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PHD IN PLANT PROTECTION - XXVI CYCLE -

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

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"CHARACTERIZATION OF NATURAL ANTAGONISTS OF *Pseudomonas syringae* pv. *actinidiae*, ISOLATED IN DIFFERENT GEOGRAPHIC WORLD AREAS"

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Summary

1. Introduction

Actinidia is a very old genus that has its geographic origin centre in the mountains and hills of South China between Chang Jiang Basin (Yangtze River) and Xi Jiang Basin, a narrow strip located between latitudes 25° and 30° N. The species of *Actinidia* can be found in Siberia, Korea and Japan, passing through China, to the Indus-China, Thailand, India and Indonesia. The genus is spread from latitude 50° N to the equator, i.e., from cold temperatures belts (arctic forests) to those of the Tropics (Ferguson, 1990).

Despite having been introduced in Europe about a century ago, fossil imprints of leaves and seeds of the *Actinidia* genus, along with plane trees and magnolias were found both in England and in the West Siberian Lowland (Zuccherelli, 1994). These paleobotanical evidences suggest that the genus could be very widespread, extending to the whole of Europe and even to Great Britain (Pearson, 1964).

Its current distribution area is bounded to the north by the 51st parallel, to the south by Borneo (18th parallel), to the west by Tibet (90th meridian) and to the East by Japan (Zuccherelli, 1994). This genus, although widely adaptable to different habitats, seem to prefer hot and humid environments (Ferguson, 1990).

While China has now become the largest producer of *Actinidia*, especially of the *Actinidia deliciosa* and *Actinidia chinensis* species, New Zealand has, instead, the merit of having made known the kiwi in the whole world and in all countries with fruit-growing tradition.

In fact, the word "kiwi", of Anglo-Saxon origin, derives from the name of the typical New Zealand bird that feeds on the fruits of this plant. Its Chinese name is "Yang Tao" (from the Yangtze River), which means "fruit of health" (Valli, 2001).

Even if it was started more recently than in other countries, the cultivation of this plant in Italy has achieved great relevance in the international markets, to the point that, excluding China, Italy has become the world leader of the market (Zuccherelli, 1994; Macchi, 2012).

1.1 Botanical and agronomic characteristics of *Actinidia* spp.

The constitution of the *Actinidia* genus dates back to 1821 made by the Danish Nathaniel Wallich, who carried out is first studies in Nepal on *Actinidia callosa* (Zuccherelli, 1994). Lindley in 1836 revised the cataloguing work of Wallich, reclassifying the specimens he studied, as belonging to a new family of *Dilleniaceae* and giving it the name of *Actinidia* (from the Greek *aktis*, "a ray") (Ferguson, 1990).

In 1899, Van Tieghem proposed the creation of the *Actinidiaceae* family, which currently includes genres *Actinidia*, *Clematiclethra*, *Sanzania* and *Sladenia*.

The first real systematic organization of the genus was carried out in 1911 by Dunn who described 24 species, the only ones known at that time. This classification was continuously updated and today it lists about 60 species and 100 taxa belonging to the *Actinidiaceae* family (Ferguson, 1990).

Among the species hitherto classified, those cultivated mostly belong to *Actinidia chinensis*, *Actinidia deliciosa* and *Actinidia arguta*.

The plants of *Actinidia* are small tree climbing-creepers (Fig. 1) that can reach a height of 8-10 m and require, for their bearing, supports for both trunk and branches.

Figure 1. *Actinidia* plants.

The root system has fleshy roots, branched and is rather superficial. The leaves are alternate, simple and petiolate, usually toothed, but sometimes with entire margins; shape and size are variable, but most commonly they are heart-shaped, leathery and

hairy on the underside.

The fruiting takes place on a year old branches (shoots), coming from the previous year ones. The shoots growing in one season, come from axillary buds grown in the previous season and are protected, during the winter, by the cortical and cicatricial tissue of the previous year leaf.

The young shoots can be glabrous or tomentose, while the older branches are covered with a gray or brown bark that often has linear lenticels.

All plants belonging to this genus are dioecious with staminate and pistillate flowers brought by different individuals (Fig. 2). The plants with female flowers produce nonviable pollen, while the male ones have a greatly reduced and underdeveloped ovary. The flowers are sometimes single, sometimes gathered in inflorescence (corymb) with thin, usually white, petals.

Figure 2. Male flower (left) and female flower (right).

The flower induction occurs during the spring, while the blossoming between May and July, depending on the variety and the place the orchard is located. Pollination, anemophilous or entomophilous, it is often difficult and must be helped with the shedding of pollen by artificial ventilation; flowers are in fact poorly attractive because they have no scent and, in some cases, of nectar.

To ensure adequate pollination normally a staminiferous individual is planted every 7-8 female individuals and it is important the presence of at least 8-10 bee hives per hectare.

Because there is a positive correlation between the number of seeds and the fruit size, on the flower stigmas are required at least 2,000 pollen grains (Pietropoli, 2004).

The fruit is an oval berry with a weight ranging from 40 to 150 g that has tomentose epicarp with soft or bristly green-brown trichomes. The mesocarp is fleshy and has many (more than 1,000 per fruit) and very small dark seeds, arranged around a white and edible heart, called columella. The flesh of the fruit is light green in *A. deliciosa*, while in *A. chinensis* there is a range of yellow, yellow-green or dark green colour (Ferguson, 1990; Testolin & Ferguson, 2009) (Fig. 3).

Figure 3. Different fruits belonging to *Actinidia* spp.

Being a sub-tropical plant, *Actinidia* requires appropriate temperatures, wide availability of water, high humidity of air and low summer temperatures. From the thermal point of view, the length of the growing season, which extends over 8-9 months, entails precise territorial limits, determined by anthesis-harvest period of about 160-180 days. This leads to exclude all areas where there is a high probability of autumn seasons with premature cold.

Planting density usually depends on the structures and the farming system adopted. The prevailing types of farming are pergola system ("tendone" in Italian), with 4x4 or 5x5 m planting layout, in southern areas, and T-bar system ("pergoletta" in Italian) in northern environments (Costa & Testolin, 1990; Dal Pane, 2002). The distance between rows should always be 4-5 m in order to ensure the passage of tractors, the one in the row varies in relation to soil fertility and vegetative vigor of the selected cultivar (Testolin *et al*., 1993).

This crop requires a medium exposure to lower temperatures (600-1200 h below

+7°C) preferring a mild climate and to avoid excessive cold and heat. In fact, it appears to be very sensitive to cold in late winter and in early spring, albeit for short periods of time, below + 5-10°C. Adult plants with very woody branches and in complete dormancy better tolerate lower temperatures. Plants that have already satisfied the cold requirements are therefore more susceptible to outbreaks of winter frost.

The high summer temperatures are usually not harmful, but are often associated with low humidity air, which must be corrected with irrigation (climatising) (Zuccherelli, 1994).

In fact, in this period, the leaves are particularly susceptible to burning and drying when the level of transpiration is very high (Xiloyannis *et al*., 1986).

The clear demonstration of the importance of irrigation practices is the development of specific irrigation techniques for this crop (No. drippers per plant, under foliage nebulisation, etc.) compared to other fruit tree crops in temperate climate (Xiloyannis *et al*., 1996).

The temperature stress can be aggravated by the wind, which causes a further reduction of air humidity leading to water deficit. The wind can also cause the breaking of branches, leaves and fruits rubbing them against pieces of wood or the support structure. It also disturbs the activity of the pollinating insects with negative effects on the fruit set (Zuccherelli, 1994).

To avoid problems of asphyxia and root rots, the soil should be dissolved and permeable, but also deep and rich in organic matter in order to ensure a sufficient supply of nutrients through the activity of the microbial flora (Marangoni *et al*., 2003).

Given the predisposition of *Actinidia* toward chlorosis phenomena related to iron deficiency, a sub-acidic reaction of soil (pH below 7.5) associated with a low content of active lime ($CaCO_3$ < 5%) is to be preferred (Rombolà *et al*., 2000; Rombolà *et al*., 2003). Although the abundant vegetation of a kiwifruit orchard requires a high nutritional support, excessive nitrogen availability could be negative because it creates a micro-environment conducive to cryptogamic attacks and risks of poor lignification that could predispose to cold damage (Marangoni *et al*., 2003; Zuccherelli, 1994).

The best soils for the planting of *Actinidia* is silty-sandy with permeable alluvial, deep, fertile, fresh subsoil and with a pH between 5.5 and 7.4 (Zuccherelli, 1994).

1.2 Cultivars of *Actinidia* spp.

Italy began to cultivate *Actinidia* plants in 1971 and since then it has considerably increased the harvested area and, consequently, its production has became one of the most important in the world (Cacioppo, 2009).

The initial development of the Italian kiwifruit industry was based mainly on the cultivars of *A. deliciosa* constituted in New Zealand, but currently also the cultivars belonging to *A. chinensis* play an important commercial role.

The cultivars mostly cultivated that belong to *A. deliciosa* are:

"Hayward" (moderately vigorous plant, later flowering than other selections, big size fruits with weight 90-150 g, green-brown tomentose epicarp, mesocarp light green in colour, good taste and exceptional preservation of fruits) (Testolin & Ferguson, 2009);

"Earligreen ®" (natural mutation of Hayward, anticipated harvest of 40-50 days compared to Hayward, all the fruits of a branch do not fill or ripen at the same time, fruits with reduced shelf life) (Bucci & Costa, 2006);

"Green Light ®" (natural mutation of Hayward, fruits with similar characteristics to those of Hayward and that fill up in advance of 30-35 days) (Spada & Spada, 2005);

"Top Star®" (less vigorous Hayward mutation, which therefore requires minimum summer pruning);

"Summer Kiwi®" (precocious selection, fruits similar to those of Hayward but having a weight of 80-90 g, short period of fruits storage);

"Abbott" (vigorous plant, precocious and productive, fruits of medium size);

"Allison" (vigorous and productive plant with later flowering compared to Abbott, fruits slightly larger than Abbott);

"Bruno" (productive and vigorous plant with flowering slightly delayed compared to Allison, large fruits of elongated-cylindrical shape);

"Monty" (very vigorous plant, very productive, sometimes excessively, oblong medium size fruits);

the two most commonly used cultivars as pollinators are "Matua" and "Tomuri".

The three cultivars of *A. chinensis*, also known as yellow kiwifruit, most cultivated are:

"Hort16A" (vigorous plant that blooms a month before Hayward, the fruits are

harvested about in the same period of Hayward, sweeter and aromatic flavour with low acidity, colour of flesh yellow-gold);

"Gold3 (G3)" (new cultivar used to replace Hort 16A that showed great susceptibility to Kiwifruit Bacterial Canker, similar to Hort 16A)

"Jintao" (less vigorous but more productive than Hayward, blooms a week before Hayward, fruits are harvested three weeks before, the flesh has a bright golden colour and a sweet taste, good preservation period but small fruits up to 90 g) (Cipriani & Testolin, 2007);

"Soreli" (fruits that ripen 30 days before Hayward, of large size, more than 100 g, flesh bright yellow in colour, good flavour but short shelf-life) (Fideghelli, 2012);

Another cultivar belonging to *A. chinensis* is the Chinese "Hong Yang" also known as red kiwifruit. More precisely, the flesh is two-tone green-yellow; the red colour is arranged radially around columella (Fideghelli, 2012; Testolin, 2014).

1.3 Commercial aspects of *Actinidia* spp.

Due to the success that this species has obtained with consumers, in recent years the cultivation of kiwifruit has seen a sharp increase in many producing countries.

Data relating to 2010 showed that the harvested area of kiwifruit in the world was close to 160,000 ha with more than 70,000 ha concentrated in China, a country that in the last 20 years has given greater impulse to the increase of cultivation.

Excluding China, the areas planted with Kiwifruit in the 90s had decreased significantly, reaching around 54,000 ha; then the interest towards this crop is gradually increased to cover 98,656 ha and reaching 1,412,455 tonnes of product in 2012 (Faostat, 2014).

The kiwifruit production is highly concentrated and the top five producing countries are China with over 490,000 t (27% of the world total), Italy with approximately 384,844 t (24% of the total and 24,327 ha harvested), New Zealand with 376,400 t (20% of the total and 12,757 ha harvested), Chile with 240,000 t (10% of the total and 10,950 ha harvested) and Greece with 161,400 t (6% of the total and 7,300 ha harvested); followed at a distance by France with 65,253 t (3,952 ha harvested), Turkey with 36,781 t (28,500 ha harvested), Iran with 32,000 t (2,900 ha harvested), Japan with 28,000 t (2,300 ha harvested), the United States with 26,853 t (1,700 ha harvested), Portugal with 25,000 t (1,600 ha harvested), Spain 16,200 t (800 ha harvested) and South Korea with 10,600 t (820 ha harvested) (Faostat, 2014).

The Italian production is concentrated in few regions: Lazio, the Italian leader for the cultivation of Kiwifruit, with over 30% of the investments, Piedmont with about 20%, Emilia-Romagna with 16%, Veneto with 15% followed by Calabria, Campania and Friuli (Macchi, 2012; Faostat, 2014).

Clearly, the attitude of a producer country is to satisfy consumer demand, increasing production and thus the supply; therefore it is necessary an extension of the sales calendar in respect of the consumers requirements.

In the EU Kiwifruit market Italy's principal competitor is Greece, which has the same seasonality of production. The southern hemisphere countries, which have a complementary seasonality of production to Italy, do not represent particular problems for Italy's export.

Chile's export calendar of kiwifruits overlaps with the tail end of the Italian one and does not affect the Italian market. However, New Zealand's export calendar clashes

with the beginning of the Italian one reducing the marketing period of the Italian product.

At present, the main problem among Kiwifruit producing countries is the outbreak and spread of Bacterial canker of Actinidia, that since 2008 has heavily affected thousands of Kiwifruit hectares worldwide, determining numerous removals of harvested areas (Macchi, 2012).

2. Diseases

Among the main reasons that have contributed to the success and, consequently to rapid expansion, of *Actinidia* orchards, not only in Italy but also in rest of the world, there is certainly the high economic convenience.

Its profitability is attributable not only to the productivity of the crop and to the price that the market confers to the fruits, but also to the sporadic phytosanitary management needed for its cultivation.

In general, fruit crops require several efforts with pesticides, in order to limit losses caused by pests.

Until a few years ago the kiwifruit cultivation required rare phytosanitary treatments. However, today the "Bacterial Canker of *Actinidia*", due to large economic loss caused, represents a tremendous parasitic problem that is still to be solved.

2.1 Main diseases of *Actinidia* spp.

Between the most well-known *Actinidia* diseases there is the "wood decay", a fungal disease with complex etiology that usually manifests in adult plants (8-10 years). It presents itself with visible symptoms on the leaf, more or less extensive necrotic areoles in the leaf surface. At the same time the wood attacked by different pathogens, is friable or necrotic mainly starting from pruning wounds distally located (Tosi *et al*., 2008).

Despite the infection progresses constantly in woody tissue, its manifestation is not progressive and constant impact over the years. Its control is related to the removal of diseased branches (Osti & Di Marco, 2011).

Other diseases of *Actinidia* spp. are caused by plant pathogenic bacteria such as *Pseudomonas syringae* pv. *syringae*, agent of floral buds necrosis, and *Pseudomonas viridiflava*, agent of bacterial blight. These were observed in Italy and recorded worldwide in the late 80s early 90s (Balestra & Varvaro, 1997; Varvaro *et al*., 1990).

P. s. pv. *syringae* is able to cause not only sporadic cankers, but also dark yellow-brown browning of flowers and buds, followed by necrosis and subsequent drop of these organs. On leaves it causes necrosis of 1-2 mm in diameter, often surrounded by chlorotic halo. Furthermore, it is able to determine frost damages, especially during the period of vegetative resumption (Rossetti & Balestra, 2007; Rossetti *et al*., 2009).

P. viridiflava causes evident symptoms mainly at leaf level with hydropic spots of angular shape that necrotize; it is also able to stop and/or alter the development of flowers and buds with subsequent fruit drop (Rossetti *et al*., 2009) and, as *P. s.* pv. *Syringae*, it is able to cause frost damages in conjunction with sudden and brief changes in temperature (Varvaro & Fabi, 1992).

The bacterial populations (INA^+ bacteria) responsible for damages and losses on *Actinidia* spp. plants are characterized by surviving at epiphytic level during different seasons, causing damages on different organs when there are unexpected decreases of temperature (Varvaro & Fabi, 1992).

2.2 Bacterial Canker of *Actinidia*

The Bacterial canker of *Actinidia* is a vascular disease caused by a phytopathogenic bacterium, *Pseudomonas syringae* pv. *actinidiae* (Psa) (Renzi *et al*., 2009).

Psa was observed for the first time, in 1984, in China in the Hunan province (Fang *et al*., 1990), but the pathogen was isolated and characterized for the first time in 1989 in Japan on *A. deliciosa* plants (Serizawa *et al*., 1989) and was subsequently classified with the nomenclature that we know today (Takikawa *et al*., 1989).

The disease was detected later in Korea (Koh *et al*., 1994; Koh *et al*., 2010) and in Italy (Scortichini, 1994).

Although present in different areas of the world, Psa was not cause of extensive damage until 2008 (Balestra *et al*., 2008). With its outbreak in Italy, a dramatic epidemic was caused by a new strain (haplotype) isolated from *A. chinensis* and *A. deliciosa.* This strain is much more aggressive than the previous one (Scortichini, 1994) and it is still affecting kiwifruit orchards all over the world.

The same Psa strain was reported in 2010 in Portugal (Balestra *et al*., 2010), France (Vanneste *et al*., 2011) and New Zealand (Everett *et al*., 2011). In 2011 it was recorded in Australia (Biosecurity Australia, 2011), Spain (Balestra *et al*., 2011), Switzerland (EPPO, 2011) and Chile (EPPO, 2011). The presence of this pathogen has also been reported in Turkey (Bastas & Karakaya, 2012) and in 2013 it reached Germany (EPPO, 2013).

The Psa strain isolated in Europe in 2008 and in New Zealand between 2010 and 2011 is very virulent and therefore it has been defined as Psa-V. This definition is based on its aggressiveness and the rapidity with which it is able to cause the disease. In New Zealand another Psa population described as less virulent has been detected. The strains belonging to this population are labelled Psa-LV (Psa-Low Virulent) and are not able to create cankers, but only foliar spots (Chapman *et al*., 2011).

This disease is, currently, widespread in most Italian (Fig. 4) and world Kiwifruit areas (Fig. 5) and has assumed pandemic dimensions with an incidence that represents a serious risk for the future of the *Actinidia* cultivation and its whole business.

Furthermore, all the *Actinidia* species and cultivars are susceptible to the Psa (Cotrut *et al*., 2013).

Figure 4. Current distribution of Psa in Italy.

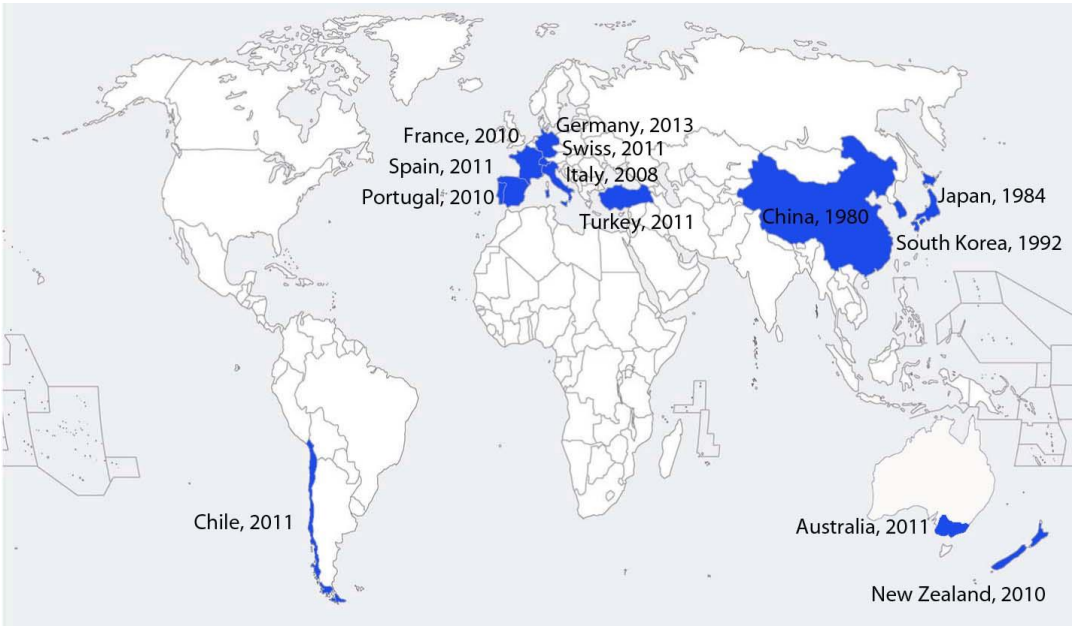


Figure 5. Current distribution of Psa in the world.

Psa is active in the orchard at temperatures between 10 and 20 °C, in any case not exceeding 25 °C, at which temperature the bacterium remains latent within the host even if the external climatic conditions are adverse (Renzi *et al*., 2009).

During the autumn, the high relative humidity and temperatures that are still

mild, in association with the entry ways determined by harvest, leaf fall and high activity of stomata and lenticels, represent an opportunity of Psa establishment that can survive within the host (Renzi *et al*., 2012).

Through winter pruning, Psa can penetrate and colonize the branches and begin its infectious cycle. In addition, frosts, hailstorms and heavy storms typical of this season are further risk factors for infections and reinfections (Mazzaglia *et al*., 2010).

At the beginning of the vegetative season, mild temperatures and relative humidity are very suitable for the multiplication of Psa, therefore, the pathogen is able to penetrate into the host through natural openings such as lenticels, stomata, natural wounds caused by emission of new shoots and leaves, or accidental wounds by wind and other meteorological events (Renzi *et al*., 2012).

