data were recorded by logger at 60-min intervals.

The extracts of garlic, pomegranate, fig and lavender EO were used at the same concentration of *in vitro* tests (1%). 24-h-old bacterial cultures of Psa LT2, Pss VT2 and Pv VT3 were suspended in SDW (sterile distillate water) and adjusted to1 $x10^8$ CFU/ml using a spectrophotometer at 600 nm.

Kiwifruit plants were sprayed with each natural extract until leaves were homogeneously wet; after 24 h, the bacterial suspensions were sprayed on plants by a CO^2-pressurized handheld sprayer equipped with a large orifice (diameter 1.4 mm, Tee-Jet 8004) nozzle operating at a pressure of 28 g cm^2 to produce fine spray droplets. In the greenhouse, 2 h before and 2 h after bacterial contamination, the relative humidity was maintained at 90% to favour stomata opening.

For each combination (bacterial pathogen/vegetal extract) 3 kiwifruit plants were used, subjected to the following treatments: 3 contaminated plants treated with vegetal extract; 3 contaminated, non-treated plants as positive control; 3 contaminated plants treated with copper oxychloride (280 g l^{-1}) as negative control (unlike the *in vitro* experiments, copper was used as positive control instead of antibiotics which are forbidden in agriculture in EU). Contaminated kiwifruit plants were daily monitored over a period of 15 days. After 1, 7 and 15 days 3 leaves for each plant were collected and washed separately in 10 ml of $MgSO^4$ buffer using stomacher equipment (Seward, model 400 Circulator). 100 µl of serial ten-fold dilution of the washing buffer were spread on NA-agar plates and incubated 48 h at 27 °C and the single colony developed were counted to obtain the CFU/cm^2 of leaves (Donegan *et al.,* 1991).

4.2.5. Field test with crude extracts

The experiments were carried out in a kiwifruit orchard sited in Latina province, 8 years-old, cv. Hayward. The plants were fertilized and watered daily by drip-irrigation system. The orchard was naturally infested by Pss and Pv.

For each combination (bacterial pathogen/vegetal extract) plot of 3 kiwifruit plants were used, repeated twice, non-treated plants as positive control; 3 contaminated plants treated with copper oxychloride (280 g l^{-1}) as negative control.

In this experiment mixtures of garlic + pomegranate (0.5% + 0.5%) and fig + lavender (0.5% + 0.5%) were used. Kiwifruit plants were sprayed with each natural extract until leaves were homogeneously wet.

After 1, 7 and 15 days 10 leaves for each plot were collected and washed separately in 10 ml of $MgSO_4$ buffer using stomacher equipment. 100 µl of serial ten-fold dilutions of the washing buffer were spread on NS-agar (Nutrient sucrose) plates supplemented with cycloexamide (100 µg/ml; Sigma) to inhibit fungal growth and incubated 48 h at 27 °C and the single colonies developed were counted to obtain the CFU/cm^2 of leaf. Recognizing of Pss and Pv colonies growth on media was morphological; after 48 hours of incubation, Pss colonies measured

3-4 mm in diameter and were convex, circular, translucent, creamy white; while Pv colonies were 2-3 mm in diameter, irregular, flat and yellow. Both showed blue luminescence under UV light on KB-agar (King B) plates.

4.2.6. Quantification of active principle

Polyphenols in *F. carica.* The total amount of polyphenols (milligrams of gallic acid equivalents -GAE- per 100 g fresh weight) in fig fruit extracts was determined by using the Folin–Ciocalteu assay (Jayaprakasha *et al.,* 2001; Marinova *et al.,* 2005). For each replicate, 1 ml of extract was added to 9 ml of sterile distilledwater (SDW), and 1 ml of gallic acid standard solution (20, 40, 60, 80 and 100 mg l 1) was similarly added to 9 ml of SDW; as control, 1 ml of SDW was used. The Folin–Ciocalteu's phenol reagent (1 ml) was added to the extract solution and to each standard solution. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to each solution. Then, 4 ml of SDW was added to each one to reach the final volume of 25 ml. After incubation for 90 min at room temperature, the absorbance was determined at 750 nm with a UV spectrophotometer. All samples were analysed in duplicate.

Allicin in *A. sativum.* Garlic clove samples (0.1 g each) were dissolved in 10 ml of SDW and incubated at room temperature for 30 min. Samples were then centrifuged at 8000 *g* for 5 min at room temperature and the supernatants filtered (diameter 0.22 mm, Fisher Scientific). The quantitative determination of allicin was obtained by chromatographic analysis using a high performance chromatograph liquid, HPLC, LC-10ATvp connected to a UV-VIS detector SPD-10Avp (Shimadzu Co, Kyoto, Japan). For molecular separation, a column nucleosil 100-5 C18 with a size 250 4 mm (Macherey-Nagel GmbH & Co, KG) was used. Elution was carried out by isocratic solvent applications of acetonitrile:H2O (30:70) (Rosen *et al.,* 2001) at a flow rate of 1 ml min 1, and then allicin was detected at 214 nm. For quantification of allicin-containing extracts, the calibration

curve was obtained with allicin (LTK Laboratories, Inc.) with more than 99.5% purity at different concentrations (1, 0.5, 0.25, 0.125 and 0.063 mg ml^{-1}) (Fujisawa *et al.,* 2008).

Gallic acid in *P. granatum* peel extract. Ethanol extract of pomegranate peel was prepared as explained in 4.2.1. paragraph. The quantitative determination of gallic acid was obtained by chromatographic analysis using a high performance chromatograph liquid, HPLC, LC-10ATvp connected to a UV-VIS detector SPD-10Avp (Shimadzu Co, Kyoto, Japan). For molecular separation, a column nucleosil 100-5 C18 with a size 250 4 mm (Macherey-Nagel GmbH & Co, KG) was used. Elution was carried out by isocratic solvent applications of acetonitrile: H2O (30:70) (Rosen *et al.,* 2001) at a flow rate of 1 ml min 1, and then gallic acid was detected at 214 nm. For quantification of gallic acid-containing extracts, the calibration curve was obtained with gallic acid (LTK Laboratories, Inc.) with more than 99.5% purity at different concentrations (1, 0.5, 0.25, 0.125 and 0.063 mg ml^{-1}) (Fujisawa *et al.,* 2008).

4.2.7. Microencapsulation of gallic and ellagic acid

For this work, a synthetic polymer was chosen: Eudragit RS 100 (IUPAC name polymethacrylate, methyl methacrylate, trimethylammonium ethyl chloride methacrylate), belonging to the family of esters methacrylate, or rather biocompatible synthetic polymers (Cortesi *et al.,* 2002; Esposito *et al.,* 2002).

The microparticles was produced by a physical method: the spray-dryer. Spray-drying is a particular technique that allows to obtain microparticles or microcapsules from the mere contact of a fluid containing the polymer and the active principle to encompass solution, suspension or emulsion, with a hot blast provoking the instantaneous evaporation of both the solvent and the polymer. In detail, the fluid is atomized in fine drops with a high specific surface, to which the hot blast gives latent heat of evaporation, resulting in a quick drying and the consequent quick cooling of the drying air (Adachi *et al.,* 2004; Glenn *et al.,* 2010).

The dimensions, the shape and the space distribution of the generated

microparticles, are determined through SEM - Scanning Electron Microscope (**Fig. 2**). The quantity and the concentration of the microencapsulated active principle, have been obtained by comparing areas from the samples' peaks to the ones gathered from a gold standard analyzed in the same operating conditions (Cortesi *et al.,* 2002).

The process led to the production of three formulations: the first one was gallic acid-based for the 4%, the second was ellagic acid-based for the 4% and in the end a mixture of gallic acid (2%) and ellagic acid (2%). Gallic acid and Ellagic acid used in this research were purchased from Sigma Company.