During the hottest periods, with lower relative humidity, Psa reduces its biological activity, but hailstorms and heavy storms may contribute to the spread of the pathogen from infected plants to healthy ones, facilitating new infections.

Spring and early summer infections are mainly characterised by necrotic angular leaf spots, surrounded by a yellowish halo (Fig. 6), which over time converge, resulting in a general wilting of the tissues and by cankers on trunks and branches almost always accompanied by discharge of a typical dark red or matt white exudate (Fig. 7), wilting of the shoots (Fig. 8) and browning of buds and flowers (Fig. 9). As far as the fruits are concerned, they are greatly wilted and often do not develop fully (Fig. 10).

Figure 6. Necrotic leaf spots with yellow halos caused by Psa.

Figure 7. Cankers with dark red (left) and matt white (right) exudates.

Figure 8. Wilting of shoots.

Figure 9. Wilting of flowers.

Figure 10. Wilting of fruits.

Differently, the autumn-winter infections present themselves with desiccation of leader canes and trunks, sometimes associated with discharge of exudate and cankers development (Rossetti *et al*., 2009; Renzi *et al*., 2009).

Moreover, Psa is able to colonize the root systems (Fig. 11) of plants affected by Bacterial Canker, proving the pathogen's ability to infect organs that are far from entry sites (Mazzagia *et al*., 2010; Renzi *et al*., 2012).

Figure 11. Bacterial Canker effects on root system (left) and whole *Actinidia* plant (right).

2.3 Control of bacterial diseases

The conventional control means are not always sufficient to fight bacterial plant diseases because, to date, there are no effective chemical molecules commercially available. Therefore, the approach is preventive rather than curative.

In the establishment of *ex-novo* orchard is important to ensure that the propagation material purchased is certified, i.e. showing the conforming label to the technical standards of the Central Plant Protection Service in accordance with Article 49 paragraph 2 of Legislative Decree N° 214/2005, issued by the same institution, after carrying out the necessary inspections and made any necessary laboratory tests.

These measures, undertaken by Italy with the MD of February 7th 2011 and updated by the EU Commission Decision of December 5th 2012, are necessary in order to prevent, control and eradicate the Bacterial canker of *Actinidia* caused by *P. s.* pv. *actinidiae*.

The rules concerning the marketing of nursery productions are essential to prevent various diseases from short and long distance spread.

In young orchards (1-2 years) it is appropriate to proceed at eradication and immediate destruction of infected plants.

At present, the fight against Kiwifruit bacterial diseases and especially the Bacterial Canker is based on appropriate technical-agronomic practices such as frequent inspections, cleaning the orchard of infected portions, continuous disinfection of all tools and closing cuts larger than 2 cm in diameter with tar or ecological adhesive (Renzi *et al*., 2009) (Fig. 12).

The emergency measures/control for the Bacterial Canker were also outlined by MIPAAF DM of February 7th 2013, which indicates to cut the plant or remove its affected parts (the cut must be made at least 70 cm from the infected portion), to uproot the affected plants and to promptly proceed with their burning or deep burial.

Together with a good conduction of the orchard, other control means can be used.

Figure 12. Tar on pruning cuts.

The most important phytosanitary treatment is the constant use of copper (e.g. after harvest, leaf fall, winter pruning and before the new season).

Besides from being constant, the copper treatments must be timely especially after important meteorological events such as frosts and hailstorms (Fratarcangeli *et al*., 2010).

The copper ion (Cu^{++}), adsorbed by bacteria in high doses, can modify the permeability of cell membranes substituting different metal ions (K^{+}, H^{+}, Ca^{++}, Mg^{++}), blocking the biosynthesis of enzymes and co-enzymes, interfering with breathing processes, blocking the redox processes and interfering with the membrane transport (Martelli, 1984; Pertot *et al*., 2005).

Moreover, due to its mechanism of multisite action, it is able to decreasing the risk to develop resistance phenomena (Brunelli & Palla, 2005).

However, for different populations of *Pseudomonas* spp., it was reported a potential development of copper resistance due to the inappropriate use of copper compounds (concentrations, No. of treatments, formulations used) (Vanneste *et al*., 2008). Copper could also determine phytotoxic effects on vegetal tissue, for this reason it is not used during the flowering phase. Because of its potential accumulation in soil, the European Union has imposed strict limitations for the use of copper (in organic farming) placing a maximum threshold of 6 Kg/ha per year or a total of 30 Kg/ha of copper in a period of five years (EU Regulation 473/2002) (Varvaro *et al*., 2001;

Asinelli *et al*., 2006).

Another strategy to control bacterial pathogens is based on the use of antibiotics. Although it could be an efficient approach, the EU has banned their use in agriculture because they easily induce forms of resistance, with serious risks of cross-resistance between phytopathogenic bacteria and human pathogens (Vanneste *et al*., 2008; Ghosh *et al*., 2008). Resistance phenomena to antibiotics used for the control of PSA have been already recorded (Nakajima *et al*., 2002).

A further control strategy, which is under evaluation, is represented by a group of molecules known as resistance inducers. These are able to stimulate the plant to implement a series of actions that cause the so-called Systemic Acquired Resistance (SAR) and Local Acquired Resistance (LAR) phenomena. The best known of these molecules is the salicylic acid, a molecule involved in induced resistance that, interacting with catalase and ascorbate peroxidase, slows the catalysis of hydrogen peroxide, ROS (Reactive Oxygen Substance), key in the occurrence of hypersensitivity reaction. Elicitors are compounds that stimulate different types of mechanisms, but in many instances with high levels of disease they proved to be much less effective. This lack of uniformity in results, together with some problems for plants development, limits their use (Walters *et al*., 2005).

Last, but not least, is the use of natural substances and natural antagonists. Both allow a good control of plant diseases in a preventive manner (Slusarenko *et al*., 2008). Appropriate technical-agronomic practices can minimize their critical aspects. In fact, while natural substances are subject to wash-out, natural antagonists may not remain sufficient in number because of their inadequate ability to substantially multiply on the plant, because of unsuitable environmental conditions, or because of the lack of nutrients.

The control adopted by these “organic strategies” is aimed at developing a biological barrier (antagonism) and to supply plants with a high level of natural resistance to infections; because of this, it is important the role played by environmental factors and the different strategies adopted by the phytobacteria (Mukerji & Garg, 1998; Pietrarelli *et al*., 2006; Slusarenko *et al*., 2008).

3. Aim of work

The cultivation of Kiwifruit is of considerable importance both in Italy and in many parts of the world, thus the Bacterial Canker of *Actinidia* causes substantial economic losses in many areas. For this reason, it seemed extremely important to verify, in Lazio (provinces of Viterbo and Latina), among the bacteria present on the phylloplane of *Actinidia* plants affected by Bacterial Canker, the presence of bacterial populations capable of antagonising not only the Italian isolates of *Pseudomonas syringae* pv. *actinidiae*, but also those present in different areas of the world areas where *Actinidia* is cultivated and affected by Kiwifruit Bacterial Canker.

4. Open field control strategies

Concerning the limited strategies against Psa and taking in account the restrictions of EU, it seemed relevant to develop alternative Psa control strategies.

For this purpose, considering a control of the Kiwifruit Bacterial Canker disease with a minimum environmental impact, has been assessed the efficacy of a natural antagonist (*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747) comparing it with a cupric compound (copper hydroxide 19.94%).

4.1 *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 in agriculture

In 1943 Fukumoto isolated from soil and described for the first time the species *Bacillus amyloliquefaciens* (Fukumoto, 1943). Initially, it was classified as a subspecies of *Bacillus subtilis*, but as a result of morphological and biochemical tests, then confirmed by molecular tests, was recognized as a separate species in 1987 (Priest *et al*., 1987).

Its name is derived from the peculiar ability to produce (*-faciens*) an amylase (*amylo*-) enzyme capable of liquefying/degrade (*-lique*-) complex carbohydrates such as starch, into simple carbohydrates.

It is a ubiquitous bacterium, present everywhere in the world, in both soil and vegetation and for its ability to produce several extracellular enzymes (α-amylase, proteases and endonucleases as the Bam HI) used in the food industry and biotechnology. Not producing dangerous toxins, the EFSA (European Food Safety Authority) considered its safe use for humans (Qualified Presumption of Safety) (EFSA, 2014).

B. amyloliquefaciens is a Gram-positive, rod-shaped, strictly aerobic, which has pericyclic flagella able to make it highly mobile during some life stages, whose cells can clump together forming long chains. Its optimum growth temperature is between 30° and 40°C.

As many species belonging to the genus *Bacillus* is spore-forming, aspect that allows him to overcome unfavourable periods and survive for long periods of time. The endospores are formed under conditions of extreme environmental stress and/or nutrition.

These characteristics are crucial in the guarantee him the persistence even in adverse conditions.

This bacterium colonizes easily roots, leaves, and each plant organ, where it multiplies rapidly, thus preventing the establishment of pathogenic bacteria and fungi. Its antagonistic activity is expressed through a complex mechanism based on the competition for the nutrient sources, for the space, but also on the production of active substances.

In particular, is proficient in producing lipopeptides and lipoproteins (subtilisin, surfactin), able to alter the permeability of the membrane, and protease, capable of degrading the components of the cell walls and other internal structures of the pathogen.

In addition, *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 (Bap) activates the mechanisms of induction of resistance towards the treated plant (Diderichsen & De Boer, 1991). It was in fact detected elicitation of Induced Resistance (ISR) on plants of *Arabidopsis thaliana*, making it resistant *Erwinia carotovora* subsp. *carotovora*) through the production of volatile substances (Biavati & Sorlini., 2008).

Considering its multi-site action and to the reduced probability that occur simultaneously in the pathogenic structural changes in all sites of interaction, there is a rare possibility of the development of resistant strains following its use (Belli, 2007).

In addition to the environmental advantages, Bap has important further positive points such as: reduced time to shortage (coverage even during the harvest time), the absence of residues on food, the absence of legislative restrictions (doses number of treatments) and possibility to use singularly or in combination/alternation with conventional fungicides (including cupric products) (Solioz *et al*., 2010; Gallipoli *et al*., 2013).

All these characteristics, confirmed by experiments *in vitro* and in field, have allowed, in Italy, to *B. a.* subsp. *plantarum* strain D747 to be recorded, on February 3rd 2012 (No. 15302), as the first microbiological fungicide/bactericide at the concentration of $5x10^{10}$ spores/g (corresponding to 25% weight/weight) against Psa during blossom time.

This natural antagonist has a broad spectrum of action, against various fungal diseases, such as *Botrytis cinerea*, *Stemphylium vesicarium*, *Bremia lactucae*, *Sclerotinia* spp. and against some bacterial diseases such as, for example, *Erwinia amylovora* and *Pseudomonas syringae* pv. *actinidiae*.

Currently it is the unique pesticide registered on *Actinidia* plants against the Psa that can be used also for organic farming during flowering phase.

4.2 Materials and Methods

The experiments were conducted on Kiwifruit plants during the biennium 2012-2013.

4.2.1 Orchard selection

In both years, the field trials were carried out in an orchard of *Actidinidia deliciosa* (cv. Hayward), Kiwi fruits with green flesh, 14 years old, located in the municipality of Montefiascone, province of Viterbo where Bacterial Canker incidence was confined and below 30%.

The orchard is located on almost flat and uniform soil, with good fertility and discretely vigorous plants. The layout system is 3,5 × 3,5 m (816 plants/ha) and the type of farming is T-bar.

4.2.2 Theses and experimental protocols

The experiment provided comparative evaluations not only between Bap and a product containing copper hydroxide, but also a combination of them at ½ of field doses during the entire vegetative season.

Previously to the field trials was done a treatment after leaf fall with copper sulphate neutralized with calcium hydroxide (10%) (field doses 8 l/ha) and a deep winter pruning proceeding to eliminate approximately 50% of fruiting branches, disinfecting all tools utilised plants by plants, by quaternary ammonium salts (benzalkonium chloride).

These preliminary practices were carried out as fundamental strategies to reduce the inoculum of further infection by Psa.

The experimental plan, in randomized block, provided the establishment of 4 experimental theses, with two replicates each:

1) **Thesis Negative Control (NC),** not subjected to any treatment;

2) **Thesis Positive Control (Cu^{++})**, treated with 2.5 l/ha of copper hydroxide (19.94%);

2) **Thesis *Bacillus amyloliquefaciens* subsp. *plantarum* (Bap),** at field dose of 1.5 kg/ha per 10 q of H_2O;

4) **Thesis with *Bacillus amyloliquefaciens* subsp. *plantarum* + Copper hydroxide (19,94%) (Bap+Cu^{++}),** mixed at ½ of field doses (0.75 kg/ha and 1.25 l/ha respectively).

In any case the volume of water used was 10 hl per hectare (equal to 1.25 litres per plant).

Among the cupric salts, was chosen a hydroxide one that provides greater promptness of action, good adhesion and uniformity of distribution on the leaf surface. In particular, this product is mixed with resins extracted and refined by conifer oils, ensuring a better adhesion, a greater persistence on the treated surfaces and a consequent reduction in the number of treatments.

These characteristics enable a greater bactericidal efficacy of the product and allow the reduction of unitary doses of copper and environmental advantages.

The thesis provided 2 replications, arranged randomly in their respective blocks, including 18 plants each. Between each repetition were left two rows of untreated plants.

The plants belonging to different experimental theses were marked by appropriate signs, showing a number and a different colour, placed at the head of each thesis, while the branches sample were made recognizable by coloured taping.

For each experimental thesis were therefore considered 36 plants and for each treatment were kept in mind the instructions reported on the label and the restrictions of law.

The use of copper-based products is scheduled since 2012 during the vegetative phase, by a temporary authorization (Article 53, paragraph 1 of Regulation (EC) No. 1107/2009), to control the bacterial diseases/canker in vegetation on kiwifruit plants by a maximum of 3 treatments within September 4^{th}. The treatments were sprayed on the Kiwifruit phylloplane by using an atomizer.

The theses were as follow:

1) **Positive Control (Cu^{++})**, a treatment every 20-25 days by a field dose (2.5 l/ha), as reported on the label, for the whole growing season except flowering and after it (before September) for 6 times;

2) ***Bacillus amyloliquefaciens* subsp. *plantarum* (Bap),** a treatment every 25-30 days by a field dose (1.5 kg/ha), before the breaking of buds, one with flowers completely opened and further 5 times after flowering;

3) ***B. a.* subsp. *plantarum* + Copper hydroxide (Bap+Cu^{++})**, at ½ of field doses (0.75 kg/ha and 1.25 l/ha respectively), a treatment every 25-30 days, twice with copper hydroxide and Bap before flowering, only Bap at flowers completely opened, 4 times after together (Bap+Cu^{++}) before September and 2 more times with only Bap at field dose until leaf fall.

4.2.3 Surveys, samplings and analysis

In both years of field trials have been performed 8 sets of phytopathological surveys, according to the following schedule:

- year 2012: (May 6th, May 31st, June 13th, June 27th, July 27th, August 25th, September 20th, October 26th);
- year 2013: (May 25th, June 10th, June 29th, July 15th, July 31st, September 12th, October 10th, October 29th).

During the records was monitored the phytosanitary status of plants and any eventual other anomalies on leaves to determine the disease incidence by a scale previously developed related to the number of leaf spots, (necrotic areas surrounded by chlorotic halo)/leaf, caused by Psa. Moreover, the vegetable samples considered were randomly analyzed in the laboratory in order to verify the presence/absence of Psa.

Finally, were considered also eventual phytotoxicity phenomena caused by copper that generally produce internerval and/or marginal leaf necrosis.

In relation to the records on the leaves to determine the disease incidence was adopted the following approach:

- Random selection of 2 branches ≥ 2nd year per plant;
- On every branch, ≥ 2nd year, 10 leaves were selected;
- Then were created classes of disease to be assigned to each leaf according to the number of spots present;
- I class: 0 spots, II class: 1-50 spots, III class: No. > of 50 spots, IV class: converged spots, V class: leaves completely withered;
- For each class of disease was assigned a value: I=0, II=0.25, III=0.50, IV=0.75, V=1;
- Using the formula (No. of leaves belonging to class I x 0 + No. of leaves belonging to class II x 0.25 + No. of leaves belonging to class III x 0.50 + No. of leaves belonging to class IV x 0.75 + No. of leaves belonging to class No. of leaves belonging to class V x 1)/total No. of leaves, was obtained the disease incidence.

The disease incidence on the leaves was measured on 36 plants of each thesis. For each plant was determined the number of spots present on the 10 basal leaves of each of the two branches chosen and every time were analyzed 72 branches per thesis, for a total of 720 leaves for each survey.

The statistical analysis of the data collected was conducted using GraphPad Prism 5.0 software. Analysis of variance was applied, one-way ANOVA followed by Tukey's post-test ($P < 0.05$).

4.3 Results

The results obtained, resulted useful and interesting, in relation to the both seasons and in respect of the different treatments performed.

4.3.1 Year 2012

The higher foliar disease index was detected in the thesis of the negative control (untreated): next 0.03 in May next, it has reached almost 0,074 in July and 0.09 in October.

The Positive Control (Cu^{++}) showed a disease index in the early part of the season relatively content (0.02 May 16th; 0.03 May 31st, 0.031 June 13th; 0.043 June 27th), so as to be statistically different compared to the results of the Negative Control. Since August, however, until the end of the reliefs, the index has increased considerably, proving not longer statistically significant.

Of both theses which included the use of Bap, the best result was found in the thesis with natural antagonist and copper hydroxide (Bap+Cu^{++}) at half doses (50 ml + 35 g), compared to the other thesis and for the whole season. More specifically, in this thesis the index never exceeded 0.04 (October), remaining for the whole season statistically lower than the untreated thesis and from August to October statistically lower also to the thesis that provided the exclusive use of copper hydroxide.

The thesis with Bap, in the first survey (May 16th) showed an index of 0.012, rising to 0.039 on July 30th and to stabilize at 0.0433 on October 26th. This thesis showed results statistically lower than the untreated thesis for the whole season, and to the Positive Control thesis from August 25th onwards.

Furthermore, the values obtained with the Bap thesis have not shown themselves significantly different from those one with combination copper-natural antagonist (Bap+Cu^{++}) (Tab. I and Fig. 13).

Vegetative season 2012				
Date	Bap	Bap + Cu^{++}	Cu^{++}	NC
16 May	0.012 ± 0.003 ^{b}	0.181 ± 0.003 ^{ab}	0.018 ± 0.004 ^{ab}	0.055 ± 0.008 ^{a}
31 May	0.027 ± 0.004 ^{b}	0.026 ± 0.005 ^{b}	0.030 ± 0.005 ^{b}	0.164 ± 0.016 ^{a}
13 June	0.034 ± 0.005 ^{b}	0.032 ± 0.006 ^{b}	0.031 ± 0.005 ^{b}	0.066 ± 0.008 ^{a}
27 June	0.034 ± 0.005 ^{b}	0.033 ± 0.006 ^{b}	0.043 ± 0.011 ^{ab}	0.070 ± 0.008 ^{a}
30 July	0.038 ± 0.005 ^{b}	0.035 ± 0.006 ^{b}	0.046 ± 0.012 ^{ab}	0.073 ± 0.007 ^{a}
25 August	0.039 ± 0.004 ^{b}	0.036 ± 0.006 ^{b}	0.073 ± 0.011 ^{a}	0.076 ± 0.007 ^{a}
20 September	0.042 ± 0.004 ^{b}	0.038 ± 0.006 ^{b}	0.073 ± 0.011 ^{a}	0.082 ± 0.007 ^{a}
26 October	0.043 ± 0.004 ^{b}	0.039 ± 0.005 ^{b}	0.075 ± 0.011 ^{a}	0.087 ± 0.008 ^{a}

Table I. Disease incidence. All parameters are expressed as mean ± SE. Values not marked with the same letter are significantly different ($P < 0.05$).

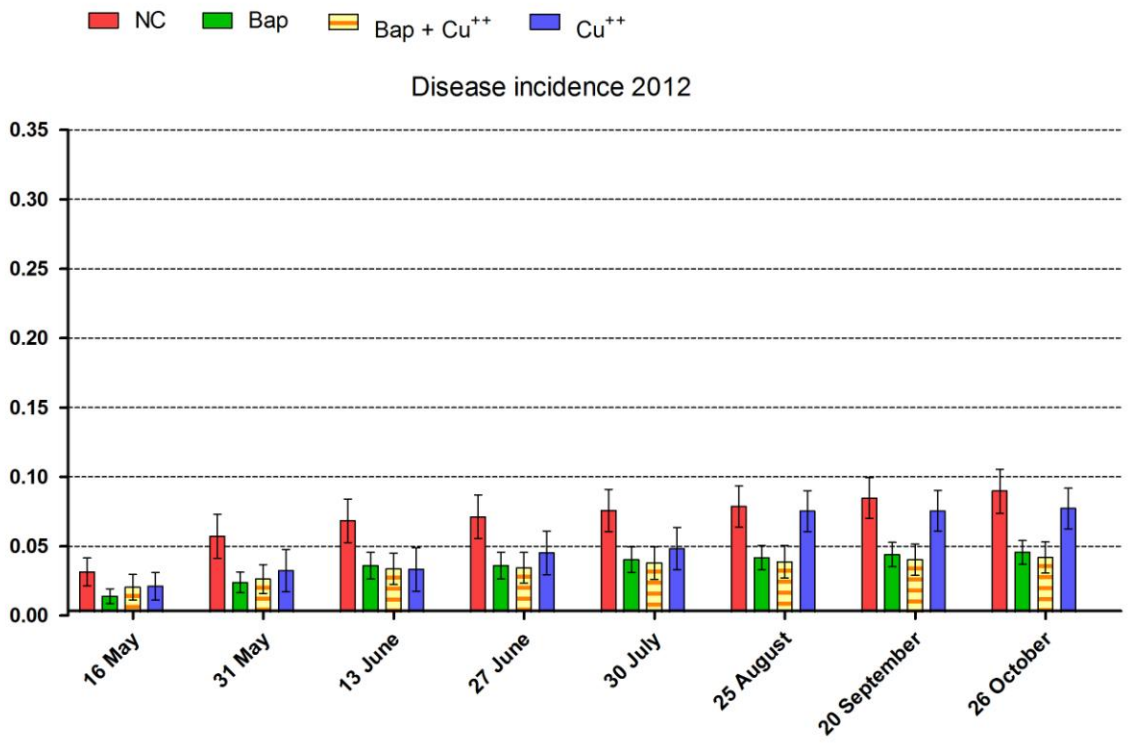


Figure 13. Bacterial Canker disease incidence during 2012. Errors bars indicated the standard error.

4.3.2 Year 2013

The higher foliar disease index was recorded in the negative control thesis (untreated): from 0.15 in May, reaching 0.193 in July and 0.32 in October.

The Positive Control (Cu^{++}) showed results statistically similar to the untreated thesis.

Both theses that included Bap use showed lower values than negative and positive control theses.

Theses with Bap produced the best results through a disease index of 0.092 in May, 0.112 on June 29th, to reach 0.165 on July 31st. From September onwards the index of this thesis was statistically lower than both controls, until the end of the season (0.240 in October).

The disease index by exclusive application of Bap was higher than Bap+Cu^{++} thesis in September (0.190) and in October (0.240), but statistically lower than the Positive Control thesis and Negative Control thesis (untreated) (Tab. II and Fig. 14) (Tab. II and Fig. 14).

Vegetative season 2013				
Date	Bap	Bap + Cu^{++}	Cu^{++}	NC
25 May	0.092 ± 0.013 ^{a}	0.105 ± 0.015 ^{a}	0.144 ± 0.017 ^{a}	0.150 ± 0.020 ^{a}
10 June	0.106 ± 0.011 ^{a}	0.124 ± 0.015 ^{a}	0.148 ± 0.015 ^{a}	0.164 ± 0.016 ^{a}
29 June	0.112 ± 0.011 ^{a}	0.125 ± 0.015 ^{a}	0.153 ± 0.015 ^{a}	0.166 ± 0.016 ^{a}
15 July	0.165 ± 0.014 ^{a}	0.150 ± 0.016 ^{a}	0.182 ± 0.016 ^{a}	0.184 ± 0.017 ^{a}
31 July	0.165 ± 0.014 ^{a}	0.150 ± 0.016 ^{a}	0.182 ± 0.016 ^{a}	0.193 ± 0.017 ^{a}
12 September	0.190 ± 0.014 ^{b}	0.175 ± 0.016 ^{b}	0.267 ± 0.016 ^{a}	0.270 ± 0.023 ^{a}
10 October	0.229 ± 0.017 ^{ab}	0.203 ± 0.019 ^{b}	0.281 ± 0.019 ^{a}	0.294 ± 0.023 ^{a}
29 October	0.240 ± 0.016 ^{bc}	0.203 ± 0.019 ^{c}	0.292 ± 0.019 ^{ab}	0.317 ± 0.022 ^{a}

Table II. Disease incidence. All parameters are expressed as mean ± SE. Values not marked with the same letter are significantly different ($P < 0.05$).

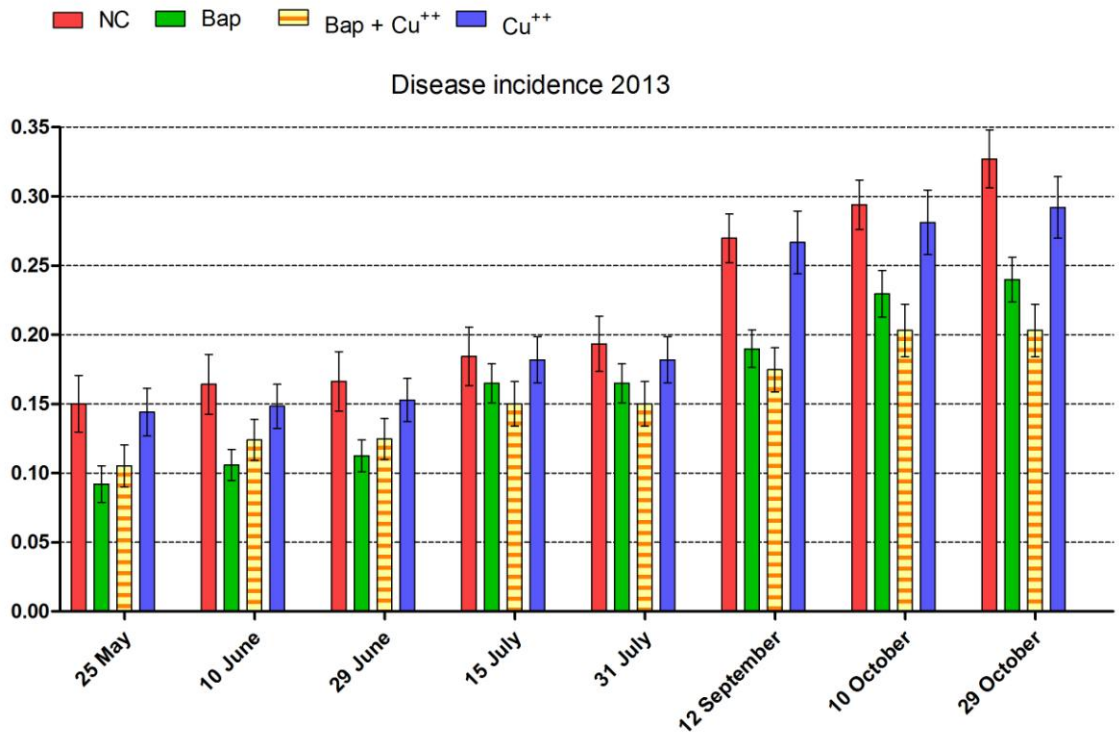


Figure 14. Graphic representation of Bacterial Canker incidence during 2013. Errors bars indicated the standard error.

5. Search of antagonists

To date, the protection of *Actinidia* plants in particular respect of the Bacterial Canker, is entrusted to control methods consisting in the application of products with low environmental impact (copper-based pesticides with a low content of copper) and to the adoption of appropriate agronomic and cultural practices that, if not promptly and continuously performed, do not guarantee an appropriate control.

Being able to isolate and to characterize bacterial populations, usually present in kiwifruit ecosystem affected by Bacterial Canker, and to verify the possibility to use them to control Psa, is an opportunity that from an ecological/phytosanitary point of view assumes remarkable importance.

5.1 Antagonism and epiphytic survival

The biological control offers an environmentally "friendly" alternative to the use of pesticides for the management of plant diseases (Völksch & May, 2001).

Over the years biological control has been defined in various ways, but the most exhaustive definition of it is the one given by Cook and Baker in 1983, which defines biological control as "the reduction of inoculum density or the ability to produce disease of a pathogen or a parasite in its dormant state or activity, through one or more important natural antagonists" (Cook & Baker, 1983).

Natural antagonists protect the plants from the action of numerous pathogens and the effectiveness of a bacterium as biocontrol agent may be linked to its ecological similarities with a pathogen.

Occupation of the same ecological niches, use of similar nutritional sources, tolerance to adverse conditions and multiplication in similar environmental conditions are of primary importance for the effectiveness of a biocontrol agent in respect of a specific pathogen (Völksch & May, 2001).

Thus, the need to isolate potential natural antagonists from the same ecosystem and from the same host plant seemed of paramount significance. In this way, bacteria can protect plants implementing several mechanisms like competing for space and for nutrient sources or producing antibiotic substances.

Because bacteria move in the environment through dispersal mechanisms, such as wind, rain, splashing water, runoff or through insects and human activity, the first point of contact between microbial cells and plants is certainly the leaf surface, in particular the cuticle (Beattie, 2002). The phyllosphere represents a niche of great agricultural and environmental significance (Whipps *et al*., 2008).

The phyllosphere is an excellent habitat for many organisms; however, due to the adverse conditions that may occur of the leaf surface, the communities that they can form on leaves vary drastically, both in size and composition, from one leaf to another (Lindow & Brandl, 2003). Microorganisms adopt different strategies to overcome distinct difficulties, such as the production of pigments to protect themselves from ultraviolet rays or the secretion of polysaccharides to prevent desiccation when water becomes scarce (Morris *et al*., 1997; Kadivar & Stapleton, 2006).

Bacteria are classified into "occasional epiphytes" (limited proliferation/survival) and "resident epiphytes" (multiplication/survival) on the bases of

their abilities to survive and multiply adapting to the different conditions relative to the changes in the micro-environment of the leaf (Suslow, 2002). In addition, not all microorganisms that arrive on the same phyllosphere are able to colonize the space and grow (Kinkel, 1997). The ability of bacteria to survive and grow depends on the environmental, physico-chemical and genetic characteristics of the plant and of the micro-organisms which together determine the structure and diversity of the microbial community (Yang *et al*., 2001; Lambais *et al*., 2006).

Another aspect that affects the *fitness* of the microorganisms present on the phylloplane is the limitation of nutrient sources, mediated by the leaf cuticle. Evidences have shown that the main microbial migrations on the phyllosphere occur towards sites of accumulation of carbonaceous sources (sucrose, fructose and glucose) (Leveau & Lindow, 2001), i.e. junction points of the wall of the epidermal cells, stomata, basis of trichomes and depression areas of the cuticle (Whipps *et al*., 2008). Unlike for carbon sources, the scarcity of other nutrients, such as nitrogen sources and iron, are not a limiting factor in the growth of microorganisms on the phyllosphere (Lindow & Brandl, 2003).

Inadequate availability of water and nutrients is the key factor limiting the growth of microorganisms; however, epiphytes have developed several mechanisms to overcome these limitations (Whipps *et al*., 2008).

For example, some *Pseudomonas* spp. may release surfactants that increase the wettability of the leaf surface (greater availability of water) and increase the solubility and diffusion of nutrients to their advantage (Bunster *et al*., 1989). Some epiphytic bacteria have also shown the ability to increase the permeability of the cuticle, favouring the availability of water and nutrients (Schreiber *et al*., 2005).

Fluorescent *Pseudomonas* spp. are capable of stimulating the growth of plants and control pathogens through the production of siderophores (Wensing *et al*., 2010) in iron-poor environments. The siderophores are low molecular weight carriers that selectively chelate iron (III) taken from the environment and making it available to the organism that produced them (Neilands, 1995).

Other bacteria may instead produce different metabolites such as antibiotics, enzymes and volatile substances that play an important role in the control of pathogens (Raaijmakers *et al*., 2002).

Among the main factors that allow some organisms to survive and colonize the harshest environments of the phyllosphere one is the formation of bacterial aggregates.

The production of extracellular polysaccharides (EPS) can protect bacteria from water stress, help the cells to anchor on the leaf surface and protect them from UV rays and reactive oxygen species (Kiraly *et al*., 1997; Whipps *et al*., 2008). This dense matrix surrounding the cells may also increase the concentration of nutrients greatly benefitting epiphytic bacteria (Costerton *et al*., 1995).

In consideration of the aforementioned characteristics, which make epiphytic bacteria very competitive in many different ways, it is appropriate to research potential natural enemies of Psa to contrast/control the Kiwifruit Bacterial Canker directly on the phyllosphere of *Actinidia* spp. plants.

5.2 Materials and Methods

5.2.1 Natural antagonists selection

For this research different orchards of *Actinidia* spp. were investigated in Latina and Viterbo provinces in Lazio.

During the vegetative season, leaves from plants of *A. chinensis* cv. Hort 16A and *A. deliciosa* cv. Hayward were randomly collected in Kiwifruit orchards affected by Bacterial Canker and analysed in the laboratory.

From each leaf in sterile conditions were obtained 4 disks of 16 mm in diameter. The samples were placed in Petri dishes with nutrient agar substrate (NA), on which Psa CFPB 7286 at a concentration of $1x10^6$ CFU/ml was previously placed.

The composition of the medium per litre of distilled water was as follow:

- Lab Lemco Broth (OXOID) 8,00 g
- Bacteriological Agar № 1(OXOID) 18,00 g.

The medium was sterilized in the autoclave at 121 °C for 20 minutes. After cooling down the medium to 50 °C, and under sterile conditions, 18 ml of it were poured in each Petri dish.

A fresh culture (24 h old) of 7286 CFPB strain was diluted in 6 ml of sterile distilled water (SDW) and centrifuged at 12,000 rpm for 10 minutes at 8 °C (Vidaver & Buckner, 1977).

Subsequently, once the supernatant was removed, the centrifuged was resuspended in 5 ml of SDW.

The optical density at 600 nm was read using a turbidimeter (21 Spetronic Mylton Roy Company®), and through appropriate dilutions it was adjusted to a value of 0.13, corresponding to a concentration of $1x10^8$ colony forming units per millilitre (CFU/ml) (Varvaro & Surico, 1978).

The concentration of $1x10^6$ CFU/ml was obtained through decimal dilutions.

Subsequently, the Petri dishes were placed at 26±1 °C for 24-48 h to allow the eventual development of bacterial colonies; the ones that developed were purified in order to be tested against Psa.

For each selected bacterial colony, 100 µl of $1x10^6$ CFU/ml of Psa 7286 were

plated in 5 NA Petri dishes. In each one of them were then placed 4 sterile disks 6 mm in diameter soaked with 15 µl of bacterial colony and 1 sterile disk with SDW.

5.2.2 Production of fluorescent pigments on King's B

Fluorescein is a fluorescent pigment (from green to blue colours) visible under ultraviolet rays (λ_{365} nm). To perform this test, it is necessary to allow the growth of bacteria on agar medium King's B (KB) (King *et al.*, 1954), so composed per litre of distilled water:

- Peptone 20,00 g
- Potassium Phosphate 1,50 g
- Magnesium Sulphate 1,50 g
- Bacteriological Agar №1 (OXOID) 18,00 g.

The medium was prepared with the same methodology previously described (see § 5.2.1).

A strain of *Agrobacterium tumefaciens* (AT 628) and a strain of *Pseudomonas fluorescens* (NCPPB 3008) were used as negative and positive control respectively., The plates were observed 24, 48 and 72 hours after being placed in the incubator at 26±1 °C, using a UV lamp at 365 nm (Sylvania Blacklite Blue Tubes F 15T8-BLB).

5.2.3 Solubility assay in KOH

A good alternative to Gram staining is represented by the KOH solubility assay; for which a drop of 3% KOH solution in sterile distilled water is placed on a glass slide.

Then, with a sterile loop, an aliquot of the bacterial culture is streaked on the glass slide to obtain a homogeneous mixture. If the latter appears to be viscous and filamentous, bacterium under investigation are Gram-, otherwise they are Gram+. This is due to the ability of KOH to degrade only the cell wall of Gram- bacteria but not the one of Gram+ bacteria (Goszczynska *et al.*, 2000).

5.2.4 LOPAT tests

Among the classical diagnostic assays, Levan production, Oxidase reaction, Potato rot, Arginine dihydrolase production and Tobacco hypersensitivity, represent a group of tests to identify bacterial profiles known as LOPAT (Goszczynska *et al*., 2000).

- **Levan production**:

Due to the action of the enzyme Levan Saccharase, many bacterial species are able to split sucrose into two simple sugars that constitute it: glucose and fructose. While Glucose is used as carbon source, Fructose is polymerized in an extracellular polysaccharidic form, called Levan, which is a polymer of β-D-Fructose. Levan colonies are characterised by whitish dome-shaped mucous (Klement *et al*., 1990).

To verify this capacity for the selected natural bacterial antagonists aliquots of their culture were streaked, through a sterile loop for bacteriology, in Petri dishes with nutrient agar medium containing 5% Sucrose (NAS) and having the following formula per litre of distilled water:

- Lab Lemco Broth (OXOID) 8,00 g
- Sucrose 50,00 g
- Technical Agar № 3 (OXOID) 18,00 g.

The NAS medium was prepared with the same methodology previously described (see § 5.2.1).

The plates were observed after 24 hours of incubation at 26±1 °C.

- **Oxidase reaction**:

Oxygen is the final electron acceptor in some energy transport chains. The presence of the Cytochrome Oxidase enzyme acts as a cytochrome reducer and ensures its transfer.

The presence of cytochrome oxidase is detectable, in some bacteria, with the use of substances that act as electron donors towards the enzyme. The reagent used is N, N, N tetramethyl-p-phenylenediamine dihydrochloride; if the test is positive and the oxidation has occurred, it changes the colour of the absorbent paper on which the bacterium was streaked to violet (Klement *et al*., 1990). For this assay, the selected bacteria were incubated for 24 hours at 26±1 °C on nutrient agar substrate NAD with the following composition per litre of distilled water:

- Lab Lemco Broth (OXOID) 8,00 g
- D-Glucose 10,00 g
- Technical Agar № 3 (OXOID) 18,00 g

The growing medium was prepared with the same methodology previously described (see § 5.2.1).

After 24 hours, using a sterile platinum loop, an aliquot of bacterial culture has been streaked on filter paper soaked with the aqueous solution of 1% (weight/volume) of N, N, N tetramethyl-p-phenylenediamine dihydrochloride. For this assay *P. fluorescens* (DPP 334) and *P. syringae* pv. *syringae* (DPP 358) were respectively used as positive and negative control.

- **Potato rot**:

The potato rot assay (Dickey & Kelman, 1988) is commonly used to detect the presence of pectic enzymes, which is able to cause the development of soft rot on different plant organs, in bacterial cells. In order to remove any residues, healthy potato tubers were washed with SDW; then they were properly disinfected with ethyl alcohol, flamed, peeled and cut into 5-6 mm slices. These, obtained under sterile conditions, were placed in Petri dishes containing sterile absorbent paper soaked with SDW. Each slice was incised and inoculated with one of the bacterial isolates. The plates were placed at 26±1 °C and monitored for 48 hours.

Clavibacter michiganensis subsp. *michiganensis* (DPP 21) and *Pseudomonas viridiflava* (PPD 321) were respectively used as negative and positive control.

- **Arginine dihydrolase production**:

The arginine dihydrolase assay allows to detect the presence of some enzymes able to produce Adenosine triphosphate (ATP) from the degradation of arginine, with the consequent formation of NH_3 and CO_2. These enzymes allow certain bacteria to grow under anaerobic conditions. In case of a positive result, the alkaline reaction resulting from the formation of NH_3 changes the colour of the substrate from yellow-orange to dark pink (Klement *et al*., 1990). Thornley 2A was used as a medium with the following formula per litre of distilled water:

- Proteose peptone 1,00 g
- Sodium chloride 5,00 g
- Potassium hydrogen phosphate 0,30 g
- Technical Agar №3 (OXOID) 3,00 g
- Phenol Red 0,01 g
- Arginine HCl 10,00 g

The growing medium was prepared with the same methodology previously described (see § 5.2.1).

200 µl of bacterial suspension $1x10^8$ CFU/ml (see § 5.2.1) of each bacterium were placed in a 1.5 ml Eppendorf, containing 1 ml of Thornley 2A. Subsequently, in order to determine anaerobic conditions, 300 µl of sterile Vaseline oil were added to all the Eppendorf, which were then placed at 26±1 °C for 24 hours. The negative control used was *C. m.* subsp. *michiganensis* (DPP 21), the positive one was *P. viridiflava* (DPP 321).

- **Tobacco hypersensitivity**:

The immediate response of a plant to a pathogenic infection with the targeted death of the invaded tissues is called hypersensitivity reaction and it is an active defence mechanism by which the infective agent is confined to a limited necrotic area (Klement, 1982).

A bacterial suspension $1x10^8$ CFU/ml (see § 5.2.1) of each selected isolate, was injected into the leaf parenchyma of tobacco plants cv. Virginia Bright at the fourth-fifth true leaf stage (Klement, 1963) using a sterile insulin syringe having needle 0,3 x 13 mm. After 24 hours it was possible to observe, in the inoculated portions of the leaf, the presence or absence of necrotic areas. The negative control used was *C. m.* subsp. *michiganensis* (DPP 21), while the positive one was *P. viridiflava* (DPP 321).

5.2.5 Molecular identification

- **Extraction of DNA**:

The genomic DNA of each isolate was extracted from individual colonies grown for 48 hours at 28 °C on KB medium. Approximately $2x10^9$ cells were used and their DNA was extracted using the PureLink™ Genomic DNA Kit (Invitrogen, USA) following the manufacturer's specifications for the Gram- and Gram+ bacteria. DNA was then resuspended in TE-buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). DNA was then stored at 4 °C (or -20 °C for longer storage times) until required.

- **Amplification by PCR (16S rDNA)**:

Amplification of the 16S ribosomal DNA gene sequence was performed by PCR (Polymerase Chain Reaction) using universal primers specific for bacteria designed on the sequence of interest:

27F (forward) 5'-AGAGTTTGATCCTGGCTCAG-3';
1492R (reverse) 5'-GGTTACCTTGTTACGACTT-3'.

In order to optimize the working times and to standardize the experiment, the PCR analysis was conducted using the GoTaq Colorless MasterMix (Promega Corporation, WI).

For each isolate a reaction solution was prepared with: 2X Go Taq MasterMix (2X Reaction Buffer, 1.25 units of Taq DNA Polymerase, 30 mM Tris-HCl pH 8.3, 125 mM KCl, 3.75 mM $MgCl_2$, 500 μM for each dNTP), 1 μl of each primer (200 nM), between 25-35 ng of genomic DNA (template DNA) and SDW for a final volume of 25 μl. For each experiment a negative control was used, i.e. in the reaction solution the template DNA was replaced with SDW.

The conditions of amplification, which was performed in a C1000 thermal cycler (Bio-Rad Laboratories srl, Milan, Italy), was characterized by an initial stage of 12 min. at 94 °C (initial denaturation of template DNA and activation of Taq polymerase), a 30 cycles intermediate stage with a denaturation step at 94 °C for 30s, *annealing* at 50 °C for 45s and extension at 72 °C for 1.5 min., and a final extension stage at 72 °C for 12 min.