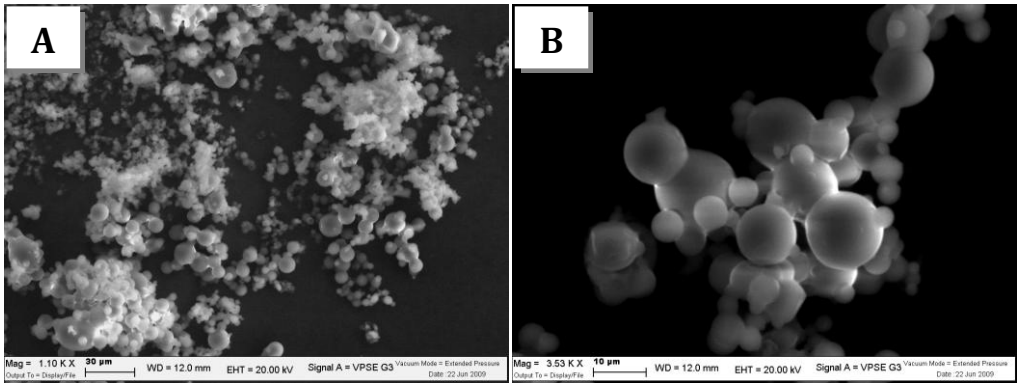

Figure 2: Scanning Electron Microscope images of gallic acid formulation (A) and its detail (B).

4.2.8. Stem test with microencapsulated

Kiwifruit stem test was carried out conserving at 4°C kiwifruit brunch collected at fall stage. After at least two months (kiwifruit dormancy period needed) the twigs were took out from the storage and cut in 20 cm long shots. The twigs were dipped in water supplemented with mineral solution and kept in a climatic chamber. Only one bud per shot was left to develope. When the new shots were 5-6 leaves old they were used for the experiment.

In this experiments gallic acid, ellagic acid and the mixture of them microencapsulated were tested. Kiwifruit shots were sprayed with each microencapsulated at a concentration of 10 g l^{-1} until leaves were homogeneously wet; after 24 h plants were contaminated with Psa LT1, Pss VT2 and Pv VT3

bacterial suspensions (1x10^8 CFU/ml) by spraying plants with a CO^2-pressurized handheld sprayer equipped with a large orifice (diameter 1.4 mm, Tee-Jet 8004) nozzle operating at a pressure of 28 g cm^2 to produce fine spray droplets. In climatic chamber, 2 h before and 2 h after bacterial inoculation, the relative humidity was maintained at 90% to favour stomata opening.

For each combination (bacterial pathogen/microencapsulated) 5 kiwifruit shots were used, while as control sterile water and copper oxychloride (280 g l^{-1}) were used. Contaminated kiwifruit plants were daily monitored over a period of 5 days. After 1 and 5 days, one leaf each branch was collected and washed separately in 10 ml of $MgSO^4$ buffer using stomacher equipment 100 µl of serial ten-fold dilutions of the washing buffer were spread on NA-agar plates and incubated 48 h at 27 °C and the single colony developed were counted to obtain the Psa, Pss and Pv CFU/cm^2 per leaves.

4.2.9. Greenhouse test with microencapsulated

Greenhouse test with microencapsulated was carried out as explained in 4.2.4. paragraph.

Potted 2 years-old kiwifruit plants cv. Hayward, were used. The three microencapsulated (gallic acid, ellagic acid and their mixture) were used at a concentration of 10 g l^{-1}. Bacterial strains were the same as those used in *in vitro* tests (Psa LT1, Pss VT2 and Pv VT3). Bacterial suspensions in sterile distilled water were prepared from 24-h-old bacterial cultures and adjusted to 10^8 CFU/ml using a spectrophotometer at 600 nm.

Kiwifruit plants were sprayed with each microencapsulated until leaves were homogeneously wet; after 24 h bacterial suspensions were sprayed on plants by a CO^2-pressurized handheld sprayer equipped with a large orifice (diameter 1.4 mm, Tee-Jet 8004) nozzle operating at a pressure of 28 g cm^2 to produce fine spray droplets. In the greenhouse, 2 h before and 2 h after bacterial inoculation, the relative humidity was maintained up 90% to favour stomata opening.

For each combination (bacterial pathogen/microencapsulated) 3 kiwifruit plants were used, subjected to the following treatments: 3 inoculated plants

treated with microencapsulated; 3 inoculated, non-treated plants as negative control; 3 inoculated plants treated with copper oxychloride (280 g l^{-1}) as positive control. Inoculated kiwifruit plants were daily monitored for 15 days. After 1, 7 and 15 days 3 leaves for each plant were collected and washed separately in 10 ml of $MgSO^4$ buffer using stomacher equipment (Seward, model 400 Circulator) 100 µl of serial ten-fold dilution of the washing buffer were plated on NA-agar plates and incubated 48 h at 27 °C and each colony developed was counted to obtain the CFU/cm^2 per leaves.

4.2.10. Field test with microencapsulated

The experiments were carried out in a kiwifruit orchard sited in Latina province, 8 years-old, cv. Hayward. The plants were fertilized and watered daily by drip-irrigation system. The orchard is naturally infested by Psa Pss and Pv.

The three microencapsulated used were the same described in 4.2.9. paragraph. Kiwifruit plants were sprayed with each microencapsulated formulation at a concentration of 10 g l^{-1} until leaves were homogeneously wet.

For each combination (bacterial pathogen/microencapsulated) plot of 3 kiwifruit plants were used, repeated twice. Non-treated plants and and plants treated with copper oxychloride (280 g l^{-1}) were used as positive and negative control.

After 1, 7 and 15 days, 10 leaves each plot were collected and washed separately in 10 ml of $MgSO_4$ buffer, using stomacher equipment; 100 µl of serial ten-fold dilutions of the washing buffer were spread on NS-agar (Nutrien sucrose) supplemented with cycloexamide (100 µg/ml; Sigma) to inhibit fungal growth and KBC-agar (King B modified; selective medium for Psa) plates and incubated 48 h at 27 °C and the single colonies developed were counted to obtain the CFU/cm^2 per leaf.

Psa colonies were counted on KBC-agar plates, whereas Pss and Pv colonies, growth on NS-agar plates, were morphologically recognized; after a 48 hours of incubation Pss colonies are 3-4 mm, white, and levan positive while Pv colonies are 2-3 mm, yellow and levan negative.

4.2.11. Statistical analysis

By drop diffusion method three replicates were developed and the mean and standard error were calculated.

By broth dilution method the mean viable counted populations of the triplicate samples was calculated.

In *in vivo* tests, population sizes were expressed per square centimetre of leaf, being estimated using "APS assess 2.0" software to determine leaf areas. Data were statistically analysed using GraphPad Prism 4 software by analysis of variance (ANOVA) and the significance of the treatments were determined using Tukey's HSD test ($p > 0.05$) (Steel *et al.,* 1997).

4.3. Results

4.3.1. Drop diffusion test

The results of 36 plant oils and extracts obtained by the agar dilution method are shown in **Tabb. 3-4**. Garlic, fig, pomegranate, laurel, olive, grapevine, lavander, mint, citronella, tea tree and sage inhibited all organisms at 1.0% (w/v). Likewise, lysozyme was effective towards all microrganisms. However garlic, fig, pomegranate and lavander showed the most significant antibacterial activity as evidenced by a zone of inhibition, larger than 20 mm.

Kiwifruit, geranium, manuka, incense, argan and neem failed to inhibit any organism at the same concentration. New Zealander native plant extracts did not show any significant inhibition against all the tested bacteria.

No significantly difference could be observed in terms of susceptibility between Psa, Pss and Pv.