- **Amplicon quantification**:

The concentration of the amplified DNA, and its quality, were determined for the subsequent sequencing. For each sample, the amplified product was separated by horizontal electrophoresis on agarose gel 1% (10 g/l) in 1X TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA). The UV detection was carried out, after electrophoresis, by staining with GelRed™ (Bioticum Inc., CA). Each experiment was repeated at least twice to confirm the reproducibility of the profiles obtained.

The concentration was estimated with the help of an optical fluorimeter (Qubit™, Invitrogen, Life Tecknologies Italy, Monza), suitably calibrated and eventually adjusted to a final concentration of 25 ng/μl.

- **Sequencing and sequence analysis**:

Amplificated products were finally sequenced by Macrogen Europe Company (Macrogen, 2014).

In order to determine their approximate taxonomic placement, the sequences, in the form of electropherograms, were edited manually and subsequently compared with those available in the GenBank database using BLAST (Basic Local Alignment Search Tool) in the NCBI website (Natiolal Center for Biotechnology Information). In cases of homology greater than 99% it was possible to attribute to the isolated its taxonomic allocation.

5.2.6 *In planta* antagonism

These experiments were carried out using potted plants (Ø 18 cm) of *Actinidia deliciosa* cv. Hayward placed in controlled environment (phytotron) set as follows:

- *first step*: 20 °C for 4 hours of light;
- *second step*: 26 °C for 4 hours of light;
- *third step*: 15 °C for 8 hours of darkness;
- *fourth step*: 22 ° C for 8 hours of light.

The relative humidity was above 75% during the whole experiments.

Each thesis was composed by six plants and as negative and positive controls, 2 theses were treated by SDW and by Psa CFPB 7286 respectively.

A bacterial suspension $1x10^7$ CFU/ml of each antagonist was manually sprayed with a vaporizer on the phyllospheres to obtain a complete wetting of the leaves. After 24 h, using the same procedure, a bacterial suspension $1x10^7$ CFU/ml of strain Psa 7286 CFPB was sprayed on the same leaves.

Samplings were carried out after 3, 7 and 15 days of the treatment with Psa. Six leaves were randomly collected from each thesis and individually washed in sterile plastic bags containing 10 ml of SDW with a Seward Stomacher 400 Circulator® (Fig. 15). Subsequently, 100 µl of bacterial suspension from each bag were plated on NA medium (see §5.2.1) using a Spiral Plater® (Fig.16). The Petri dishes were placed at 26±1 °C for 24 hours and then observed with a stereomicroscope in order to count the colonies and, thus, obtain the CFU with the Spiral Plater®'s algorithmic system.

The colony forming units (CFU) per unit of surface (cm^2) were obtained on the basis of leaf surface calculated with a dedicated software (Leaf Area software), (Donegan *et al*., 1991).

Data obtained were subjected to statistical analysis two-way ANOVA followed by Bonferroni post-test, using the GraphPad Prism 5.0 software.

Figure 15. Seward Stomacher 400 Circulator®.

Figure 16. Spiral Plater®.

5.2.7 Epiphytic survival of antagonists

This experiment was carried out using potted plants (Ø 18 cm) of *Actinidia deliciosa* cv. Hayward placed in controlled environment (phytotron) with the following photoperiod:

- *first step*: 20 °C for 4 hours of light;
- *second step*: 26 °C for 4 hours of light;
- *third step*: 15 °C for 8 hours of darkness;
- *fourth step*: 22 ° C for 8 hours of light.

The relative humidity was above 75% during the whole experiments. Each thesis was constituted of nine plants.

A bacterial suspension ($1x10^7$ CFU/ml) of each antagonist was manually distributed with a vaporizer on the phyllospheres to obtain a complete wetting of the leaves.

Samplings were carried out after 4 hours, 3, 7 and 15 days of the treatment with Psa. Nine leaves were randomly collected from each thesis and individually washed in sterile plastic bags containing 10 ml of SDW with a Seward Stomacher 400 Circulator®.

Subsequently, 100 µl of bacterial suspension from each bag were plated on NA medium (see §5.2.1) using Spiral Plater®.

The Petri dishes were placed at 26±1 °C for 24 hours and then observed with a stereomicroscope in order to count the colonies and, thus, obtain the CFU with the Spiral Plater®'s algorithmic system.

The colony forming units (CFU) per unit of surface (cm^2) were obtained on the basis of sample leaf surface calculated with dedicated software (Leaf Area software), (Donegan *et al*., 1991).

Data obtained were subjected to statistical analysis two-way ANOVA followed by Bonferroni post-test, using the GraphPad Prism 5.0 software.

5.3 Results

5.3.1 Natural antagonists selection

During the first stage of the research 60 potential natural enemies were selected from all isolations and *in vitro* tests carried out. Subsequently, on the basis of antagonism assays, 6 of them (A1, A2, A3, A4, A5, A6) were chosen as potential biocontrol agents (BCAs).

5.3.2 Production of fluorescent pigments on King's B

Bacterial isolates A1, A4 and A6 showed production of fluorescentpigments; vice versa A2, A3 and A5 isolated resulted negative (Fig. 17).

Figure 17. Strain positive for the production of fluorescent pigments on KB. A6 isolate on the top of Petri dish; positive control *P. fluorescens* on the left; negative control *A. tumefaciens* on the rigth.

5.3.3 Solubility assay in KOH

Bacterial isolates A1, A4 and A6 showed increase of viscosity, proving to be Gram-; A2, A3 and A5 proved to be Gram+ (Fig. 18).

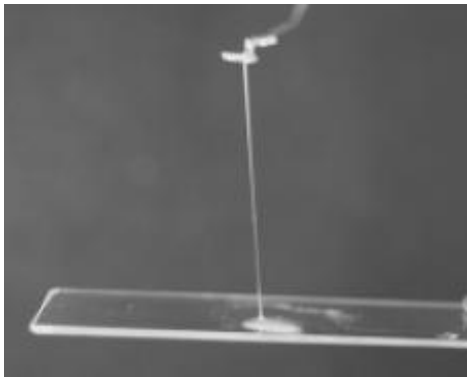


Figure 18. Solubility test in KOH; Gram- strain (from Goszczynska *et al*., 2000).

5.3.4 LOPAT tests

Bacterial isolates A2, A3 and A5 were negative for all five tests of LOPAT; A1 and A6 isolates were negative for the production of levan colonies, potato rot and tobacco hypersensitivity, while they were positive for oxidase reaction and arginine dihydrolase production. A4 was negative for potato rot and tobacco hypersensitivity, while it was positive for levan colonies, oxidase reaction and arginine dihydrolase production (Tab. III, Fig. 19-23).

	Biocontrol agents (BCAs)					
	A2	A3	A5	A1	A6	A4
Levan	-	-	-	-	-	+
Oxidase	-	-	-	+	+	+
Potato	-	-	-	-	-	-
Arginine	-	-	-	+	+	+
Tobacco	-	-	-	-	-	-

Table III. LOPAT profiles of natural antagonists.

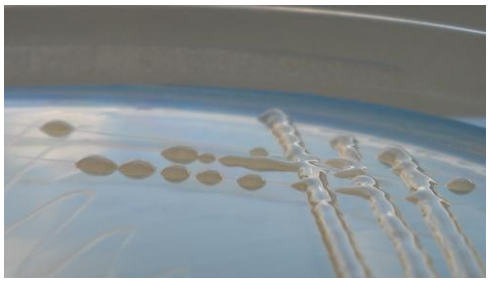


Figure 19. Positive bacterial strain for Levan production.

Figure 20. Oxidase reaction test. Positive (left) and negative (right) bacterial strains.

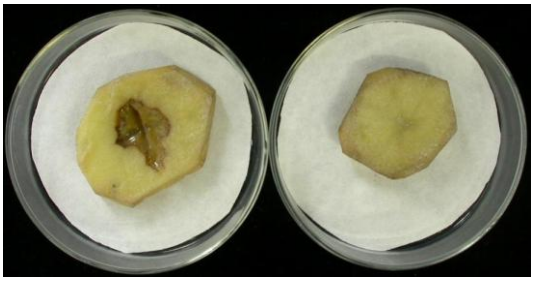


Figure 21. Potato rot test. Positive (left) and negative (right) bacterial strains.

Figure 22. Arginine dihydrolase production test. Positive (left) and negative (right) bacterial strains.

Figure 23. Tobacco hypersensitivity reaction test. Positive (left) and negative (right) bacterial strains.

5.3.5 Molecular identification

The comparative analysis in GenBank of the profiles obtained from the sequencing of the bacteria of interest has indicated that the 6 isolates belong to the following species:

Isolate A1 - similarity of 99.9% (1382/1383 identical nucleotides) with different deposited sequences, belonging to the species *Pseudomonas fulva* (PfI);

Isolate A2 - similarity of 99.9% (1366/1367 identical nucleotides) with different deposited sequences, belonging to the species *Bacillus amyloliquefaciens* (BAR);

Isolate A3 - similarity of 99.9% (1348/1349 identical nucleotides) with different deposited sequences, belonging to the species *Bacillus amyloliquefaciens* (BAY);

Isolate A4 - the sequence obtained was readable only in one direction (forward) with only 695 nucleotides; they nevertheless showed a similarity of 100% (695/695 identical nucleotides) with different deposited sequences, all belonging to the genus *Pseudomonas*, the species of which has not been defined; however most of them have been isolated from aqueous matrices (Psp);

Isolate A5 - similarity of 100% (1272/1273 identical nucleotides) with different deposited sequences, belonging to the species *Bacillus subtilis* (Bs);

Isolate A6 - similarity of 99.9% (1389/1390 identical nucleotides) with different deposited sequences, belonging to the species *Pseudomonas fulva* (PfII);

Being there a high homology of the sequences (99-100%) with those present in the NCBI international database, it has been possible to assign isolates to different species with reasonable certainty (Tab. IV).

№	Biocontrol agents (BCAs)	Origin	Year	Host
1	*Pseudomonas fulva* (PfI)	Italy	2012	Hort 16A
2	*Pseudomonas fulva* (PfII)	Italy	2012	Hort 16A
3	*Pseudomonas* sp. (Psp)	Italy	2012	Hayward
4	*Bacillus amyloliquefaciens* (BAR)	Italy	2012	Hayward
5	*Bacillus amyloliquefaciens* (BAY)	Italy	2012	Hayward
6	*Bacillus subtilis* (Bs)	Italy	2012	Hayward

Table IV. Identification of the 6 potential BCAs for Psa.

5.3.6 *In planta* antagonism

The *in planta* antagonism tests have shown that, at 3, 7 and 15 days post inoculation, all 6 antagonists (vs. Psa 7286 CFPB) showed a significant decrease of Psa's CFU compared to the control test (only PSA 7286 CFPB).

As shown in Figures 24, 25 and 28, 3 days after the distribution of the bacteria, in theses treated with BAR, BAY and PfII BCAs, CFU of Psa showed a decrease, compared to control, between 19% and 20%.

After 3 days PfI had obtain a Psa reduction of 18.11% compared to the control thesis (Fig. 27). Conversely, in the theses with Bs and Psp, the reduction of Psa was respectively of 11.32% and 12.45% (Fig. 26, 29).

After 7 days Bs BCA showed the same activity (11.81%) in respect of the pathogen (Fig. 24). After 7 days a decrease of about 18% was observed in the theses with BAY, PfII and Psp BCAs (Fig. 25, 28, 29).

The PfI and BAR BCAs, after 7 days, were able to determine a reduction of Psa of 24.41% and 33.90%, respectively (Fig. 24, 27). In all the theses where the antagonists were still present 15 days after inoculation, Psa was dead, while in the control thesis the record of Psa was about $1x10^1$ CFU/cm^2 (Fig. 24-29).

In each thesis the BCA at 3, 7 and 15 days showed a population density of around $1x10^3$ CFU/cm^2. However, the *Bacillus* strains, in respect of Gram- BCAs, showed a slight decrease 15 days after the inoculation (Fig. 24-29).

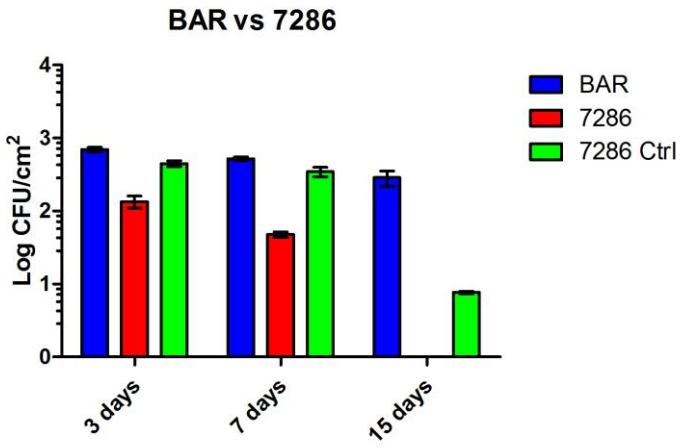


Figure 24. Natural antagonist BAR vs. 7286 CFPB *in planta*. Errors bars indicated the standard error.

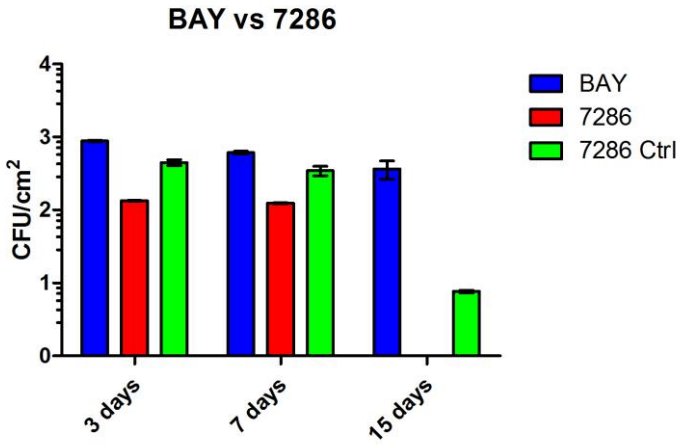


Figure 25. Natural antagonist BAY vs. 7286 CFPB *in planta*. Errors bars indicated the standard error.

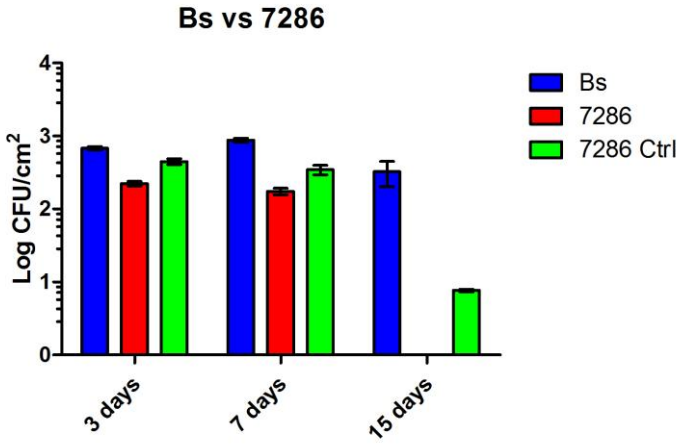


Figure 26. Natural antagonist Bs vs. 7286 CFPB *in planta*. Errors bars indicated the standard error.

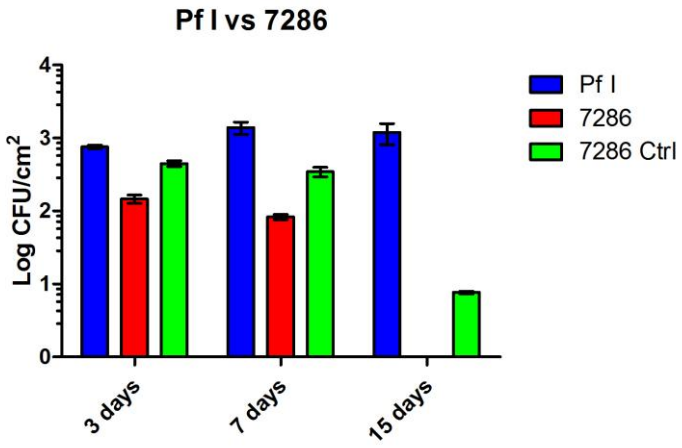


Figure 27. Natural antagonist PfI vs. 7286 CFPB *in planta*. Errors bars indicated the standard error.

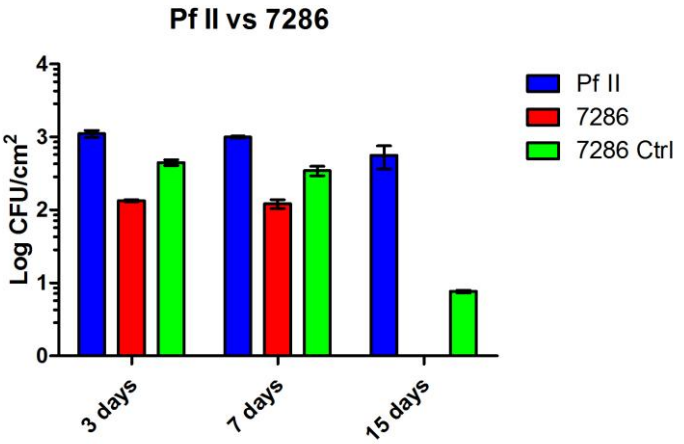


Figure 28. Natural antagonist PfII vs. 7286 CFPB *in planta*. Errors bars indicated the standard error.

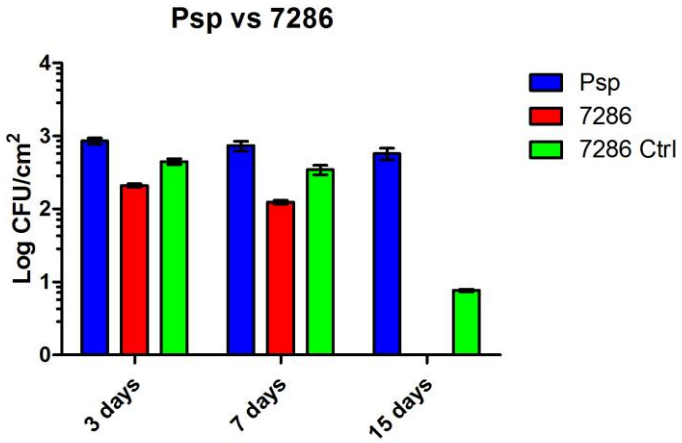


Figure 29. Natural antagonist Psp vs. 7286 CFPB *in planta*. Errors bars indicated the standard error.

5.3.7 Epiphytic survival of antagonists

From these tests emerged the 6 BCAs ability to survive on the phylloplane for more than 15 days.

The three Gram+ strains, BAR, BAY and Bs (Fig. 30), 4 h after the inoculation had a density between $1x10^4$ and $1x10^5$ CFU/cm^2. After 3 days they showed a decrease of about 1-2 log units which; however, this density remained costant until day 15 (range: $1x10^3$ – $1x10^4$ CFU/cm^2). Among them, BAR showed a better trend with an increase in population between days 7 and 15 (Fig. 30).

Gram- strains, PfI, PfII and Psp, 3 days after the treatment showed a reduction of CFU/cm^2 of 1 log unit compared to the detection performed 4 h after the treatment. They also maintained their population density around $1x10^3$ CFU/cm^2 at days 7 and 15 (Fig. 31).

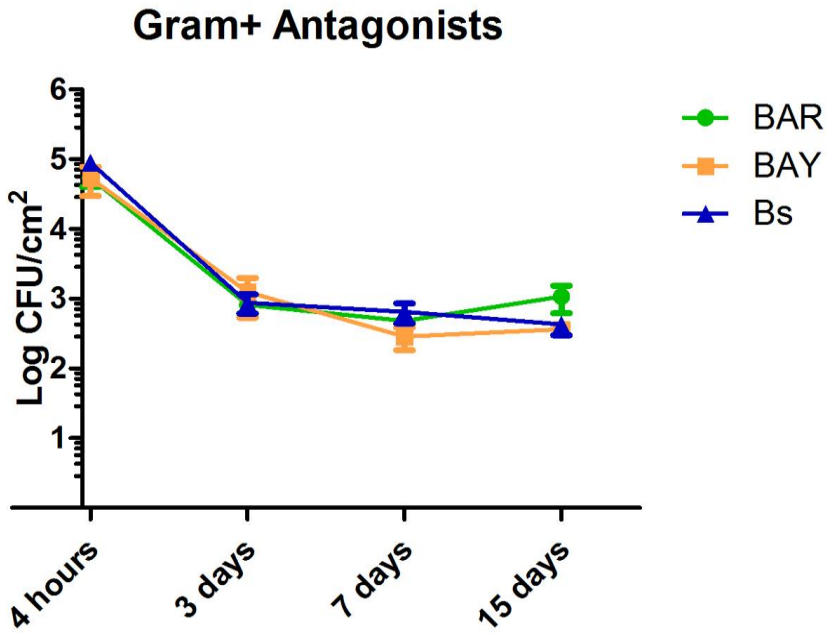


Figure 30. Epiphytic survival of Gram+ antagonists at 4 h, 3,7 and 15 days. Errors bars indicated the standard error.

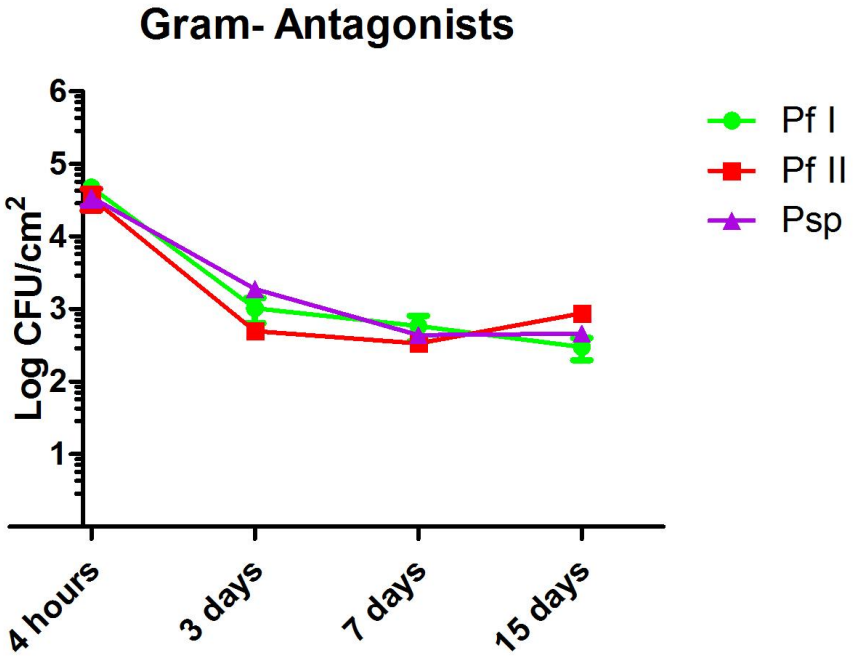


Figure 31. Epiphytic survival of Gram- antagonists at 4 h, 3, 7 and 15 days. Errors bars indicated the standard error.

6. Search of *P. s.* pv. *actinidiae* in China

In order to understand the similarities and/or eventual differences related to Psa populations from International Kiwifruit areas, a stage of study and research was carried out in the "Key Lab of Pomology", Horticulture Department, Anhui Agricultural University, located in Hefei City (People's Republic of China).

Thanks to a collaboration between DAFNE and Anhui University, it was possible to carry out, under the supervision of Prof. Li Wu Zhu, Director of Horticulture Department, a stage of six months (April-September 2012) in the laboratories of Phytobacteriology.

There, have been a deep study about the causal agent of Bacterial canker of *Actinidia*, in Asia its differences with Psa strains from Europe and control strategies utilised against this plant pathogenic bacterium.

China, as well as being the country with the highest production of kiwifruit, constitutes the centre of origin of the latter.

The knowledge-cultivation of *Actinidia* plant in China, the large number of its varieties (Zhixue *et al*., 2002) and recent acquisitions about Psa that indicate China as the country of its origin (Mazzaglia *et al*., 2012), made the site chosen particularly suitable to investigate the issues relating to our researches.

6.1 Materials and Methods

The experiments were carried out in orchards of *Actinidia* spp., of different cultivars and in different provinces of Republic of China.

6.1.1 Isolation of Psa

The organs collected in field (leaves and branches), from plants affected by Bacterial Canker of Actinidia, were immediately transported to the laboratory.

In order to isolate the pathogen, was developed a semi-selective medium for Psa having the following composition:

- Deionized water 450 ml
- Glycerol 4.5 g
- Pseudomonas Agar f (Merck) 17.1 g
- Technical Agar № 3 (Oxoid) 4.05 g
- The 1.5% solution of boric acid 50 ml
- Solution of cycloheximide in ethanol at 2.5% 4 ml
- Solution of cephalexin in water at 1% 4 ml

The medium, was sterilized by autoclaving at 121 °C for 20 minutes.

In the meantime were added, by sterile filters, 4 ml of cycloheximide solution and 4 ml cephalexin solution, respectively in 50 ml of boric acid solution. The latter was then added to the medium previously cooled up to 50 °C.

Under sterile conditions, 18 ml of medium were poured per Petri dish. At occurred solidification the plates were placed for 24 h, at a temperature of 26±1 °C and then stored at 4 °C in sterile bags.

From infected organs collected, portions at the edge of the symptoms, in leaves and woody parts, were obtained.

The leaf tissues were disinfected through immersion in an aqueous solution of sodium hypochlorite at 0.5% for 2-5 min. and then rinsed few times in SDW in order to remove all traces of disinfectant. After washing, the tissues were macerated, by using a sterile scalpel, in 200 µl of SDW. An aliquot of this suspension was then streaked on the semi-selective medium previously described (see § 6.1.1).

After the plates were incubated at 26±1 °C for 24-48 h. the developed bacterial colonies morphologically similar to Psa were selected and restreaked on KB medium in order to be purified and then they were subjected to molecular analysis for their identification.

6.1.2 Molecular identification

- **Extraction of DNA**:

The genomic DNA of each isolate was extracted from individual colonies grown for 48 hours at 28 °C on nutrient agar substrate KB. Approximately $2x10^9$ cells were used and their DNA was extracted using the PureLink™ Genomic DNA Kit (Invitrogen, USA) following the manufacturer's specifications for the Gram-.

The DNA concentration was estimated through the use of fluorimeter (Qubit™, Invitrogen, Life Tecknologies Italy, Monza) and adjusted to a final concentration of 50 ng/µl with TE-buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). DNA was then stored at -20 °C until required.

- **Amplification by PCR**

For the identification of PSA were used two pairs of pathovar specific primers, described by Rees-George *et al*. (2010):

PsaF1 (5'-TTTTGCTTTGCACACCCGATTTT-3')

PsaR2 (5'-CACGCACCCTTCAATCAGGATG-3')

and described by Koh & Nou (2002):

F (5'-ACAGATACATGGGCTTATGC-3')

R (5'-CTTTTCATCCACACACTCCG-3').

Every reaction consisted in a total volume of 50 µl containing 25 µl of 2X GoTaq Colorless MasterMix (Promega Corporation, WI), 1 µl of template DNA, 1 µM of each primer and sterile distilled water to bring to volume.

In each experiment was added a positive control, represented by Psa 7286 CFPB, and a negative control, reaction without DNA template.

The condition of amplification applied, performed in C1000 thermal cycler (Bio-Rad Laboratories srl, Milan, Italy), was characterized for the pair of primers of Rees-George *et al*. from an initial stage at 95 °C for 2 min (initial denaturation of template

DNA), an intermediate stage of 30 cycles having a denaturation step at 95 °C for 30s, *annealing* at 65 °C for 30s, and extension at 72 °C for 30s, and a stage of final extension at 72 °C for 5 min.

For the pair of primers of Koh & Nou has been set one initial stage at 94 °C for 5 min., 33 cycles of an intermediate stage having a denaturation step at 94 °C for 30s, *annealing* at 60 °C for 30s, and extension at 72 °C for 45s, and a stage of final extension at 72 °C for 5 min.

The two pairs of primers specific for Psa have an expected amplicon of 280 bp and 492 bp respectively (Fig. 32-33).

- **Electrophoresis**

For each sample, the amplified product was separated by horizontal electrophoresis on a 2% agarose gel (20 g/l) in 1X Tris-acetate-EDTA buffer. The UV detection was carried out, after electrophoresis, by staining with GelRed™ (Bioticum Inc., CA) (Fig. 32-33).

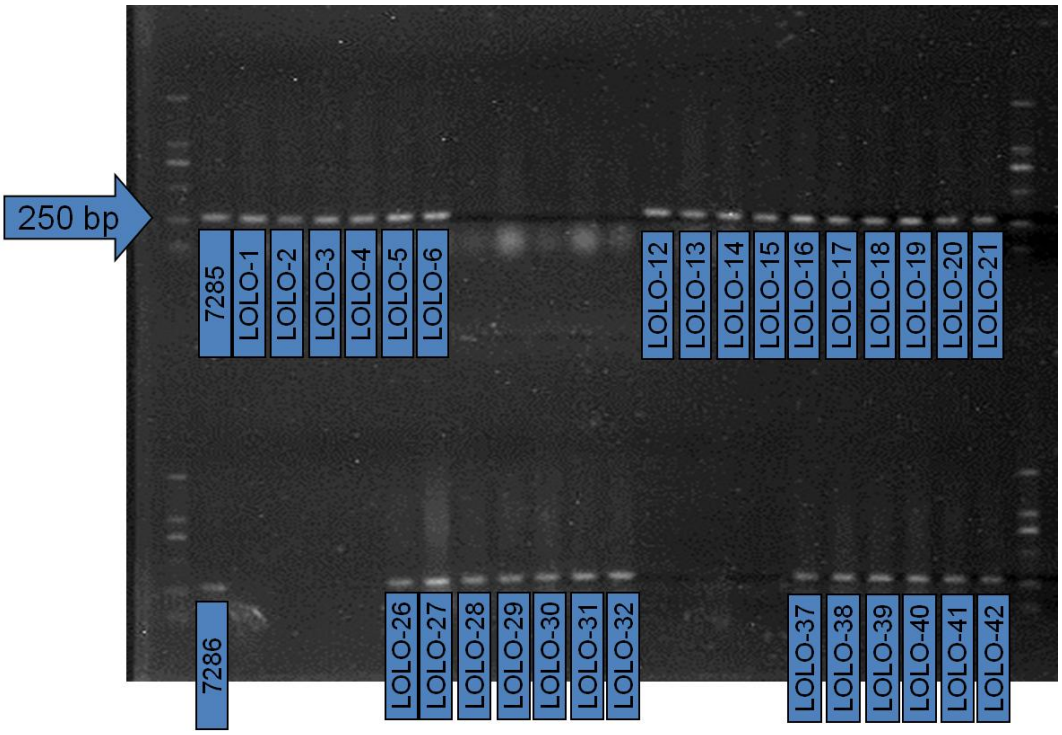


Figure 32. Molecular identification of Psa LOLO group by use of Rees-George *et al*. primers.

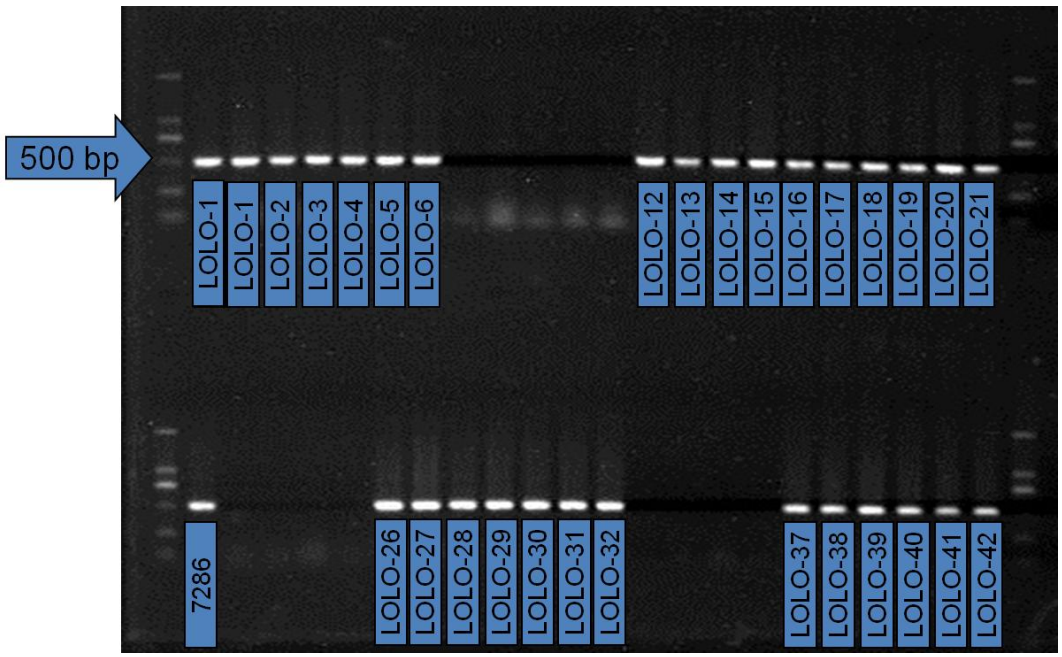


Figure 33. Molecular identification of Psa LOLO group by use of Koh & Nou primers.

6.2 Results

From the samplings it was possible to isolate more than 400 bacterial strains and, through their molecular identification, 52 of them, from different areas of China, *Actinidia* species and cultivars, were identified as Psa (Fig. 34):

- In Anhui province, (Yuexi city) were obtained 10 isolates of Psa from *Actinidia chinensis* cv. Jin Feng (4 from branches, 6 from leaves), 1 isolated from *Actinidia* wild type (Fig. 35) (from branch) and 1 from *Bothrogonia ferruginea* F. [thanks to Dr. Liu Pu, Anhui Agricultural University, Hefei City (People's Republic of China), for identification] (Group JILO).

- In Shaanxi province, (Xi'an city) were obtained 8 isolates of Psa from *Actinidia deliciosa* cv. Hayward (from leaves) (Group HAXA).

- In Sichuan province, (Mian Yang city) were obtained 4 isolates of Psa from *Actinidia chinensis* cv. Hong Yang (from leaves) (Group HYM).

- In Gui Zhou province, (Xiu Wen city) were obtained 29 isolates of Psa from *Actinidia deliciosa* cv. Guichang (23 from branches and 6 from leaves) (Group LOLO).

1: Anhui province, Yuexi city, cv. JinFeng.
2: Shaanxi province, Xi'an city, cv. Hayward.
3: Sichuan province, Mian Yang city, cv. Hong Yang.
4: Guizhou province, Xiu Wen city, cv. Guichang

Figure 34. Map of China with areas in which were isolated Psa strains.

Figure 35. *Actinidia* wild type plant.

7. Selection of *P. s.* pv. *actinidiae* from different geographical areas and *in vitro* antagonism

To evaluate the possible differences between Psa from different geographic areas, was done a selection among the 52 Chinese Psa strains and from those of the collection at DAFNE, University of Tuscia (Italy).

The choice of strains was based primarily on their geographic origin, as well as in respect of their year of isolation.

Psa strains were selected from the follow areas:

- Asian area (origin of the pathogen) including Japan, China, Korea and Turkey;
- European area represented by Italy, France, Spain, Portugal and Switzerland;
- Oceanic area including New Zealand and Australia;
- South American area with Chile.

In detail they were:

2 Japanese strains, 1 of 1984 (KW11) and 1 of 2011 (2818), 3 Koreans, 23663 of 1989, Psa K2 of 1997 and K6 of 2010, 11 Chinese isolates 2 of 2010 (VI and M23) and 9 of 2012 from different provinces (JILO 4, JILO 30, INS, LOLO 4, LOLO 26, HAXA 1, HAXA 3, HYM 2, HYM 3), 1 Turkish strain of 2011 (K2 T), 1 Italian 7286 CFPB of 2008 , 1 French of 2010 (1F), 1 Spanish (827), 1 Portuguese (349), 1 Swiss (3817) all of 2011; 1 New Zealand Low Virulent of 2010 (18804), 2 New Zealand Virulent strains 2011 (18839 and 19200), 1 Australian Low Virulent strain of 2011 (19440) and 2 Chileans of 2012 (19439 and 19456) (Tab. V).

№	Strains	Origin	Year
1	KW 11	Japan	1984
2	2818	Japan	2011
3	23663	Korea	1989
4	K6	Korea	2010
5	Psa K2	Korea	1997
6	VI (CH2010-6)	China	2010
7	M 23	China	2010
8	JILO 4	China	2012
9	JILO 30	China	2012
10	INS	China	2012
11	LOLO 4	China	2012
12	LOLO 26	China	2012
13	HAXA 1	China	2012
14	HAXA 3	China	2012
15	HYM 2	China	2012
16	HYM 3	China	2012
17	K2 T	Turkey	2011
18	7286	Italy	2008
19	1F	France	2010
20	827	Spain	2011
21	349	Portugal	2011
22	3817	Swiss	2011
23	18804 (LV)	New Zealand	2010
24	18839 (V)	New Zealand	2011
25	19200 (V)	New Zealand	2011
26	19440 (LV)	Australia	2011
27	19439	Chile	2012
28	19456	Chile	2012

Table V. Selected strains of Psa. V = Virulent strain; LV = Low Virulent strain.

7.1 Materials and Methods

7.1.1 Production of fluorescent pigments on King's B

It was used the same procedure described in the paragraph § 5.2.2.

7.1.2 LOPAT tests

For these assays were applied the same protocols described in paragraph § 5.2.4.

7.1.3 *In vitro* antagonism

With the availability of Psa isolates from different world Kiwifruit areas, it was possible to evaluate the effectiveness of the 6 selected natural antagonists (PfI, PfII, Psp, BAR, BAY, Bs) against the 28 Psa selected strains (Tab. V).

The different Psa strains, were placed, by using a Spiral Plater®, on NA medium (100 µl of each bacterial suspension at $1x10^6$ CFU/ml).

Subsequently, five spots of 10 µl each were made, of which 4 were filled with the bacterial suspension ($1x10^8$ CFU/ml) of the BCA and 1 with SDW, as a control. 5 plates (20 replicates) per each BCA were developed.

The plates were then placed at 26±1 °C. At 24 and 48 h were observed, by a stereomicroscope, to measure the inhibition areas in respect of each Psa strain.

7.2 Results

7.2.1 Production of fluorescent pigments on King's B

The results showed that the strain isolated in Turkey (K2T) and the 2 Low Virulent (LV) strains, New Zealand 18804 and Australian 19440, had a positive response to the production of fluorescent pigments, while, the other Psa strains resulted negative.

7.2.1 LOPAT tests

All 28 selected strains were positive for the production of levan colonies and tobacco hypersensitivity.

Instead gave a negative response for oxidase reaction, potato rot and arginine dihydrolase production (+ - - - +).

7.2.3 *In vitro* antagonism

Among the Gram+ antagonists, the BAR (Fig. 43) and BAY strains showed their activity on all 28 Psa strains of different geographic areas; they determined an average Zone Of Inhibition (ZOI) of 3.029 ± 0.125 mm and 3.080 ± 0.097 mm, respectively (Tab. VI; Fig. 39-40). Bs showed activity in respect of KW 11, 2818, JILO 4 and 7286 with an average ZOI of 3.884 ± 0.642 mm (Tab. VI; Fig. 41).

Gram- BCAs PfI and PfII (Fig. 42) had effectiveness to inhibit the growth of all isolates of Psa with an average ZOI of 5.411 ± 0.371 mm and 5.879 ± 0.416 mm respectively (Tab. VI; Fig. 36-37). Psp was able on KW 11, 2818, 23663, K6, Psa K2, VI, JILO 4, JILO 30, INS, LOLO 4, LOLO 26, HAXA 3, 7286, 1F, 827, 349 and 19456 Psa strains with an average ZOI of 4.209 ± 0.465 mm (Tab. VI; Fig. 38).

BCA's / Psa	Zone of Inhibition (ZOI) Mean ± Std. Error					
	PfI	PfII	Psp	BAR	BAY	Bs
KW 11 (320)	6.594 ± 0.366 [a]	7.750 ± 0.296 [a]	2.250 ± 0.470 [c]	2.844 ± 0.118 [c]	2.625 ± 0.133 [c]	4.406 ± 0.251 [b]
2818	4.188 ± 0.241 [bc]	5.000 ± 0.064 [ab]	4.500 ± 0.456 [abc]	3.813 ± 0.187 [c]	2.563 ± 0.120 [d]	5.250 ± 0.199 [a]
23663	4.625 ± 0.290 [b]	6.031 ± 0.264 [a]	4.969 ± 0.226 [b]	2.500 ± 0.102 [c]	3.250 ± 0.158 [c]	0.00 [d]
K6	4.188 ± 0.237 [b]	5.000 ± 0.219 [a]	4.406 ± 0.184 [ab]	3.531 ± 0.191 [bc]	3.438 ± 0.170 [bc]	0.00 [d]
Psa K2	4.156 ± 0.245 [b]	6.563 ± 0.232 [a]	4.813 ± 0.437 [b]	2.500 ± 0.129 [c]	2.375 ± 0.107 [c]	0.00 [d]
VI (CH2010-6)	8.969 ± 0.414 [a]	9.938 ± 0.373 [a]	5.188 ± 0.437 [b]	2.531 ± 0.133 [c]	3.188 ± 0.213 [c]	0.00 [d]
M23	6.156 ± 0.315 [b]	8.094 ± 0.303 [a]	0.00 [e]	2.281 ± 0.120 [d]	3.625 ± 0.174 [c]	0.00 [e]
JILO 4	2.563 ± 0.101 [c]	2.281 ± 0.102 [c]	7.656 ± 0.493 [a]	4.500 ± 0.262 [b]	4.281 ± 0.193 [b]	3.656 ± 0.118 [b]
JILO 30	5.688 ± 0.520 [a]	5.656 ± 0.553 [a]	5.875 ± 0.397 [a]	4.125 ± 0.155 [b]	3.375 ± 0.168 [b]	0.00 [c]
INS	7.906 ± 0.441 [a]	2.813 ± 0.143 [d]	5.719 ± 0.384 [b]	3.969 ± 0.220 [c]	3.719 ± 0.228 [cd]	0.00 [e]
LOLO 4	4.000 ± 0.241 [b]	5.875 ± 0.161 [a]	2.125 ± 0.085 [c]	2.656 ± 0.109 [c]	2.563 ± 0.101 [c]	0.00 [d]
LOLO 26	6.281 ± 0.546 [a]	5.719 ± 0.398 [a]	2.406 ± 0.138 [b]	2.875 ± 0.133 [b]	3.094 ± 0.114 [b]	0.00 [c]
HAXA 1	3.219 ± 0.158 [a]	2.969 ± 0.107 [a]	0.00 [b]	3.063 ± 0.208 [a]	2.938 ± 0.157 [a]	0.00 [b]
HAXA 3	9.250 ± 0.773 [a]	9.531 ± 0.464 [a]	5.469 ± 0.358 [b]	3.219 ± 0.129 [c]	2.719 ± 0.158 [c]	0.00 [d]
HYM 2	3,750 ± 0.151 [a]	3.281 ± 0.137 [ab]	0.00 [d]	2.594 ± 0.131 [c]	3.250 ± 0.137 [b]	0.00 [d]
HYM 3	2.875 ± 0.202 [a]	3.219 ± 0.214 [a]	0.00 [b]	2.906 ± 0.104 [a]	2.844 ± 0.118 [a]	0.00 [b]
K2T	7.844 ± 0.218 [b]	9.063 ± 0.376 [a]	0.00 [e]	2.281 ± 0.091 [d]	3.813 ± 0.203 [c]	0.00 [e]
7286	6.375 ± 0.432 [a]	7.031 ± 0.294 [a]	2.438 ± 0.136 [b]	2.594 ± 0.114 [b]	2.625 ± 0.125 [b]	2.225 ± 0.126 [b]
1F	6.719 ± 0.353 [b]	8.313 ± 0.312 [a]	4.144 ± 0.204 [c]	2.719 ± 0.091 [d]	2.813 ± 0.143 [d]	0.00 [e]
827	7.906 ± 0.382 [a]	8.688 ± 0.335 [a]	1.125 ± 0.324 [c]	4.031 ± 0.372 [b]	3.250 ± 0.121 [b]	0.00 [d]
349	2.219 ± 0.120 [c]	7.031 ± 0.2641 [a]	1.563 ± 0.699 [c]	3.938 ± 0.269 [b]	2.531 ± 0.125 [c]	0.00 [d]
3817	3.719 ± 0.209 [a]	3.094 ± 0.146 [b]	0.00 [c]	3.000 ± 0.158 [b]	3.050 ± 0.165 [ab]	0.00 [c]
18804 (LV)	5.594 ± 0.210 [a]	6.250 ± 0.306 [a]	0.00 [c]	2.031 ± 0.372 [b]	2.344 ± 0.127 [b]	0.00 [c]
18839 (V)	4.500 ± 0.194 [b]	6.313 ± 0.228 [a]	0.00 [d]	3.500 ± 0.241 [c]	3.563 ± 0.262 [c]	0.00 [d]
19200 (V)	5.969 ± 0.161 [b]	6.750 ± 0.241 [a]	0.00 [d]	3.050 ± 0.216 [c]	3.469 ± 0.235 [c]	0.00 [d]
19440 (LV)	4.781 ± 0.376 [a]	4.438 ± 0.176 [a]	0.00 [c]	2.375 ± 0.097 [b]	2.281 ± 0.091 [b]	0.00 [c]
19439	3.625 ± 0.174 [a]	3.344 ± 0.1628 [a]	0.00 [c]	2.250 ± 0.079 [b]	2.688 ± 0.164 [b]	0.00 [c]
19456	7.844 ± 0.2176 [a]	4.594 ± 0.238 [b]	6.906 ± 0.404 [a]	3.125 ± 0.226 [c]	3.531 ± 0.185 [c]	0.00 [d]

Table VI. Antagonism *in vitro* test. All parameters are expressed in mm as mean ± SE. Values not marked with the same letter are significantly different ($P < 0.05$).