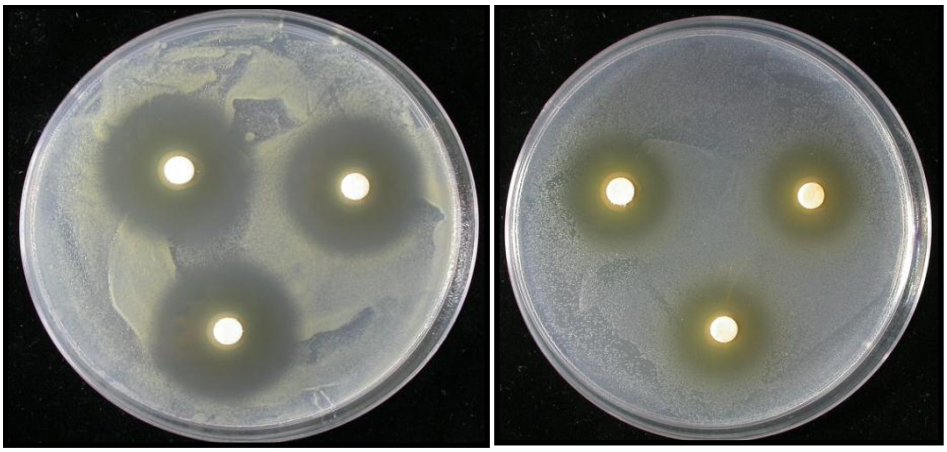

Figure 2: Inhibition zones of garlic extract on *Pseudomonas viridiflava* VT3 (left) and lavender essential oil on *P. s.* pv. *syringae* VT2.

4.3.2. Broth dilution test

Natural extracts added in NB were able to reduce the total viable counts of Psa, Pss and Pv. In particular, after 24 hours, garlic, pomegranate, lavender and the mixtures lavender+garlic and lavender+pomegranate extracts caused a reduction of 6-7 logs CFU compared to NB alone (**Tab. 5**).

Analyzing the bacterial dynamics growth within 24 hours, the mixtures lavender+garlic and lavender+pomegranate, garlic, lavender and pomegranate extracts caused a rapidly devrement of viable populations of Psa, Pss and Pv, while adding bay tree, lysozyme, tea tree and mint extracts bacterial populations were relatively unvarying. None activity was observed by fig, grapevine and sage extracts (**Fig. 3**).

Table 3 – Antimicrobial activity of natural extracts to *Pseudomonas syringae* pv. *actinidiae* (Psa) strain LT2, *P. s.* pv. *syringae* (Pss) strain VT2 and *P. viridiflava* (Pv) strain VT3.

Natural extracts	Inhibition (mm)[1]		
	Psa	Pss	Pv
Allium sativum	24.2 ± 1.3 [a]	23.3 ± 1.9 [a]	25.0 ± 0.9 [a]
Ficus carica	16.8 ± 1.2 [a]	15.3 ± 1.4 [b]	16.5 ± 1.2 [a]
Actinidia deliciosa	0	3.1 ± 0.2 [c]	0
Punica granatum	22.3 ± 2.7 [a]	21.6 ± 1.7 [a]	21.7 ± 1.6 [a]
Laurus nobilis	15.2 ± 1.0 [a]	12.8 ± 0.9 [a]	15.2 ± 1.1 [a]
Olea europea	3.2 ± 0.3 [c]	4.0 ± 0.2 [c]	3.6 ± 0.2 [c]
Lysozyme	14.5 ± 1.1 [b]	13.6 ± 1.0 [b]	15.1 ± 0.9 [b]
Vinis vinifera	11.8 ± 1.4 [b]	10.0 ± 0.8 [b]	11.3 ± 1.2 [b]
Lavanda hybrida	24.2 ± 1.3 [a]	21.8 ± 1.6 [a]	22.3 ± 1.5 [a]
Mentha piperita	16.5 ± 0.9 [b]	14.9 ± 0.8 [b]	12.7 ± 0.7 [b]
Cymbopogon nardus	4.4 ± 0.3 [c]	5.4 ± 0.4 [c]	4.8 ± 0.3 [c]
Malaleuca alternifolia	11.7 ± 1.0 [b]	11.3 ± 0.6 [b]	10.9 ± 0.7 [b]
Pelargonium zonale	0	0	0
Leptospermum scoparium	0	0	0
Salvia officinalis	8.1 ± 0.7 [c]	11.1 ± 0.6 [b]	10.0 ± 0.7 [b]
Boswellia sacra	0	0	0
Argania spinosa	0	0	0
Azadiracta indica	0	0	0
Sterile water	0	0	0
Streptomycin (100 µg/ml)	28.3 ± 1.2	27.8 ± 1.3	28.1 ± 1.1

0 = no inhibition

[1]Values expressed are mean ± S.D. of eight experiments. Means within column followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's HSD test.

Table 4 – New Zealander plant extracts with antimicrobial activity to *Pseudomonas syringae* pv. *syringae* (Pss) strain VT2 and *P. viridiflava* (Pv) strain VT3.

Natural extracts	Inhibition (mm)[1]	
	Pss	**Pv**
Alectryon excelsus	0	0
Aristotelia serrata	0	0
Arthropodium cirratum	0	0
Cordyline australis	0	0
Corynocarpus laevigatus	0	0
Griselinia littoralis	0	0
Hebe pauciramosa	2.5 ± 0.3	3.5 ± 0.4
Olearia paniculata	0	0
Phormium tenax	0	0
Pittosporum crassifolium,	0	0
Pittosporum eugenioides	0	0
Podocarpus totara	0	0
Pseudopanax gilliesii	0	0
Pseudopanax laetus	0	0
Pseudopanax lessonii	0	0
Alectryon excelsus	0	0
Aristotelia serrata	0	0
Arthropodium cirratum	0	0
Sterile water	0	0
Streptomycin (100 µg/ml)	27.8 ± 1.3	28.1 ± 1.1

0 = no inhibition

[1]Values expressed are mean ± S.D. of eight experiments

Table 5 – Mean bacteria viable growth on nutrient broth of *Pseudomonas syringae* pv. *actinidiae* (Psa) strain LT2, *P. s.* pv. *syringae* (Pss) strain VT2 and *P. viridiflava* (Pv) strain VT3, after 24 h incubation at 27°C in presence of different natural extracts.

Natural extracts	Viable count (CFU/ml)		
	Psa	**Pss**	**Pv**
Allium sativum	6.3×10^3	$2{,}4 \times 10^1$	$2{,}4 \times 10^1$
Ficus carica	6.9×10^7	$1{,}3 \times 10^7$	$7{,}1 \times 10^7$
Punica granatum	2.8×10^4	4.7×10^4	1.3×10^3
Laurus nobilis	3.9×10^6	4.0×10^4	2.8×10^6
Lysozyme	1.9×10^2	1.9×10^2	$1{,}0 \times 10^4$
Vinis vinifera	6.7×10^8	1.7×10^7	3.6×10^7
Lavandula hybrid	3.7×10^1	3.7×10^1	1.0×10^4
Mentha piperita	9.1×10^4	8.7×10^4	4.6×10^4
Malaleuca alternifolia	5.6×10^2	3.7×10^2	3.7×10^2
Salvia officinalis	2.4×10^6	2.8×10^6	1.0×10^6
L. hybrida + A. Sativum	1×10^1	1×10^1	1×10^1
L. hybrida + P. granatum	1×10^1	1×10^1	1×10^1
Streptomycin	0	0	0
NB-broth alone	1.3×10^8	7.5×10^7	1.4×10^8

The values shown are the mean of four experiments. ($p \leq 0.05$, Tukey's HSD test)