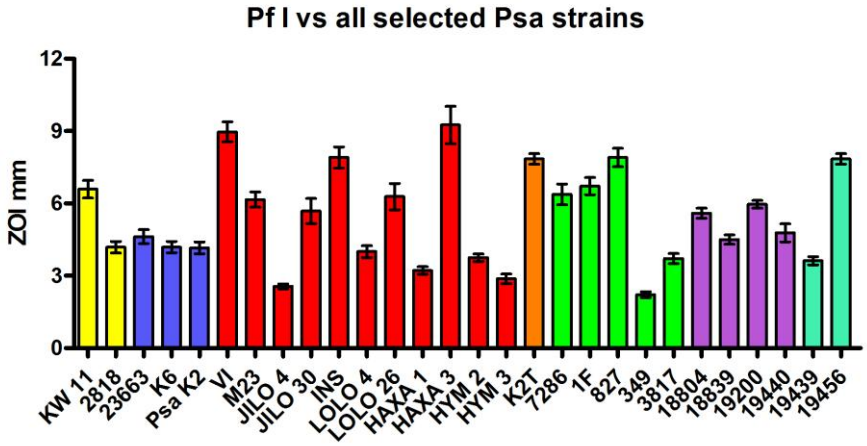


Figure 36. Antagonism *in vitro* test. Zone Of Inhibition (ZOI) of PfI against all selected Psa strains. Errors bars indicated the standard error.

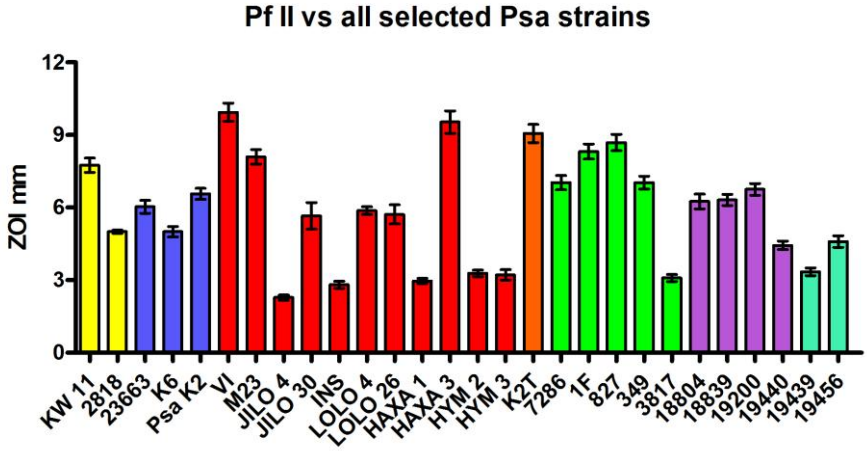


Figure 37. Antagonism *in vitro* test. Zone Of Inhibition (ZOI) of PfII against all selected Psa strains. Errors bars indicated the standard error.

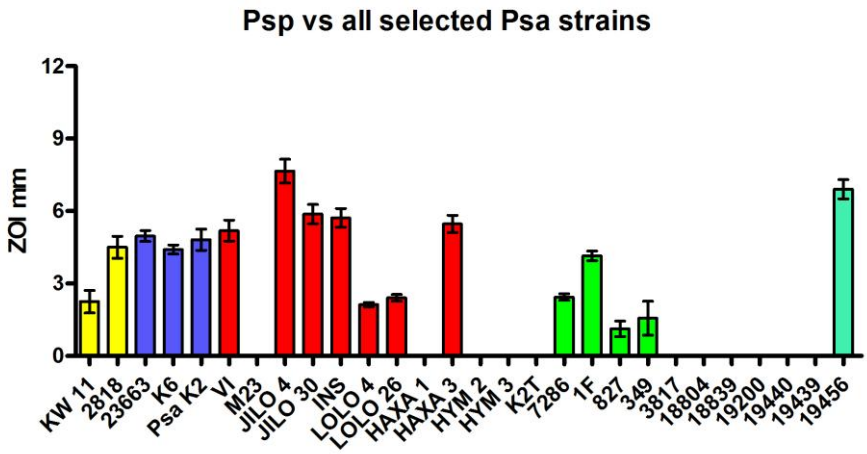


Figure 38. Antagonism *in vitro* test. Zone Of Inhibition (ZOI) of Psp against all selected Psa strains. Errors bars indicated the standard error.

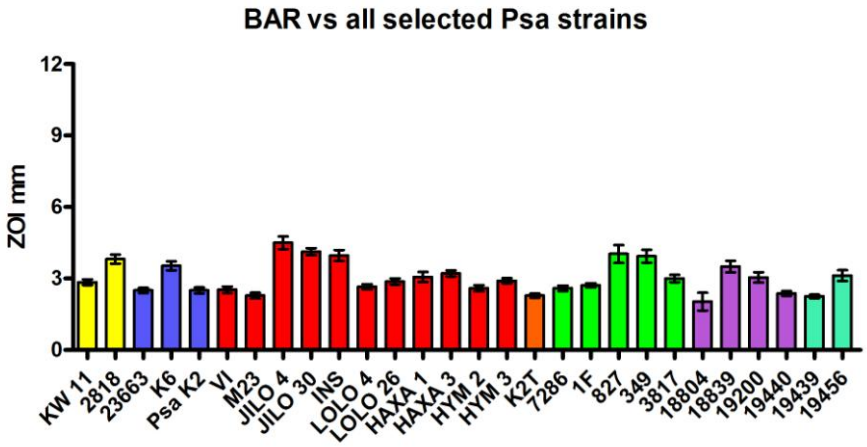


Figure 39. Antagonism *in vitro* test. Zone Of Inhibition (ZOI) of BAR against all selected Psa strains. Errors bars indicated the standard error.

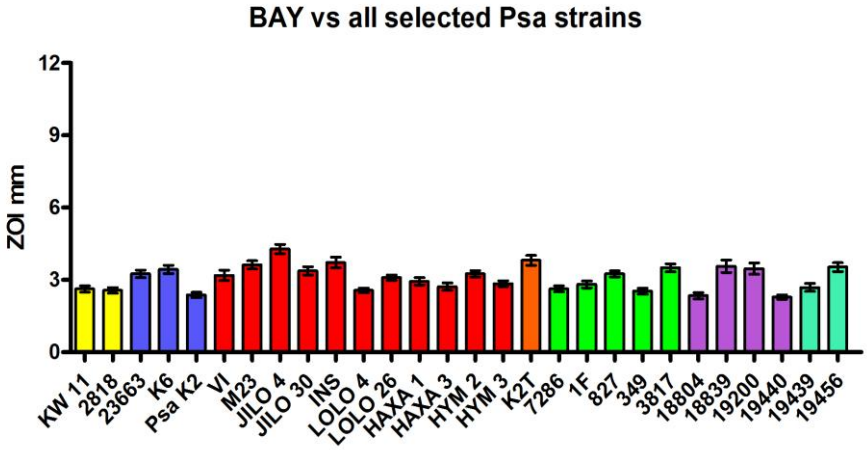


Figure 40. Antagonism *in vitro* test. Zone Of Inhibition (ZOI) of BAY against all selected Psa strains. Errors bars indicated the standard error.

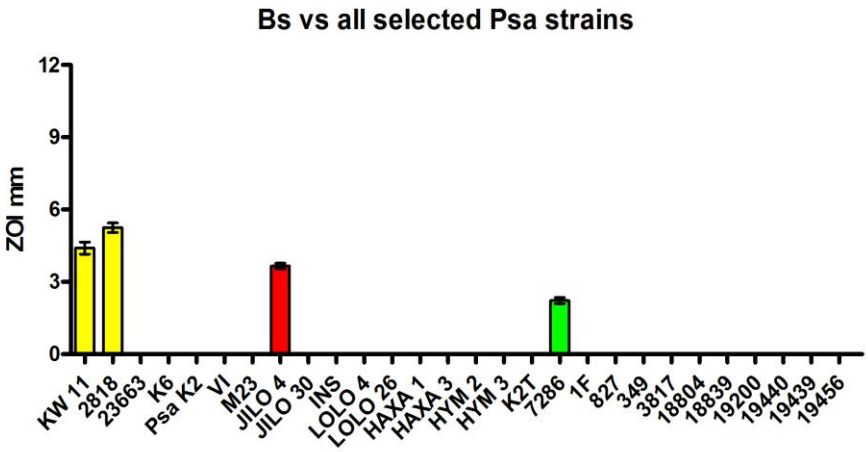


Figure 41. Antagonism *in vitro* test. Zone Of Inhibition (ZOI) of Bs against all selected Psa strains. Errors bars indicated the standard error.

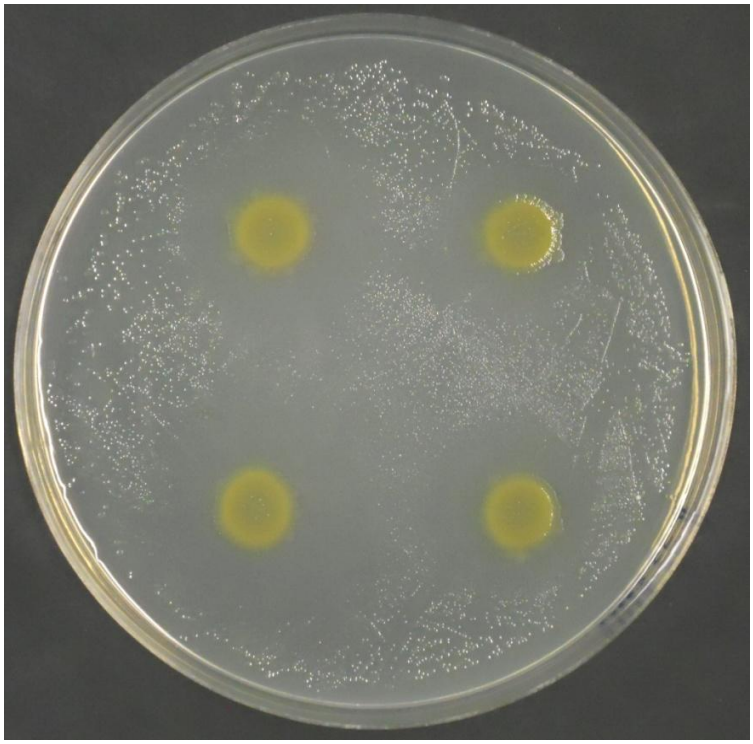


Figure 42. Inhibition halos of PfII.

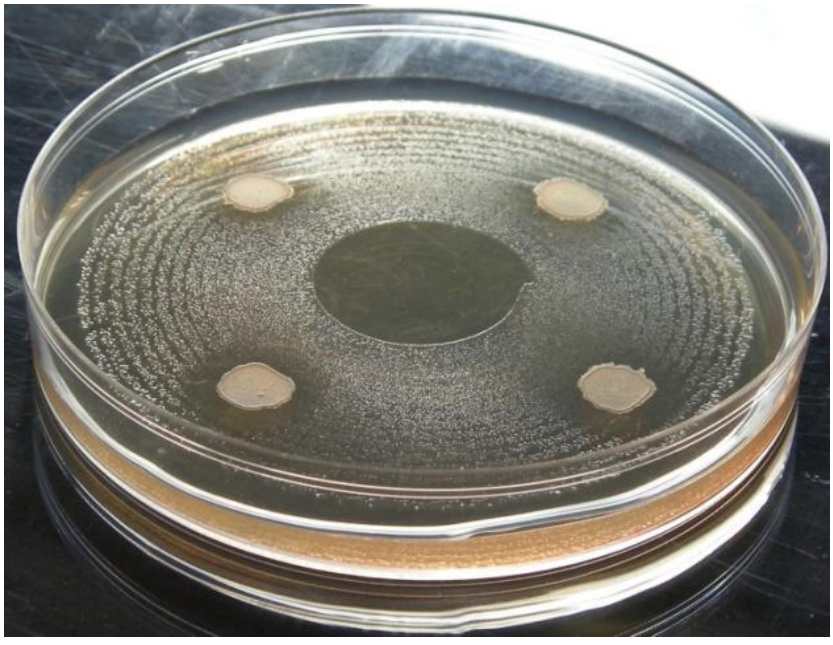


Figure 43. Inhibition halos of BAR.

8. Evaluation of antagonists

Natural antagonists conducted their biocontrol activity mainly through the production of antimicrobial substances and the competition for space and nutrients. If applied on the plants before they are colonized by other pathogen bacteria, antagonistic bacteria with the above mentioned capacities, can rapidly multiply and compete for available resources (carbon sources), and/or activate the production of antimicrobial compounds (Lindow & Brandl, 2003). There are evidences that this type of bacteria allows the formation of numerous and heterogeneous aggregates on the surface of the plants (Lindow, 2002).

The production of extracellular polysaccharides (EPS), which are considered the main components of bacterial aggregates' (biofilms) matrix, favours the epiphyte bacterial populations on the phyllosphere (Costerton *et al*., 1995).

Some bacteria are able to effectively modify their microhabitats, not only through the production of a *biofilm*, but also increasing the availability of nutrients on the phylloplane by producing compounds with *surfactants* properties that can improve the wettability of the leaf surfaces (Buster *et al*., 1989; Schreiber *et al*., 2005).

8.1 Biofilm Formation

With the term bacterial biofilms are indicated community structures of microbial cells included in a self-produced polymeric matrix adherent to an inert or living solid surface (Whitchurch *et al*., 2002).

The free cell growth in the aqueous phase is labelled planktonic; conversely the formation of sessile communities promotes the development of biofilms that act as cooperative consortia (McDougald *et al*., 2012).

Many bacterial species, such as the *Staphylococcus*, *Vibrio*, *Listeria*, and in particular the *Bacillus* and *Pseudomonas* genera, are capable of forming sessile microcolonies.

The microbial cells in biofilms, being in close contact maintain an interdependent behaviour and adopt collective strategies, acting as integral parts of a cooperative consortium (Kjelleberg & Givskov, 2007).

The biofilm microbial cells are able to sense the density of the cellular population, *quorum-sensing*, and accordingly change the expression of specific genes.

To sense the population density of a colony, bacteria use autoinducers (AI), signal molecules produced in very small quantities. AI are diffusible molecules that accumulate and are usually formed chemical mediators known as *N*-acyl homoserine lactones (AHL).

AHL are constituted by a lactone group linked to an acyl chain (amide bond) whose length (4-16 carbon atoms), substitutions and the presence of one or more unsaturated bonds determine the specificity of the signal (Fuqua *et al*., 1996). The most frequent substitutions were found on the third carbon atom of the chain where there may be a carbonyl group, a hydroxyl group or an oxygen atom (Dong *et al*., 2000; Leadbetter & Greenberg, 2000). Homoserine lactones are chemically stable at neutral pH or in acid aqueous solution (Schaefer *et al*., 2000), while in a basic environment the lactone ring is subject to alkaline hydrolysis (Voelkert & Grant, 1970).

The biological functions mediated by cellular communication systems are generally related to numerous responses such as the production of exopolysaccharides (EPS) with possible formation of biofilms, the synthesis of antibiotics, the luminescence, the motility and pathogenicity characteristics (Swift *et al*., 1994; Quinones *et al*., 2003, Shrout *et al*., 2006).

It was also demonstrated that AHL are involved in the regulation of production

of biosurfactants compounds (Ron & Rosemberg, 2001; Di Mattia, 2011).

The development of biofilms occurs in 5 steps:

1- conditioning of the surface with organic and inorganic molecules that can be adsorbed in inert materials such as glass, polystyrene and on plant tissues such as rhizoplane and phylloplane;
2- Translocation of planktonic cells on the surface;
3- Adhesion of the first layer of cells through pili, surface proteins, flagella and flagellar adhesins;
4- Irreversible anchorage of cells, which multiplying fix themselves to the substrate through the production of extracellular material, adhering to the surface through covalent chemical bonds. The produced EPS contribute to the cellular microaggregation as well as to the substrate adherence;
5- Colonization of the surface and formation of mature biofilms. Starting with the first layer of cells that proliferate, stratify and gradually die, the biofilms mature releasing disaggregated parts and planktonic cells.

The bacterial cells are in contact with the solid substrate through interactions involving electrostatic balances and hydrophobicity. Some bacteria are able to improve superficial adherence, at the level of the liquid/solid interface, through the extracellular secretion of biosurfactants, a class of surfactants having polar hydrophilic and nonpolar hydrophobic domains. These cells are able to interact with and adhere much more easily to a hydrophobic surface if the biosurfactant exposes its hydrophobic portion on the external surface of the cell; in fact, the role of superficial hydrophobic interactions for adhesion and biofilm formation is widely recognized (Di Mattia, 2011). Structurally the biofilm is formed by a series of cells incorporated and stratified in EPS with a system of pores and horizontal channels between each layer that allow the transfer of fluids and nutrients (Di Mattia, 2011).

The biofilm, besides cells, is essentially constituted by polysaccharides, lipopolysaccharides, proteins, lipoproteins, lipids and enzymes (Flemming & Wingender, 2010). The biofilm matrix moderates pH and gas exchanges, helps the anchorage of cells to the leaf surface and ensures protection from ultraviolet rays, water stress, antibiotics, disinfectants and ROS (Mah & O'Toole, 2001; Kiraly *et al*., 1997).

8.2 Biosurfactants

Surfactants can be classified into two large groups: synthetic surfactants and biosurfactants. Synthetic ones are produced through organic chemical reactions, while biosurfactants, being extracellular secretions of microorganisms such as bacteria, fungi and yeasts starting from low cost substrates or waste materials, are produced through biological processes (Pornsunthorntawee *et al*., 2008).

In fact, biosurfactants are natural molecules deriving from secondary and fermentative metabolic processes of many microbial genera and they act as surfactants substances (SURFace ACTive AgeNTS), better known as surfactants (Ron & Rosemberg, 2001).

These substances are generally constituted by a polar hydrophilic group, defined head, and a non-polar hydrophobic group, defined tail (Karanth *et al*., 1999; Rosemberg & Ron, 1999).

Bacteria synthetise surfactants during all the phases of their life, but mostly during their growth phase. Their synthesis is a very complex process that is influenced by various nutritional, environmental, biotic and abiotic factors. The conditions that favour the biosurfactant biosynthetic activity of a bacterial species may inhibit that one of another species. Carbon sources are the basic element for biosurfactants biosynthesis, however, their induction is linked to the presence of hydrophobic insoluble compounds in water, in order to solubilise and make them more available (Abouseoud *et al*., 2008; Onwosi & Odibo, 2012).

Another important element for the synthesis biosurfactants is Nitrogen, but even more significant is the C/N ratio in the surrounding environment (Abouseoud *et al*., 2008).

On the basis of microbiological origin and structural characteristics, these substances can be divided into lipopeptides, lipoproteins, neutral lipids, phospholipids, fatty acids, polymeric surfactants and glycolipid (Karanth *et al*., 1999; Rosemberg & Ron, 1999). Lipopeptidic biosurfactants (surfactin, iturin, subtilisin), produced mainly by the bacterial genus *Bacillus*, and glycolipids (rhamnolipids), produced by *Pseudomonas*, deserve particular attention.

Lipopeptides are molecules consisting of a lipid linked to a peptide. Of these the best known one is the surfactin lipopeptide, which is formed by 7-membered amino acids ring (L-asparagine, glycine, 2 L-leucine, L-valine and 2 D-leucine) linked to a

fatty acid (hydrophobic) of 13-15 carbon atoms (Priya & Usharani, 2009). Thanks to their biosurfactant power they are used as antibiotics and for their strong antimicrobial and antagonistic attitude they are able to improve the growth of bacteria able to produce them and to represent a form of defence against microorganisms coexisting in the same environment (Peypoux *et al*., 1999; Singh & Cameotra, 2004).