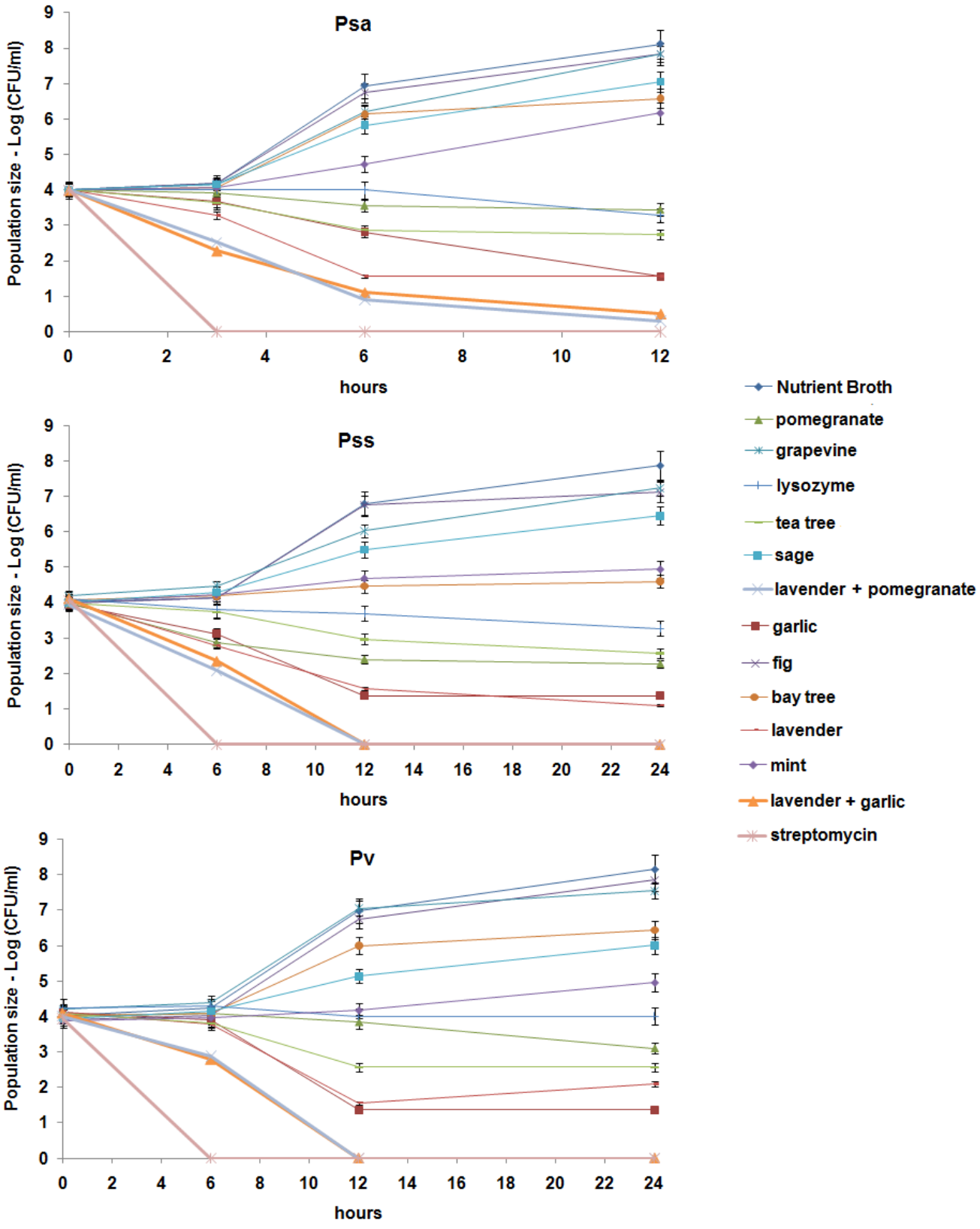

Figure 3 – Viable count of *Pseudomonas syringae* pv. *actinidiae* (Psa), *Pseudomonas syringae* pv. *syringae* (Pss) and *Pseudomonas viridiflava* (Pv) in nutrient broth added with different Natural extracts. Bars indicate standard errors of the mean.

4.3.3. Greenhouse test with crude extracts

The antimicrobial effects of the substances contained in some natural extracts were confirmed in *in vivo* tests on plants contaminated by suspension of the strains Psa, Pss and Pv (**Fig. 4**). Garlic, pomegranate and lavender extracts, when compared to the untreated control, reduced Psa, Pss and Pv populations of to 1 log unit. After 7 and 14 days, bacterial population in all treatment were raising.

Garlic was as effective as copper and significantly more effective than pomegranate and lavender extracts on Pss, while on Psa and Pv there was not difference between different treatments and none was effectiveness as copper.

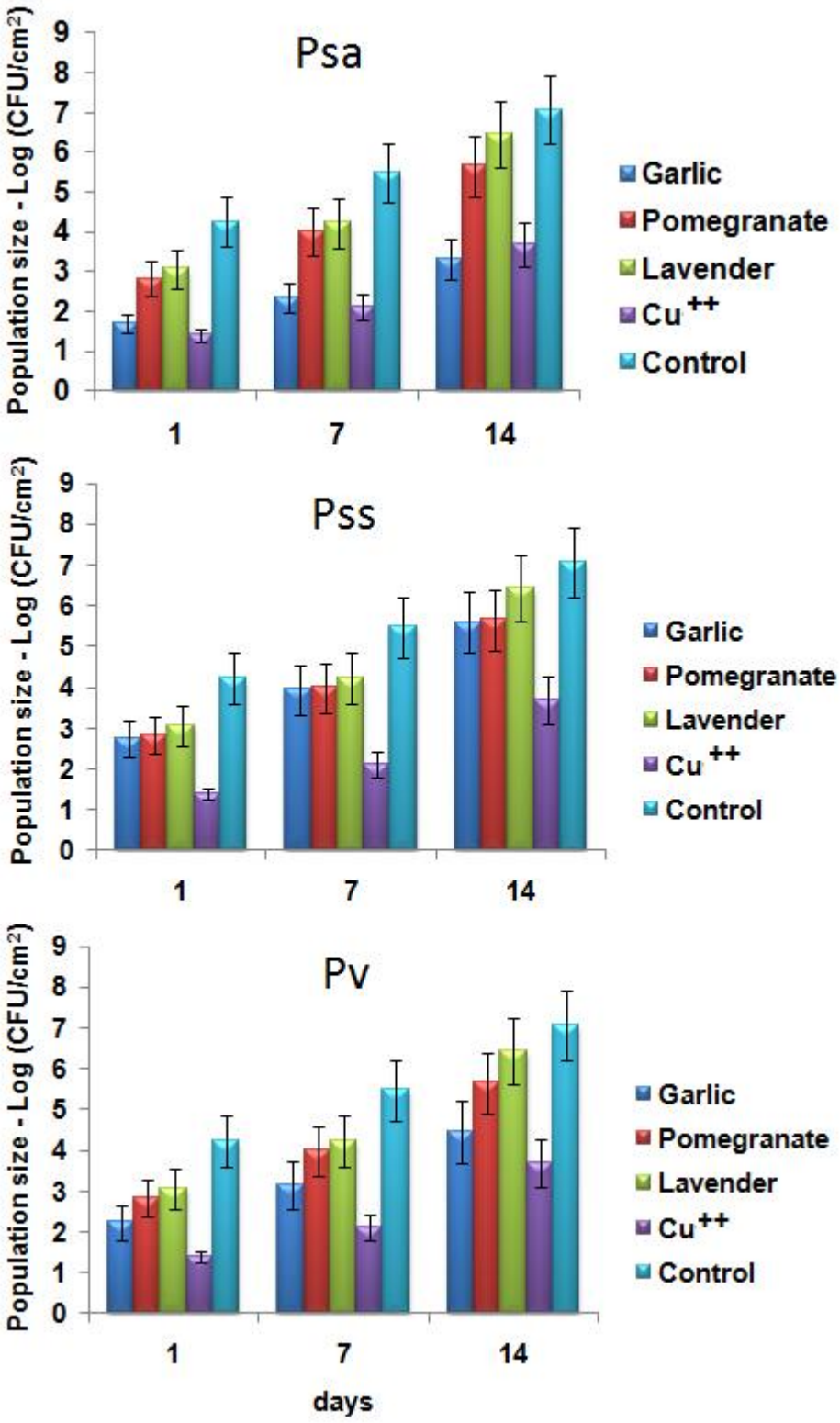

Figure 4 – Dynamics epiphytic population on kiwifruit leaves of *Pseudomonas syringae* pv. *actinidiae* (Psa), *P. s.* pv. *syringae* (Pss) and *P. viridiflava* (Pv) on kiwifruit leaves treated with natural crude extracts in greenhouse experiments. Bars indicate standard errors of the mean.

4.3.4. Field test with crude extracts

Field tests by using crude natural extracts as lavender, garlic and pomegranate used in combination showed their ability to reduce Pss and Pv epiphytic populations 1 day after the treatment (**Fig. 5**). The efficacy of the treatment with plant extracts decreased after 7 and 14 days.

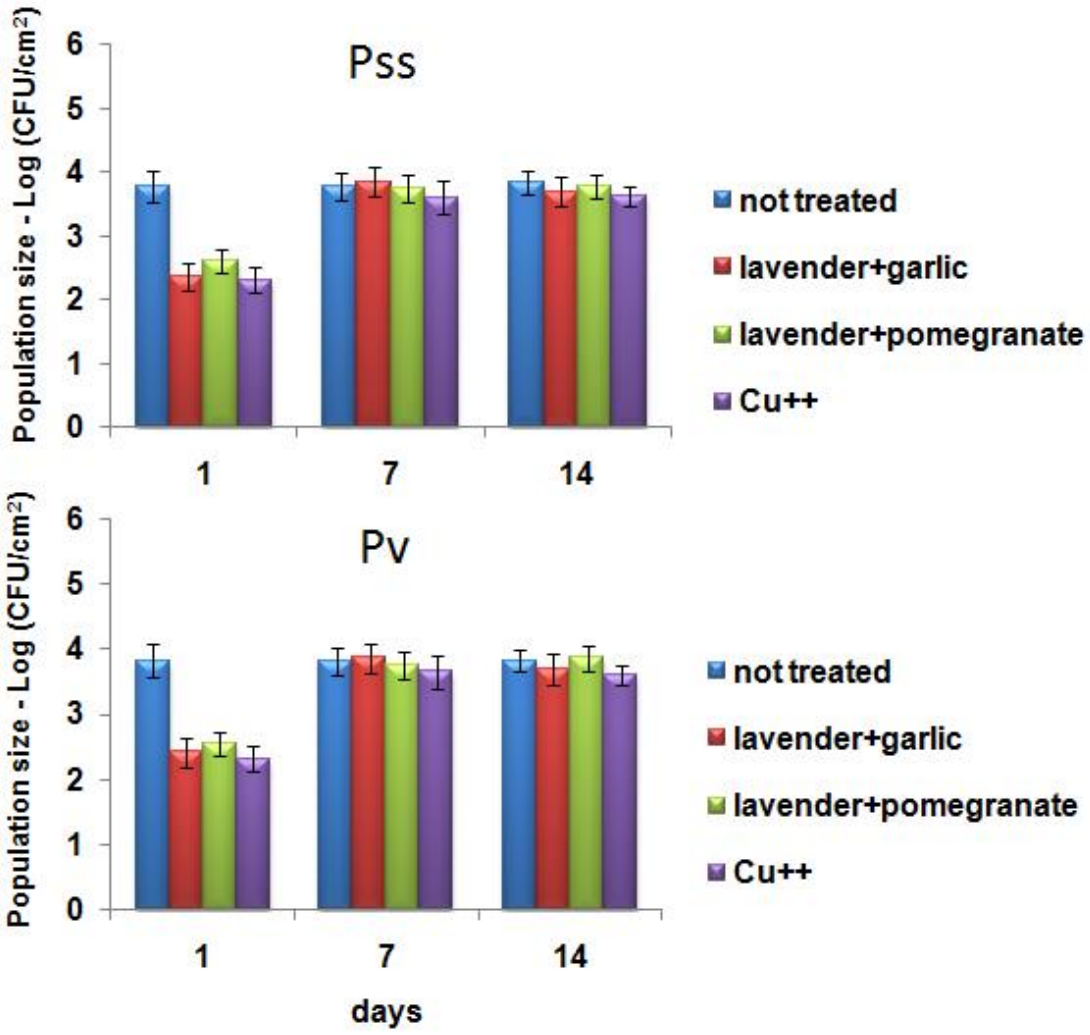

Figure 5 – Dynamics epiphytic populations on kiwifruit leaves of *Pseudomonas syringae* pv. *syringae* (Pss) and *P. viridiflava* (Pv) treated with natural crude extracts in field experiments. Bars indicate standard errors of the mean.

4.3.5. Quantification of active principle

Polyphenols in *F. carica.* The polyphenol content in fig fruit extracts was 72.8 ± 1.1 mg GAE 100 g^{-1}.

Allicin in *A. sativum.* Allicin from garlic cloves was detected as a single peak (retention time 7.5 min), its optical absorbance measured at 214 nm (**Fig. 6**), and its concentration resulted 0.032 mg ml^{-1}.

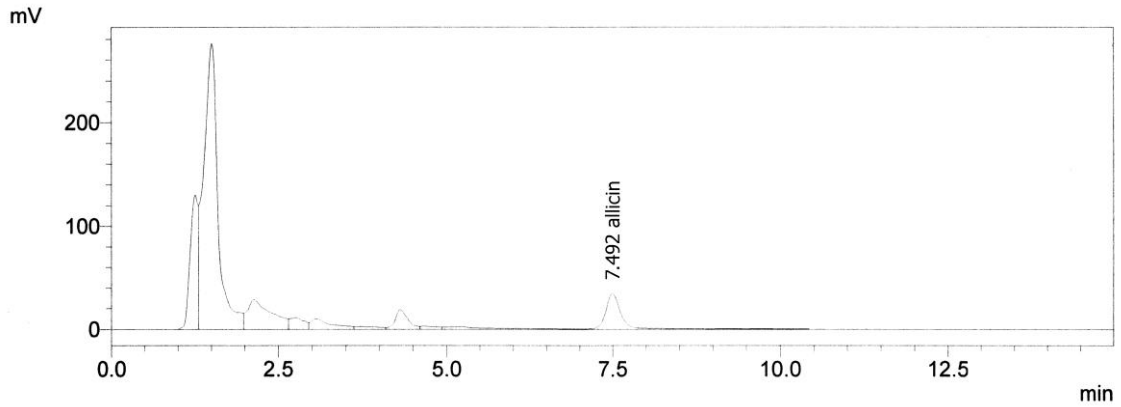

Figure 6 - HPLC chromatogram of *Allium sativum* bulb extract.

Gallic acid *P. granatum*. Gallic acid from pomegranate peel extract was detected as a single peak (retention time 11.0 min)(**Fig. 7**), and its concentration resulted 0.01292 mg ml^{-1}.

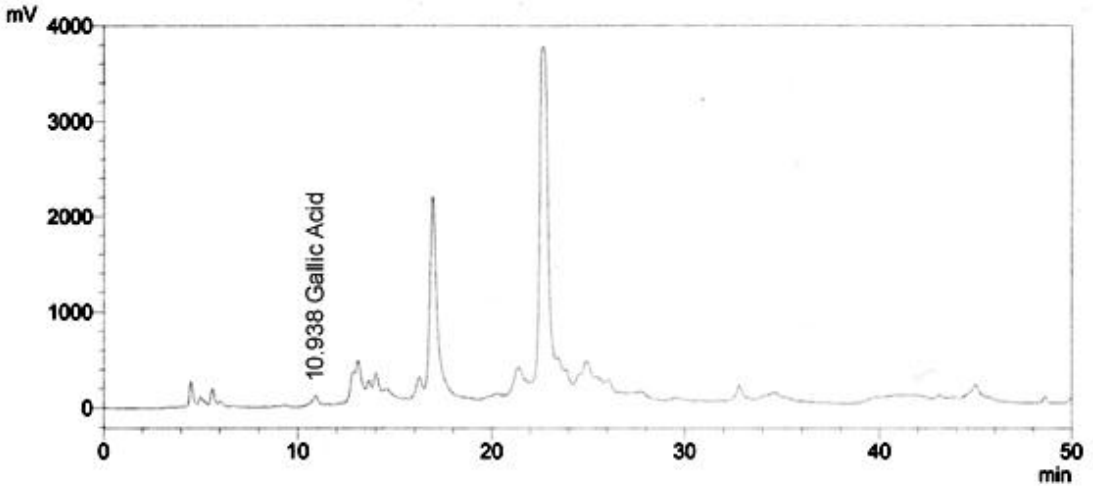

Figure 7 - HPLC chromatogram of *Punica granatum* peel extract.