Rhamnolipids are some of the most important glycolipid biosurfactants; they formed by one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid (Priya & Usharani, 2009). Rhamnolipids show antibacterial ability against many plant pathogens; their mechanism of action is based on the activities of cell permeabilization and induction of direct lysis of the cells (De Jonghe *et al*., 2005).

Thanks to the physical and chemical characteristics of biosurfactants, the microbial cells are able to improve the surface adherence of solid-liquid interface and to solubilise substrates that would be insoluble in water making them more available (Suresh Chander *et al*., 2012).

Moreover, the production of these natural surfactants is able to vary towards alkalinity the pH of the environment in which they are located (Abouseoud *et al*., 2008). These substances are also known to have inhibitory capacity towards fungal and bacterial pathogens due to their production of antibiotics lipopeptide (subtilisin, surfactin, iturin) and glycolipid (rhamnolipids), as shown by several strains of *Bacillus* spp. and *Pseudomonas* spp. (Ron & Rosemberg, 2001; Stein, 2005).

Reducing the surface tension of a liquid facilitates the wettability of surfaces and the miscibility between different liquids; in this way the *swarming* motility of the organisms that are able to produce biosurfactants is promoted (Chrzanowski *et al*., 2012).

In fact, for *Pseudomonas aeruginosa*, it has been demonstrated the involvement of the operon *rhlAB* in the synthesis of rhamnolipids and this confirmed the necessity of the latter for the formation of biofilms and swarming motility (Deziel *et al*., 2003).

Rhamnolipids and lipopeptides were also found to have the peculiarity to be elicitors of induced systemic response (ISR) in plants (Ongena *et al*., 2007; Vasileva-Tonkova *et al*., 2010). These surfactants are also adept at reducing the toxicity of heavy metals (Sandrin *et al*., 2000).

Finally, they are able to promote the production of biofilm (diffusion of *quorum sensing* signal molecules) and to facilitate the formation of the biofilm matrix because

they are able to be integral part of the exopolysaccharidic structure (Davey *et al*., 2003).

The existence of horizontal transfers of surfactants with high molecular weight from the bacteria that produced them to heterologous bacteria has significant involvements in the microbial communities, in co-aggregation and in biofilms formation (Ron & Rosemberg, 2001).

8.3 Materials e Methods

The experiments were carried out on 6 BCAs (PfI, PfII, PSP, BAR, BAY, Bs) and on 28 selected and characterized Psa strains. The evaluated variables were: the biofilm production, the ability to vary pH and the production of biosurfactants (for the 6 BCAs).

8.3.1 Biofilm production

- **Congo Red Agar method**

The production, in qualitative terms, of biofilm was carried out following the method used and described by Freeman *et al*., 1989.

The production was determined by using Congo Red Agar with the following formula per litre of distilled water:

- Brain Heart Infusion Broth (OXOID) 37 g
- Sucrose 50 g
- Bacteriological Agar №1 (OXOID) 3,00 g
- Congo Red 0,8 g

Congo Red was prepared as concentrated aqueous solution and autoclaved at 121°C for15 minutes separately from other medium constituents.

Remaining constituents, after warm dissolution, were sterilized by autoclaving them at 121 °C for 20 minutes.

After cooling down the medium to 55 °C, and under sterile conditions, the solution containing the Congo Red was added to it, subsequently 18 ml of the medium and Congo Red mixture were poured per Petri dish. Then the plates were streaked with a bacterial strain each and incubated for 24-48 at 26±1 °C.

Positive results were indicated by dark red colonies, weak biofilm producers usually presented pink results, while negative strains produced white or very light pink results (Dag *et al*., 2010).

- **Microtiter plate method**

The quantification of biofilm production was carried out modifying the microtiter plate method described by Stepanovic *et al*., 2004.

Biofilm formation was performed using Brain Heart Infusion Broth (BHI, OXOID 37g/l of distilled water) in a sterile 96-well flat-bottomed polistyrene microtiter plate.

Each well was filled with 230 µl of BHI and 20 µl of bacterial culture grown for 24 h.

Each bacterial strain used was assayed three times and placed for 24 hours at 26±1 °C. The negative control wells were filled only with the culture medium.

After 24 hours, the wells were emptied of their contents and washed three times using 300 µl of SDW. In order to fix the remaining bacteria adhering to the wall of each well, these were filled with 250 µl of methanol and, after 15 minutes, emptied and air dried.

When dried, the wells were stained with 250 µl of Crystal Violet for 5 min. After the removal of the dye, the microtiter plate was allowed to dry.

The dye bound to the bacterial cells adhering to the wall of the wells was resolubilized with 250 µl of glacial acetic acid at 33% (V/V).

For the quantification of biofilm production, the reading of coloured plate was performed using a spectrophotometer for 96-well plates capable to read at a wavelength of 590 nm. The bacterial strains were selected and classified into the following categories:

- $O.D._{Strain} \leq O.D._{Negative\ Control}$ = no biofilm producer;
- $O.D._{N.C.} < O.D._{S.} \leq (2 * O.D._{N.C.})$ = weak biofilm producer;
- $(2 * O.D._{N.C.}) < O.D._{S.} \leq (4 * O.D._{N.C.})$ = moderate biofilm producer;
- $(4 * O.D._{N.C.}) < O.D._{S.}$ = strong biofilm producer.

Three replicates were made for each isolate and the data obtained were subjected to one-way ANOVA statistical analysis followed by Tukey's post test, by using the GraphPad Prism 5.0 software.

8.3.2 Variation of pH

Nutrient Phenol Red Agar (NPRA) and Nutrient Bromothymol Blue Agar (NBBA), two media able to change their colour according to the variation of the pH, were used to verify the ability of the selected strains to change the pH.

NPRA composition per litre of distilled water was the following:

- Lab Lemco Broth (OXOID) 8,00 g
- Bacteriological Agar № 1 (OXOID) 18,00 g
- Phenol Red 0,025 g

The medium was prepared with the same methodology previously described (see § 5.2.1).

This medium, containing Phenol-Red as pH indicator, is red in alkaline environment, orange in neutral environment and yellow in acid environment. Its variation range in which this dye acts is yellow pH 6.4 and red pH 8.2.

NBBA formula per litre of distilled water was the following:

- Lab Lemco Broth (OXOID) 8,00 g
- Bacteriological Agar № 1 (OXOID) 18,00 g
- Bromothymol Blue 0,03 g

The medium was prepared with the same methodology previously described (see § 5.2.1).

Bromothymol Blue, the pH indicator used in the NBBA culture medium, is blue in alkaline environment, green in neutral environment and yellow in acid environment. Its range of action is yellow pH 6.0 and blue pH 7.5.

In both types of media, the selected bacterial strains were placed at the concentration of $1x10^8$ CFU/ml (see §5.2.1) on 4 four sterile disks of 6 mm in diameter, used for sensibility test. They were placed inside Petri dishes at the vertices of a virtual square and soaked with 15 µl of each bacterial suspension.

The inoculated plates were then placed at a temperature of 26±1 °C for 24 hours, subsequently the pH variation was observed.

In parallel, a measure of the pH variation was performed through a pH-meter using a liquid medium with the following composition per litre of distilled water:

- Lab Lemco Broth (OXOID) 8,00 g

This broth was aliquoted (9 ml) in tubes for bacteriology and subsequently inoculated with the selected bacteria to obtain a final concentration of $1x10^8$ CFU/ml (see §5.2.1). The experiment was performed twice and the obtained values averaged.

pH of all media used was placed in condition of neutrality.

8.3.3 Biosurfactants production

To assess their production of biosurfactants, the BCAs' emulsifying capacity, was measured using a surfactant essay, expressed for a system of immiscible phases, called E24 (Emulsification Index E24).

1.5 ml of each fresh bacterial culture (24 h old) were placed in Lab Lemco Broth (OXOID 8 g/l of distilled water), with the same amount of a common vegetable oil (sunflower oil) in a tube for bacteriology.

After mixing it with a *vortex* at high speed and for 5 min., each sample was left to stand for 24 hours. At the end of this period, the height of the formed emulsion layer (mm) was measured and then divided by the total height of the column (mm).

The emulsifying capacity is expressed as a percentage increase in comparison with the negative control (nutrient medium without bacteria and vegetable oil) (Abouseoud *et al*., 2008). Each experiment was conducted in triplicate and the data obtained were subjected to one-way ANOVA statistical analysis followed by Tukey's post-test ($P < 0.05$) with the GraphPad Prism 5.0software.

8.4 Results

8.4.1 Biofilm production

All isolates of the selected Psa tested with the Congo Red Agar method produced pink results and were, therefore, classified as weak biofilms producers strains.

All 6 BCA' results were dark red, highlighting their strong ability to produce biofilm (Fig. 44).

Furthermore, the classification of the 28 Psa as weak biofilm producers (Tab. VIII; Fig. 46) and the 6 BCAs as strong biofilm producers (Tab. VII; Fig. 45) was confirmed by the polistyrene microtiter plate essay that as well as being a qualitative method, such as Congo Red Agar, is also quantitative.

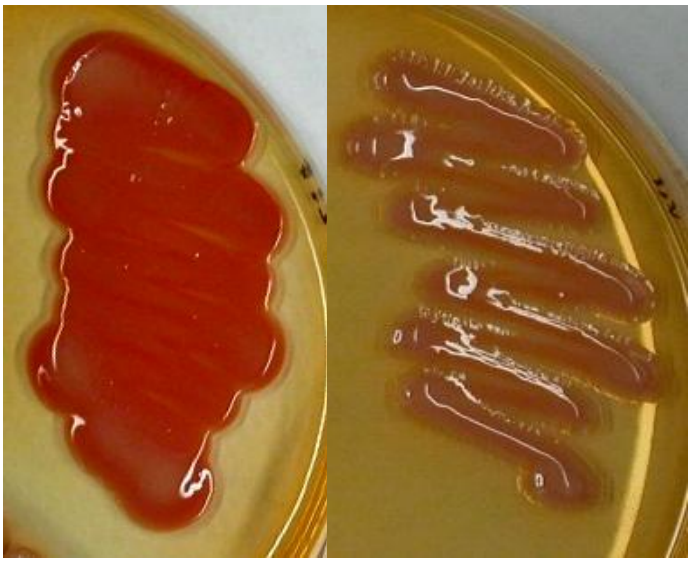


Figure 44. Biofilm production with Congo Red Agar Method. Strong biofilm producer (left) and weak biofilm producer (right) strains.

№	Strains	Origin	Year	Mean ± Std. Error
0	Control	-	-	0.077 ± 0.005 ^{d}
1	Pf I	Italy	2012	1.233 ± 0.056 ^{bc}
2	Pf II	Italy	2012	1.411 ± 0.090 ^{b}
3	Psp	Italy	2012	1.203 ± 0.084 ^{bc}
4	BAR	Italy	2012	1.913 ± 0.179 ^{ab}
5	BAY	Italy	2012	2.167 ± 0.123 ^{a}
6	Bs	Italy	2012	2.147 ± 0.184 ^{a}

Table VII. Biofilm quantification of BCAs with microtiter plate method. All parameters are expressed in O.D. at 590 nm as mean ± SE. Values not marked with the same letter are significantly different ($P < 0.05$).

№	Strains	Origin	Year	Mean ± Std. Error
0	Control	-	-	0.077 ± 0.001 ^{bc}
1	KW 11 (320)	Japan	1984	0.085 ± 0.002 ^{abc}
2	2818	Japan	2011	0.080 ± 0.002 ^{abc}
3	23663	Korea	1989	0.095 ± 0.001 ^{a}
4	K6	Korea	2010	0.087 ± 0.003 ^{abc}
5	Psa K2	Korea	1997	0.094 ± 0.004 ^{ab}
6	VI (CH2010-6)	China	2010	0.078 ± 0.001 ^{bc}
7	M23	China	2010	0.080 ± 0.003 ^{abc}
8	JILO 4	China	2012	0.082 ± 0.004 ^{abc}
9	JILO 30	China	2012	0.078 ± 0.003 ^{bc}
10	INS	China	2012	0.078 ± 0.001 ^{bc}
11	LOLO 4	China	2012	0.088± 0.004 ^{abc}
12	LOLO 26	China	2012	0.080 ± 0.002 ^{abc}
13	HAXA 1	China	2012	0.079 ± 0.004 ^{bc}
14	HAXA 3	China	2012	0.092 ± 0.002 ^{abc}
15	HYM 2	China	2012	0.081 ± 0.003 ^{abc}
16	HYM 3	China	2012	0.080 ± 0.002 ^{abc}
17	K2T	Turkey	2011	0.088 ± 0.004 ^{abc}
18	7286	Italy	2008	0.079 ± 0.001 ^{bc}
19	1F	France	2010	0.079 ± 0.002 ^{bc}
20	827	Spain	2011	0.078 ± 0.001 ^{bc}
21	349	Portugal	2011	0.079 ± 0.001 ^{bc}
22	3817	Switzerland	2011	0.081 ± 0.004 ^{abc}
23	18804 (LV)	New Zealand	2010	0.079 ± 0.004 ^{bc}
24	18839 (V)	New Zealand	2011	0.078 ± 0.001 ^{bc}
25	19200 (V)	New Zealand	2011	0.078 ± 0.003 ^{bc}
26	19440 (LV)	Australia	2011	0.079 ± 0.001 ^{abc}
27	19439	Chile	2012	0.084 ± 0.004 ^{abc}
28	19456	Chile	2012	0.078 ± 0.005 ^{bc}

Table VIII. Biofilm quantification of Psa with microtiter plate method. All parameters are expressed in O.D. at 590 nm as mean ± SE. Values not marked with the same letter are significantly different ($P < 0.05$).

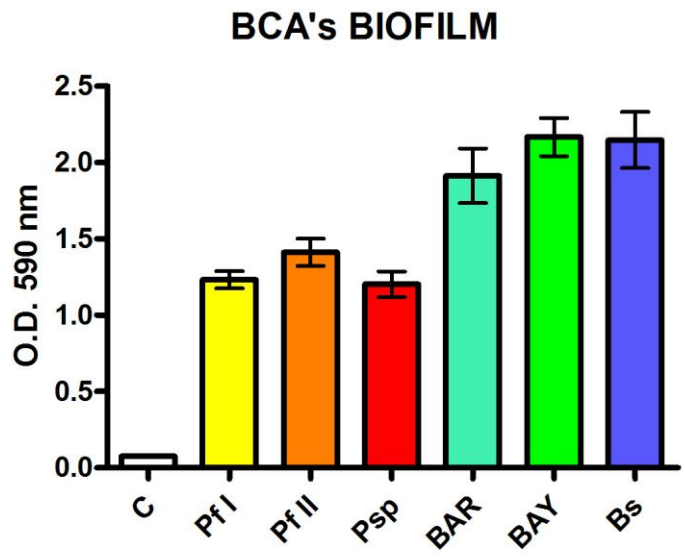


Figure 45. Biofilm production of natural antagonists with microtiter plate method. Errors bars indicate the standard error.

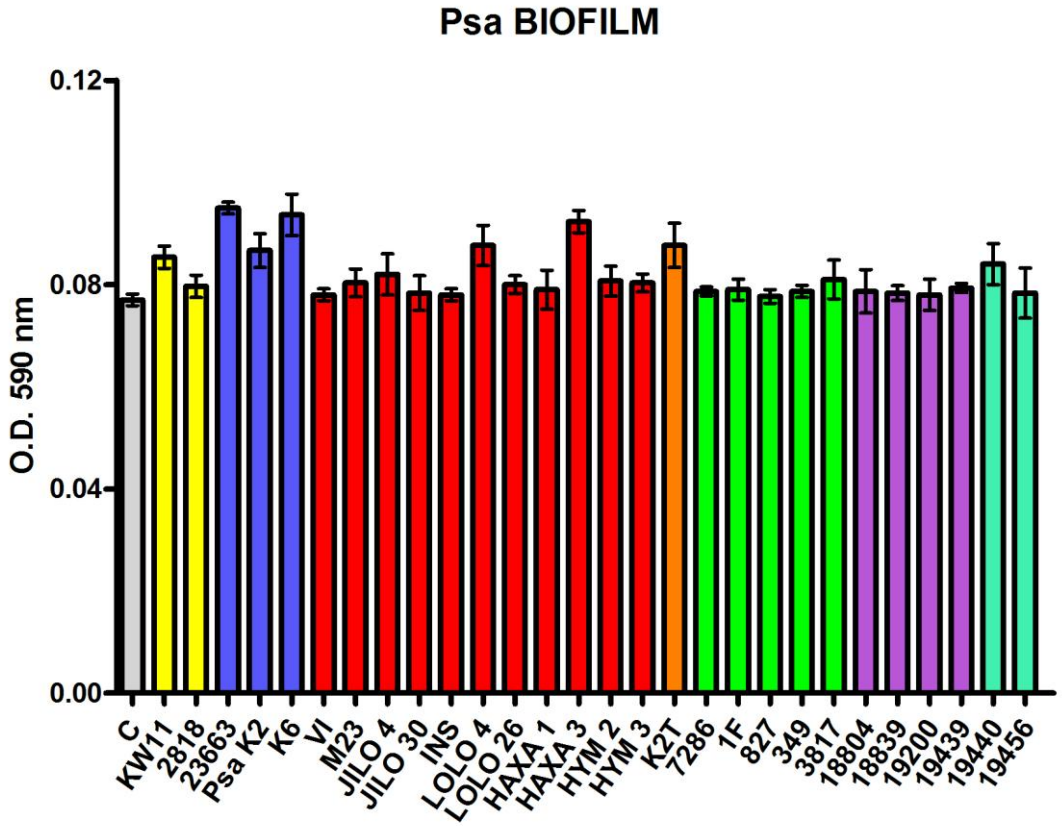


Figure 46. Biofilm production of Psa strains with microtiter plate method. Errors bars indicated the standard error.

8.4.2 Variation of pH

All pathogen strains and all BCAs showed the ability to change the pH from the initial condition of neutrality toward alkalinity.

This was observed both in NPRA medium, which changed from orange to red (Fig. 47), and in NBBA, which turned from green to blue (Fig 48). These results were also confirmed by the measurements registered with the pH-meter. After 24 h of incubation, the pH of all isolates was comprised in the 8.435-8.700 range, as shown in Tables IX and X.

№	Strains	Origin	Year	Mean ± Std. Error
1	Pf I	Italy	2012	8.545 ± 0.005
2	Pf II	Italy	2012	8.485 ± 0.015
3	Psp	Italy	2012	8.480 ± 0.040
4	BAR	Italy	2012	8.490 ± 0.010
5	BAY	Italy	2012	8.500 ± 0.010
6	Bs	Italy	2012	8.505 ± 0.005

Table IX. pH values modified by natural antagonists after 24 h expressed as mean ± SE.

№	Strains	Origin	Year	Mean ± Std. Error
1	KW 11 (320)	Japan	1984	8.495 ± 0.005
2	2818	Japan	2011	8.485 ± 0.005
3	23663	Korea	1989	8.485 ± 0.005
4	K6	Korea	2010	8.505 ± 0.005
5	Psa K2	Korea	1997	8.515 ± 0.015
6	VI (CH2010-6)	China	2010	8.655 ± 0.015
7	M23	China	2010	8.645 ± 0.015
8	JILO 4	China	2012	8.485 ± 0.005
9	JILO 30	China	2012	8.645 ± 0.015
10	INS	China	2012	8.695 ± 0.025
11	LOLO 4	China	2012	8.620 ± 0.020
12	LOLO 26	China	2012	8.635 ± 0.035
13	HAXA 1	China	2012	8.595 ± 0.015
14	HAXA 3	China	2012	8.540 ± 0.010
15	HYM 2	China	2012	8.555 ± 0.005
16	HYM 3	China	2012	8.600 ± 0.010
17	K2T	Turkey	2011	8.635 ± 0.005
18	7286	Italy	2008	8.585 ± 0.015
19	1F	France	2010	8.575 ± 0.005
20	827	Spain	2011	8.655 ± 0.005
21	349	Portugal	2011	8.555 ± 0.015
22	3817	Switzerland	2011	8.655 ± 0.005
23	18804 (LV)	New Zealand	2010	8.435 ± 0.005
24	18839 (V)	New Zealand	2011	8.560 ± 0.000
25	19200 (V)	New Zealand	2011	8.515 ± 0.015
26	19440 (LV)	Australia	2011	8.520 ± 0.010
27	19439	Chile	2012	8.700 ± 0.010
28	19456	Chile	2012	8.620 ± 0.020

Table X. pH values modified by the selected Psa strains after 24 h expressed as mean ± SE.

Figure 47. Nutrient Phenol Red Agar (NPRA). Neutral environment (left) and alkaline environment (right) after 24 h.

Figure 48. Nutrient Bromothymol Blue Agar (NBBA). Neutral environment (left) and alkaline environment (right) after 24 h.

8.4.3 Biosurfactants production

The Emulsification Index E24, showed that all biocontrol agents tested, BAR, BAY, Bs, PfI, PfII and Psp, were able to produce biosurfactants. The average percentage increase observed was of 34.63%, compared to the control (NB).

Among the Gram+, BAY and BAR had the greater emulsifying capacity with 41.11% and 36.67%, respectively.

Between *Pseudomonas* strains, PfII was the best biosurfactants producer with 35.56% (Table XI).

№	Strains	Origin	Year	Mean ± Std. Error
0	NB	-	-	0 ^{c}
1	BAR	Italy	2012	36.67 ± 1.925 ^{a}
2	BAY	Italy	2012	41.11 ± 2.222 ^{a}
3	Bs	Italy	2012	33.33 ± 1.925 ^{ab}
4	Pf I	Italy	2012	34.44 ± 1111 ^{ab}
5	Pf II	Italy	2012	35.56 ± 2.940 ^{ab}
6	Psp	Italy	2012	26.67 ± 1.925 ^{b}

Table XI. Average percentage of Emulsifying Index ± SE.