Composition of *Lavandula spica* EO. The supplier of lavender EO (Muller & Koster spa), provide the product with gas chromatography analysis. The main components are linalool (42.10%), linalyl acetate (14.69%), camphor (7.90%), terpinen-4-ol (3.79%) and 3,7-dimethyl-2,6-octadienyl acetate (3.08%).

4.3.6. Stem test with microencapsulated

Bacteria population of Psa, Pss and Pv was similar, lower than 1 x 10^4 CFU/cm^2, 1 day after sprayed and developed after 5 days reaching values close to than 1 x 10^6 CFU/cm^2. The antimicrobial effects of microencapsulated were higher after 5 days, when populations were lower than 1 x 10^4 CFU/cm^2. No significant differences were observed between different microencapsulated (**Fig. 8**).

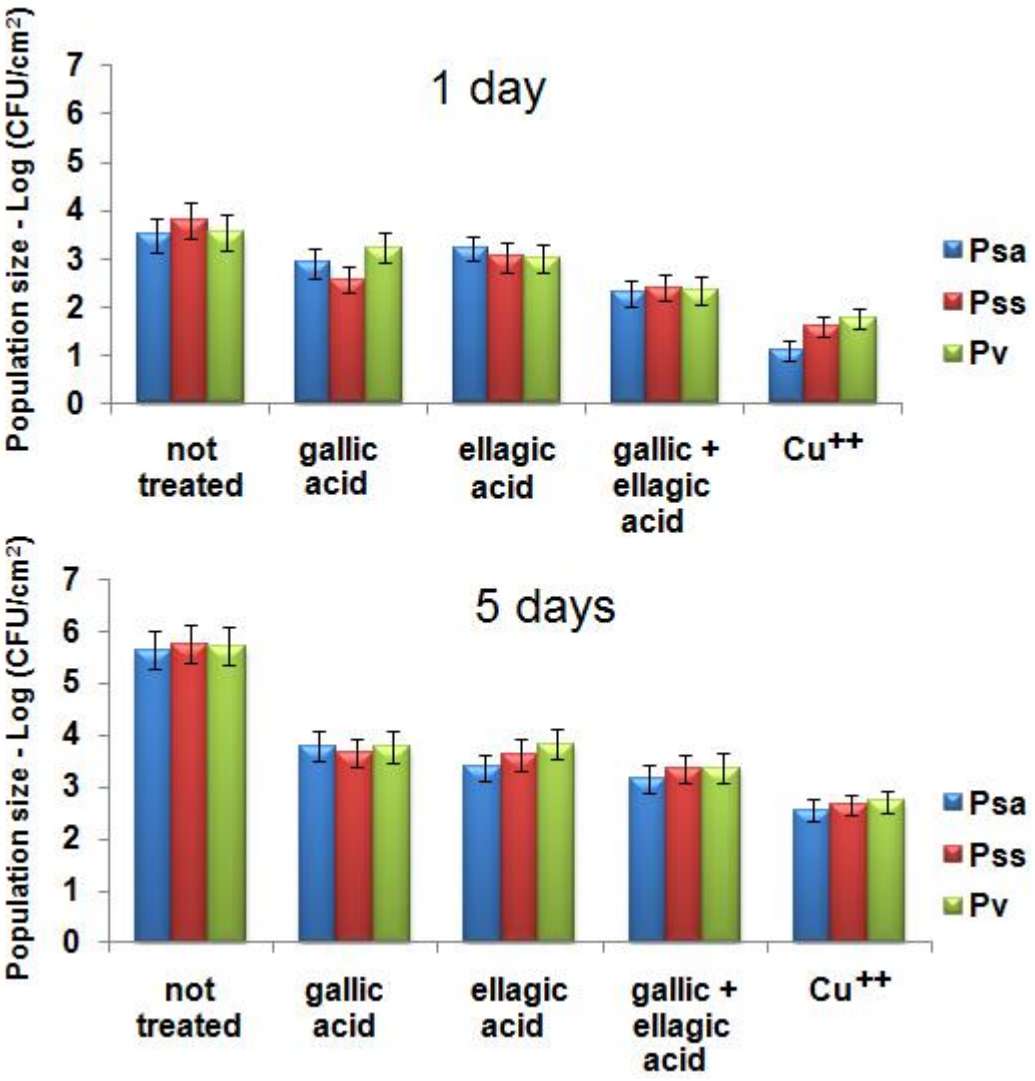

Figure 8 - Dynamics epiphytic population on kiwifruit leaves of *Pseudomonas syringae* pv. *actinidiae* (Psa), *P. s.* pv. *syringae* (Pss) and *P. viridiflava* (Pv) treated with natural compounds microencapsulated in stem experiment. Bars indicate standard errors of the mean.

4.3.7. Greenhouse tests with microencapsulated

Population sizes of Psa, Pss and Pv were lower shortly after inoculation and increased along the time. Population sizes of the introduced strains ranged from 10^3 to 10^5 CFU/cm^2 on kiwifruit leaves during the 15 days after inoculation (**Fig. 9**).

On kiwifruit plants sprayed with microencapsulated, population size of Psa, Pss and Pv either remained constant or slowly increased along the time. However, the population size of the treated plants decreased more than 100-fold (two logs unit) over the 15-day sampling period. Mean bacterial population sizes of kiwifruit plants treated with different microencapsulated did not vary substantially among plant species at a given sample time.

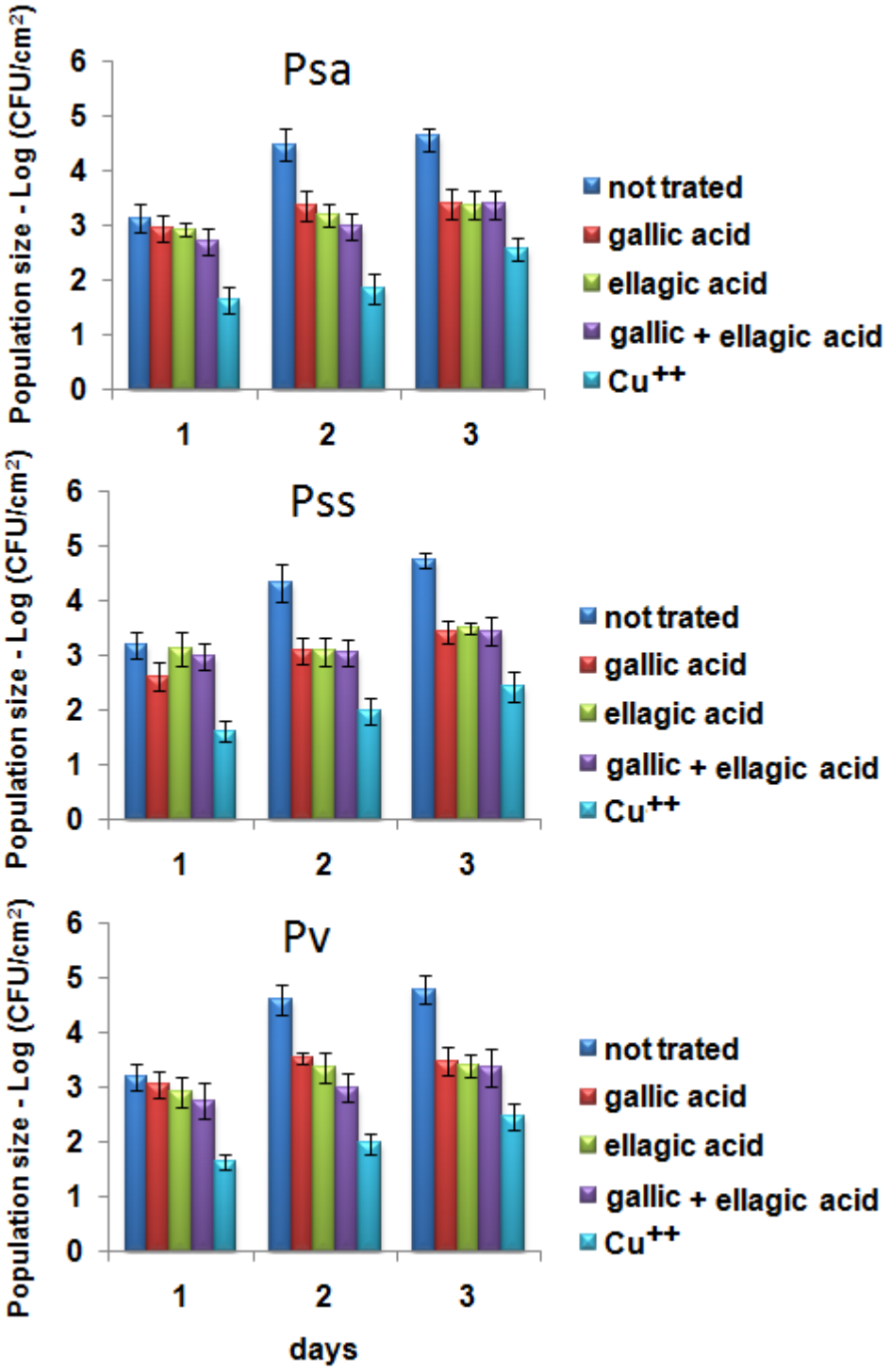

Figure 9 - Dynamics epiphytic population on kiwifruit leaves of *Pseudomonas syringae* pv. *actinidiae* (Psa), *P. s.* pv. *syringae* (Pss) and *P. viridiflava* (Pv) treated with microencapsulated in greenhouse experiment. Bars indicate standard errors of the mean.

4.3.8. Field test with microencapsulated

In field test, with Psa, Pss and Pv naturally established on kiwifruit plants, bacterial population size remained constant over the 15-day experiment.

The mixture gallic+ellagic microencapsulated, when compared to the untreated control, reduced Pss and Pv populations of 1 log unit (**Fig. 10**).

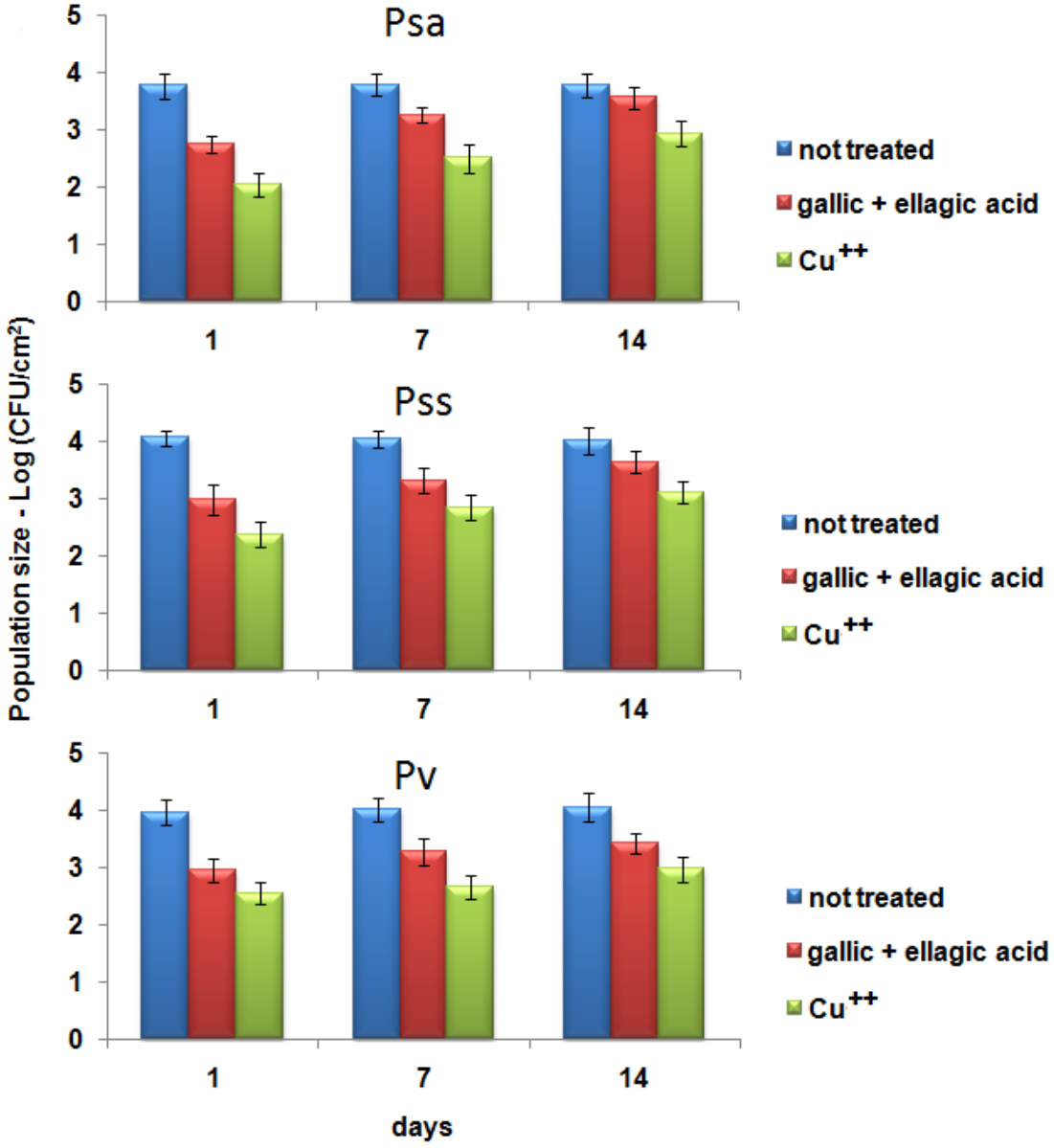

Figure 10 – Dynamics epiphytic population on kiwifruit leaves of *Pseudomonas syringae* pv. *actinidiae* (Psa), *P. s.* pv. *syringae* (Pss) and *P. viridiflava* (Pv) treated with microencapsulated in field experiments. Bars indicate standard errors of the mean.

4.4. Discussion

Bacterial diseases of kiwifruit are widespread and extremely harmful, especially in warm-humid climates (Balestra *et al.,* 2009). Copper compounds were used mainly to reduce losses foliar bacterial diseases, but frequent use of copper represent a problem due to their phytotoxic effect, soil accumulation and establishment of copper-resistant pathogen strains. Besides a EU regulation (Reg. EU n. 473/2002) restricts the use of copper in organic agriculture (Varvaro *et al.,* 2001). Thereby there is a great interest to control important diseases with natural compounds.

The antimicrobial activity of essential oils and of plant extracts used in this research has been already proved against both human and plant pathogens (Harris *et al.,* 2001; Prashanth *et al.,* 2001; Cavanagh & Wilkinson, 2002; Reddy *et al.,* 2007; Slusarenko *et al.,* 2008) but never to control kiwifruit bacterial pathogens, while extracts coming from New Zealander native plant had never been investigated before against bacterial plant pathogens.

In vitro tests provided useful information: in particular garlic, pomegranate and lavender extracts had a remarkable antibacterial potential against Psa, Pss and Pv in drop diffusion test and their efficiency was not significantly different. Fig mint, bay tree and lysozyme had a less significant antibacterial potential. The other extracts, including all New Zealander native plant extracts, did not show any antibacterial potential against kiwifruit bacterial pathogens.

Broth dilution test partially confirmed the previous results: garlic, pomegranate, lavender and the mixture lavender+garlic and lavender+pomegranate gave a significant reduction in bacterial growth, while bay tree, lysozyme, tea tree and mint showed bacteriostatic activity. Fig and grapevine extracts, which showed antibacterial activity in the drop diffusion test did not confirm antibacterial activity in broth test.

Garlic, pomegranate and lavender extracts provided the highest antimicrobial activity in *in vitro* assays. However differences in results obtained may differ depending on many factors of variation; these include differences in microbial growth, exposure of micro-organisms to plant oil, the solubility of oil or oil

components, and the use and quantity of an emulsifier (Janssen *et al,.* 1987; Hili *et al.,* 1997; Hammer *et al.,* 1999). Moreover modification in plant composition caused by different provenience and harvesting time may develope differences in antimicrobial activity of essential oils and natural extracts (Marino *et al.,* 2001).

It's not well defined the relation between bacterial pathogens inoculation and the symptoms appearance on kiwifruit plants. Some preliminary tests confirmed the difficulty to obtain standardized symptoms in simulated condition. Consequently the *in vivo* antagonist activity of natural extracts was evaluated using the phytopathogenic bacteria epiphytic population sizes, which according to Hirano & Upper (1983) is a good predictor of foliar disease.

Tests *in vivo* were performed both in greenhouse and in field. The conditions are different: in greenhouse tests, the pathogens are sprayed once at the beginning of the experiment and the climatic conditions remain stable, whereas in field the weather conditions are variable and the pathogen spreads itself repeadetly. In both tests garlic, pomegranate and lavender crude extracts were successfully able to reduce epiphytic population of phytobacterial kiwifruit pathogens and no negative (phytotoxic) effects were recorded on kiwifruit plants. Moreover a synergic effect of the mixtures lavander+garlic and lavander+pomegranate was observed. The efficacy of treatments was lower than that one by copper as standard, decreasing within 7 days. In particular in field results, with changeable climatic conditions, remarked the ability of natural extracts to control kiwifruit bacterial pathogens.

The results obtained by use of crude extract pointed out the demand for a more effective formulation. The isolation of the fractions with potential antimicrobial activity which were microencapsulated, has been targeted with the aim of improving the effectiveness and the application of natural extracts. In this study two phenolic substances were selected and microencapsulated with Eudragit RS 100, gallic and ellagic acid. The results obtained with their use alone and in mixture showed how all microencapsulated products were effective against Psa, Pss and Pv in greenhouse experiment. In field experiments the gallic and ellagic acid mixture was able to reduce epiphytic population of Psa, Pss and Pv within 15 days. Therefore microencapsulated formulation was effective in reducing the washing off effect of extracts and allowed to extend efficacy.

These findings remarked results obtained by previous investigation and those here obtained suggest the potential use of natural extracts as alternatives to or in combination with a reduced amount of copper compounds to effectively protect host plant respevt to phytobacterial populations. Moreover, they are inexpensive, easily obtainable and without any negative effects on plants as on human health.

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CHAPTER 5

CONCLUSIONS

Control of phytobacterial pathogens is an important issue due to their heavy damages caused especially during the last few years. Kiwifruit is a clear example: bacterial canker is rapidly spreading from Asia to Europe and New Zealand, badly damaging the orchards. In Italy, economic and trade-related losses due to bacterial canker on kiwifruit have been estimated at around € 2 million.

Past control strategies all over the world, especially against Psa, have not been particularly successful. At present, appropriate cultural practices and preventive copper treatments are suggested. In addition, new pesticides based on biocontrol agents and natural extracts could provide new control opportunities. However, these will need careful testing to determine their efficacy.

Use of biocontrol agents is a promising strategy in order to reduce chemicals and to prevent phenomena of pathogenic bacteria resistance.

Some epiphytic bacteria naturally-occurring on kiwifruit leaf surface collected in New Zealand were selected for their antagonist activity in lab experiments. In particular strain hr63, belonging to *Pseudomonas fluorescens* species, was able to control Psa, Pss and Pv in lab experiments and to reduce bacterial populations present on leaf surface. All these results are promising in hr63 use in biological control of bacterial kiwifruit pathogens, nevertheless further studies are needed concerning ecology, leaf colonization and way of action of BCA strain hr63.

Part of the experiments regarding natural antagonists were carried out in New Zealand, at the Plant and food Research center (Hamilton) supervised by Dr. Joel Vanneste. This experience gave me the chance to deepen my knowledge of antagonisms and improve my experience in molecular biology, and it has provided a complete connection between the different possibilities of biological control of kiwifruit bacterial pathogens investigated in this Thesis.

The possibility to control kiwifruit bacterial pathogens by vegetal extracts,

appears of particular interest considering the antimicrobial potentiality largely unstudied.

Several natural extracts tested in our study had a protective effect against bacterial diseases of kiwifruit. Garlic, pomegranate and lavender extracts, selected after several *in vitro* tests, were useful in biocontrol of bacterial pathogens of kiwifruit plants. Microencapsulation of selected fractions with antibacterial activity allowed to improve their effect in Psa, Pss and Pv biocontrol for longer time.

The bactericidal activity of these vegetal extracts gives new opportunities to improve control against different kiwifruit bacterial pathogens that cause heavy losses in kiwifruit orchards; moreover, these extracts are also suggested for a preventive biocontrol of these bacterial pathogens in kiwifruit orchards.

So, to conclude, can be affirmed that biological control strategies carried out in this research by using natural antagonists and natural extracts, had highlighted their potential effectiveness in biocontrol of kiwifruit bacterial pathogens and that, by counting these researches, these bacterial diseases will be effectively controlled during next years.

APPENDIX

Media, broths and buffers

NS-agar

Nutrient broth (Oxoid)	8 g
Sucrose	50 g
Agar	18 g
Distilled water	1000 ml
(pH=7.0)	

NA-agar

Nutrient broth (Oxoid)	8 g
Agar	18 g
Distilled water	1000 ml
(pH=7.0)	

NG-agar

Nutrient broth (Oxoid)	8 g
Glycerol	20 g
Agar	18 g
Distilled water	1000 ml
(pH=7.0)	

KB-agar

Proteose Peptone	20.0 g
K_2HPO_4	1.5 g
$MgSO_4 \times 7H_2O$	1.5 g
Glycerine	8.16 ml
Agar	18.0 g
Distilled water	1000 ml
(pH= 7.2)	

KBC-agar

Proteose Peptone	20.0 g
K_2HPO_4	1.5 g
$MgSO_4$ x $7H_2O$	1.5 g
Glycerine	8.16 ml
Agar	18.0 g
Boric acid	1.5 g
Distilled water	1000 ml

LB-agar

Bacto Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Technical agar	18 g
Distilled water	1000 ml
(pH=7.0)	

LB- broth

Bacto Tryptone	10 g
Yeast extracts	5 g
NaCl	10 g
Distilled water	1000 ml
(pH=7.0)	

NB-broth

Nutrient broth	8 g
Distilled water	1000 ml
(pH=7.0)	

MM Salt (stock solution 20 X)

K_2HPO_4	35.10 g
KH_2PO_4	15.10 g
L-Asparagin	15.00 g
$(NH_4)_2SO_4$	10.00 g
Nicotinic Acid	2.50 g
Glucose	20.00 g
Sodium citrate	2,50 g
$MgSO_4$ x $7H_2O$	0.05 g
Distilled water	up 500 ml

MM-agar

MM Salt SS	50 ml
Agar	18.0 g
Distilled water	950 ml

MM soft agar

MM Salt SS	50 ml
Agar	7.0 g
Distilled water	950 ml

MM-broth

MM Salt SS	50 ml
Distilled water	950 ml

$MgSO_4$ buffer (10 mM)

Distilled water	500 ml
S.S. 1M $MgSO_4$	5 ml