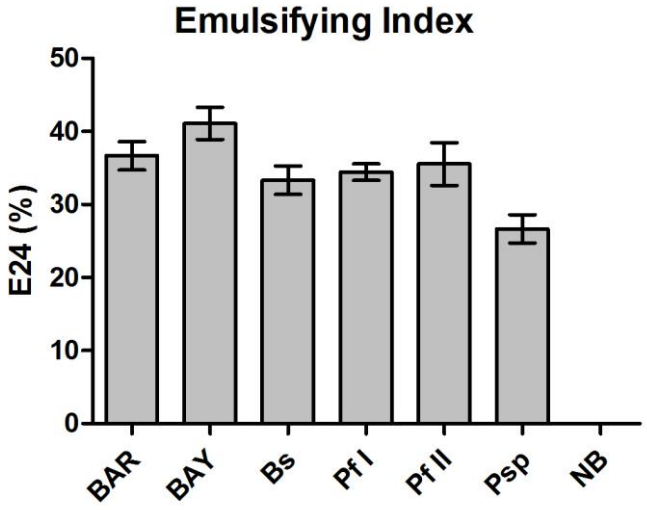


Figure 49. Percentage of Emulsifying Index. Errors bars indicated the standard error.

9. Metabolic profiles with the Biolog System™

This method is conventionally used for the identification of bacterial strains and it is based on a comparison of the metabolic profile obtained from a particular strain with the profiles registered in the database of the Biolog System™.

The total conformity or similarity to a profile allows the identification of the genus, the species of bacterium and, in the best case, the pathovar. In the Biolog database are recorded over 2100 microorganisms including Gram+ and Gram-, aerobic and anaerobic bacteria, yeasts and fungi.

The operating principle is based on the use by the microorganism of a series of 95 organic substrates, specific GP2 for Gram+ and GN2 for Gram- bacteria, subdivided in lyophilized form (Fig. 50-51).

The microbial suspension is inoculated into microplates with 96 flat-bottom wells each containing tetrazolium violet, a growth detector, and a different source of nutriment.

The tetrazolium salts may, in fact, be used as colorimetric indicator of respiratory activity at cellular level (Bochner, 1989). The eventual use of the substrate is observed through the metabolic activity of an organism that results in a redox reaction of the indicator, which, being reduced, veers towards violet.

The biological oxidation of an organic substrate by a microorganism originates reduced NADH. If the electrons are donated to a transport chain, the tetrazolium salt can function as artificial final acceptor, reducing and forming a coloured compound, the formazan. The intensity of staining is directly proportional to the use of the organic substrate. After a suitable incubation period, positive or negative biochemical profile of microorganism is obtained on the basis of biochemical tests (Fig. 52).

The reading of the coloured plate is performed using a spectrophotometer for 96-well plates capable of reading at a wavelength of 590 nm and 750 nm (Fig. 53).

In the specific case, the Biolog system was used exclusively for a “metabolic” characterization of the selected bacterial strains because it is known that two microbial species, having the same metabolic profile, do not necessarily correspond to the same microorganism, because different organisms can use the same sources (genetic redundancy) (Campbell *et al*., 1997; Microlog™ System, 2001).

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Water	α-Cyclodextrin	Dextrin	Glycogen	Tween 40	Tween 80	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	Adonitol	L-Arabinose	D-Arabitol	D-Cellobiose
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
i-Erythritol	D-Fructose	L-Fucose	D-Galactose	Gentiobiose	α-D-Glucose	m-Inositol	α-D-Lactose	Lactulose	Maltose	D-Mannitol	D-Mannose
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D-Melibiose	β-Methyl-D-Glucoside	D-Psicose	D-Raffininose	L-Rhamnose	D-Sorbitol	Sucrose	D-Trehalose	Turanose	Xylitol	Pyruvic Acid Methyl Ester	Succinic Acid Mono-Methyl Ester
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
Acetic Acid	Cis-Aconitic Acid	Citric Acid	Formic Acid	D-Galactonic Acid Lactone	D-Galacturonic Acid	D-Gluconic Acid	D-Glucosaminic Acid	D-Glucuronic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyryc Acid	γ-Hydroxybutyryc Acid
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
p-Hydroxy-phenylacetic Acid	Itaconic Acid	α-Ketobutyric Acid	α-Ketoglutaric Acid	α-Ketovaleric Acid	D,L-Lactic Acid	Malonic Acid	Propionic Acid	Quinic Acid	D-Saccharic Acid	Sebacic Acid	Succinic Acid
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Bromosuccinic Acid	Succinamic Acid	Glucuronamide	L-Alaninamide	D-Alanine	L-Alanine	L-Alanyl-Glycine	L-Asparagine	L-Aspartic Acid	L-Glutamic Acid	Glycyl-L-Aspartic Acid	Glycyl-L-Glutamic Acid
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
L-Histidine	Hydroxy-L-Proline	L-Leucine	L-Ornithine	L-Phenylalanine	L-Proline	L-Pyroglutamic Acid	D-Serine	L-Serine	L-Threonine	D,L-Carnitine	γ-Aminobutyric Acid
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Urocanic Acid	Inosine	Uridine	Thymidine	Phenylethyl-amine	Putrescine	2-Aminoethanol	2,3-Butanediol	Glycerol	D,L-α-Glycerol Phosphate	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate

Figure 50. Metabolic sources in GN2 microplates (for Gram-).

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Water	α-Cyclodextrin	β-Cyclodextrin	Dextrin	Glycogen	Inulin	Mannan	Tween 40	Tween 80	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	Amygdalin
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
L-Arabinose	D-Arabitol	Arbutin	D-Cellobiose	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	α-D-Glucose	m-Inositol
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
α-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	α-Methyl D-Galactoside	β-Methyl D-Galactoside	3-Methyl-D-Glucose	α-Methyl-D-Glucoside
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
β-Methyl-D-Glucoside	α-Methyl-D-Mannoside	Palatinose	D-Psicose	D-Raffinose	L-Rhamnose	D-Ribose	Salicin	Sedoheptulosan	D-Sorbitol	Stachynose	Sucrose
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
D-Tagatose	D-Trehalose	Turanose	Xylitol	D-Xylose	Acetic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyric Acid	γ-Hydroxybutyric Acid	p-Hydroxyphenyl-acetic Acid	α-Ketoglutaric Acid	α-Ketovaleric Acid
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Lactamide	D-Lactic Acid Methyl Ester	L-Lactic Acid	D-Malic Acid	L-Malic Acid	Pyruvic Acid Methyl Ester	Succinic Acid Mono-Methyl Ester	Propionic Acid	Pyruvic Acid	Succinamic Acid	Succinic Acid	N-Acetyl-L-Glutamic Acid
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
L-Alaninamide	D-Alanine	L-Alanine	L-Alanyl-Glycine	L-Asparagine	L-Glutamic Acid	Glycyl-L-Glutamic Acid	L-Pyroglutamic Acid	L-Serine	Putrescine	2,3-Butanediol	Glycerol
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Adenosine	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Adenosine-5'-Monophosphate	Thymidine-5'-Monophosphate	Uridine 5'-Monophosphate	D-Fructose-6-Phosphate	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate	D,L-α-Glycerol Phosphate

Figure 51. Metabolic sources in GP2 microplates (for Gram+).

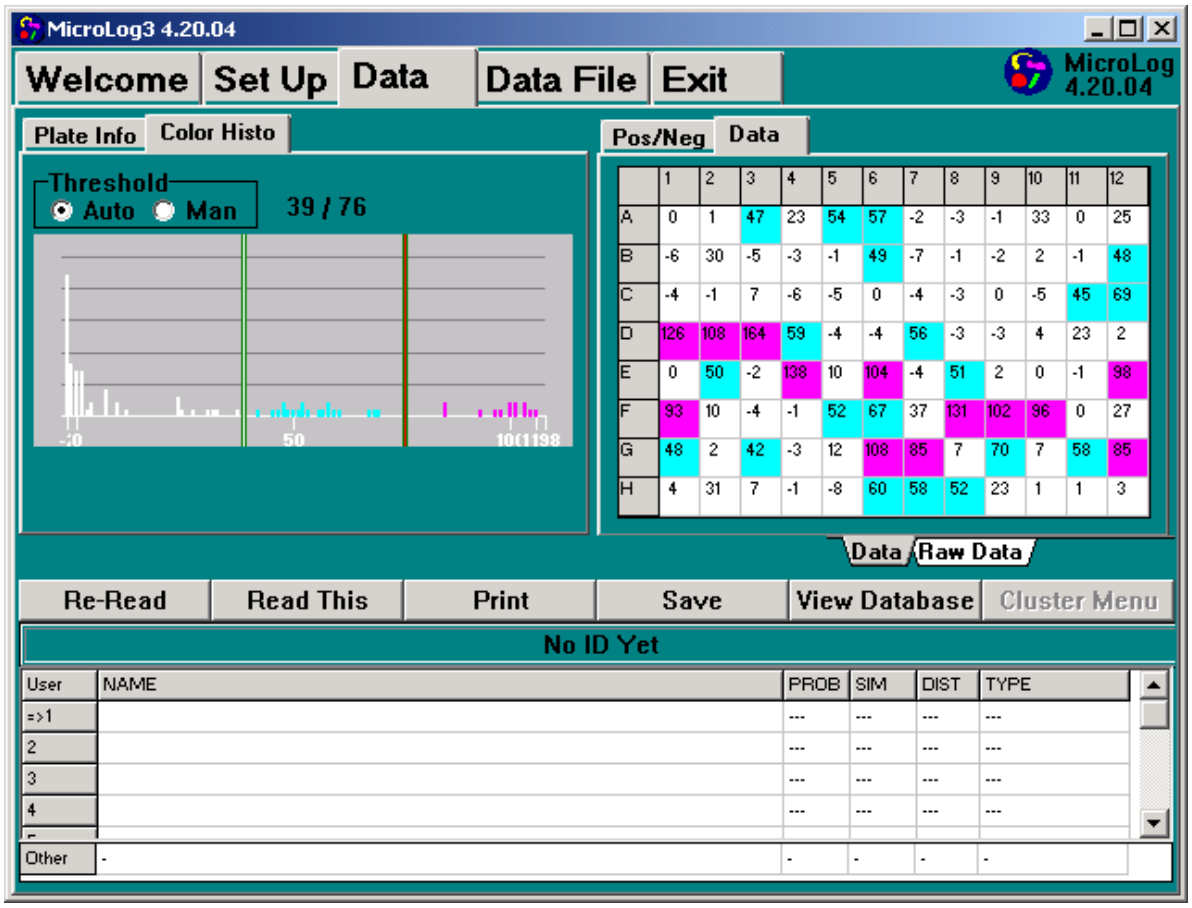


Figure 52. Example of metabolic profile obtained with Biolog™ system.

Figure 53. Biolog Microstation.

9.1 Materials and Methods

Both the 6 BCAs and the 28 selected Psa, were initially streaked onto Petri dishes containing Tryptone Soya Agar (TSA) with the following composition per litre of distilled water:

- Tryptone Soya Broth (OXOID) 30 g
- Technical Agar №3 (OXOID) 18 g

The Petri dishes were then incubated at 26±1 °C for 48 hours. The medium was prepared with the same methodology previously described (see § 5.2.1).

Subsequently, the bacteria were plated on BUG™ Agar (Biological Universal Growth; Biolog Bug Agar 70101 57 g/l of distilled water) and placed in a thermostat for 24 hours at 26±1 °C, in order to obtain bacterial colonies in log phase of growth. The medium was prepared with the same methodology previously described (see § 5.2.1).

According to the Biolog protocol, Thioglycolate was placed on the BUG Agar before inoculation it with the Gram+ strains (Microlog™ System, 2001).

After that, using a sterile swab (Biolog InoculatorZ™ 3323) each bacterial culture was suspended in a sterile saline solution (Biolog GN/GP Inoculating fluid 72101). It was then measured the optical density of this suspension with the Biolog turbidimeter 3531 reading at a wavelength of 590 nm. Through appropriate dilutions, each suspension was adjusted to an absorbance of 0.52 for Gram- and of 0.28 Gram+ bacteria. Subsequently, with a multichannel pipette, 150 µl of the bacterial suspension were placed in each one of the 96 well of the microplates.

The microplates were then incubated for 24 hours at a temperature of 30±1 °C. The reading of the plates was performed using a spectrophotometer (Biolog Microstation) capable of reading at a wavelength of 750 nm and 590 nm.

A metabolic profile, or useful profile of the substrates, was obtained for each tested bacterial strain. The nutrients profiles were obtained with the specific software Microlog™ System 4.2 (Microlog™ System, 2001).

9.2 Results

The use of the Biolog® System, through the Microlog™ System 4.2 software, has generated, for each isolate, a matrix that expresses the capacity to utilize the 95 different metabolic sources.

The matrices obtained were used to generate, with the use of the JMP Pro 10 software, dendrograms that divide the strains used (Psa and antagonists) into groups with different metabolic capabilities. The hierarchical clustering was performed using multivariate analysis with a single method.

Gram- BCAs PfI and Psp are very similar in the use of metabolic sources with a hierarchical distance of 122.99, while PfII is at a hierarchically distance of 213.02 from them (Fig. 54) as it uses more L-Arabinose, D-galactose, α-D-Glucose, D-Sorbitol, Sucrose, D-Trehalose, D-Galacturonic acid, D-Gluconic acid, D-Saccharonic acid, L-Asparagine, L-Urocanic acid and Proline.

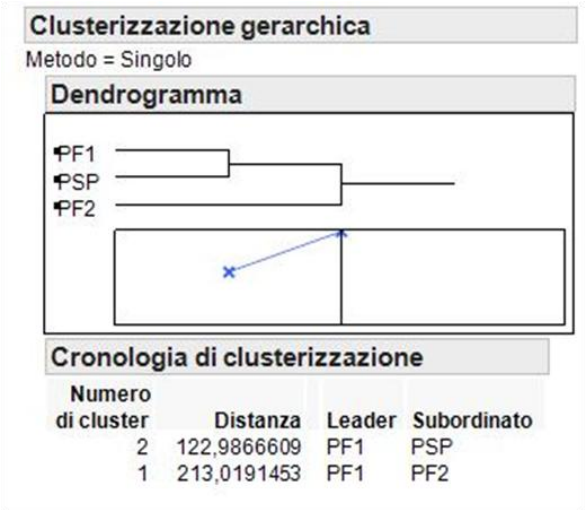


Figure 54. Hierarchical Cluster of Gram- natural antagonists.

Also in the case of Gram+ BCAs (Fig. 55) there were 2 distinct clusters. The first one consisting of BAR and Bs, which are very similar and have a distance 289.90; the second one consisting only of BAY, which is metabolically different with a distance of 405.39 due to the greater use of D-Gluconic, α-Keto-Glutaric, Lactic, Malic, Succinic, Glutamic and Pyroglutamic acids, nitrogen sources D- and L-Alanine, Glycine, Asparagine, Serine, Inosine and Arabinose.

On the other hand, the consumption of Dextrin, Maltotriose, Palatinose and Turanose was lower than BAR and Bs.

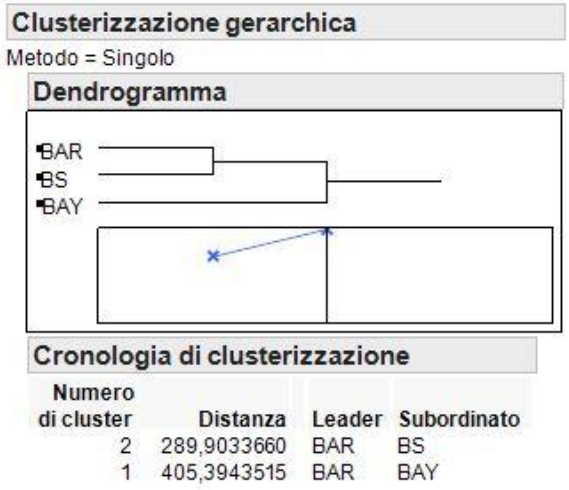


Figure 55. Hierarchical Cluster of Gram+ natural antagonists.

Different considerations have to be made for the 28 Psa strains for which the hierarchical organization is represented in three clusters (Fig. 56).

The first one, Group A, is represented by the Japanese strain KW11 and the Korean strain 23663 isolated, respectively, in 1984 and 1989. Group B includes two New Zealand (18804 and 18839), one Australian (19440), one Spanish (827) and one Portuguese (349) strains. All the other strains of Psa (20) are included in a single large group, Group C (Table XII).

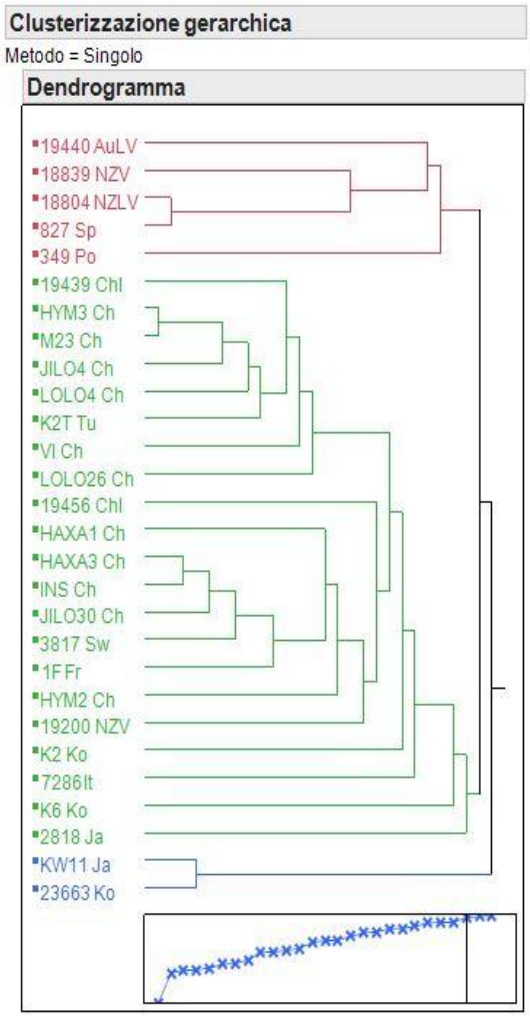
Psa of the latter group are capable of metabolizing the main carbonaceous and nitrogenous sources without showing any preference. A greater selection of nutrient sources is adopted by the other two groups.

The Psa strains in Group B prefer to use D-Saccharic acid, D-Gluconic acid, Citric acid, Turanose, Sucrose and D-galactose and they are the only strains to use α-Ketobutirric and α-Ketoglutaric acids. Within this group, the Portuguese strain 349 is distinguished by the greater use of all the sources listed above.

Psa of Group A differed from the others by a greater use of Cis-Aconitic, D-Gluconic, D-Saccharic and β-hydroxybutyric acids, D, L, α-Glycerol and nitrogen sources, particularly Inosine, Uridine and L-Serine (used exclusively by Group A).

Groups	Strains	Origin	Year
A	KW 11 (320)	Japan	1984
	23663	Korea	1989
B	827	Spain	2011
	349	Portugal	2011
	18804 (LV)	New Zealand	2010
	18839 (V)	New Zealand	2011
	19440 (LV)	Australia	2011
C	2818	Japan	2011
	K6	Korea	2010
	Psa K2	Korea	1997
	VI (CH2010-6)	China	2010
	M23	China	2010
	JILO 4	China	2012
	JILO 30	China	2012
	INS	China	2012
	LOLO 4	China	2012
	LOLO 26	China	2012
	HAXA 1	China	2012
	HAXA 3	China	2012
	HYM 2	China	2012
	HYM 3	China	2012
	K2T	Turkey	2011
	7286	Italy	2008
	1F	France	2010
	3817	Switzerland	2011
	19200 (V)	New Zealand	2011
	19439	Chile	2012
	19456	Chile	2012

Table XII. Identified groups edited from Hierarchical Cluster of selected Psa.



Cronologia di clusterizzazione

Numero di cluster	Distanza	Leader	Subordinato
27	12,1655251	HYM3 Ch	M23 Ch
26	223,5508891	18804 NZLV	827 Sp
25	241,2819927	HAXA3 Ch	INS Ch
24	245,0204073	KW11 Ja	23663 Ko
23	258,5053191	HAXA3 Ch	JILO30 Ch
22	283,3725463	HYM3 Ch	JILO4 Ch
21	288,2932535	HAXA3 Ch	3817 Sw
20	316,0031645	HYM3 Ch	LOLO4 Ch
19	366,8337498	HYM3 Ch	K2T Tu
18	370,1891408	HAXA3 Ch	1F Fr
17	380,2486029	19439 Chl	HYM3 Ch
16	394,3323471	19439 Chl	VI Ch
15	435,8199169	19439 Chl	LOLO26 Ch
14	449,6242876	HAXA1 Ch	HAXA3 Ch
13	452,5317668	HAXA1 Ch	HYM2 Ch
12	478,2551620	18839 NZV	18804 NZLV
11	502,2678568	HAXA1 Ch	19200 NZV
10	510,0490173	19456 Chl	HAXA1 Ch
9	531,7649857	19439 Chl	19456 Chl
8	534,7167474	19439 Chl	K2 Ko
7	549,5479961	19439 Chl	7286 It
6	572,4639727	19440 AuLV	18839 NZV
5	576,9800690	19440 AuLV	349 Po
4	578,5127483	19439 Chl	K6 Ko
3	610,5825088	19439 Chl	2818 Ja
2	617,1215439	19440 AuLV	19439 Chl
1	621,0475022	19440 AuLV	KW11 Ja

Figure 56. Hierarchical Cluster of selected Psa.

10. Discussion

The control of plant pathogenic bacteria is particularly complex due to the restrictions on the use of copper and the prohibition of the use of antibiotics in Europe in order to avoid the development of resistances.

The lack of effective alternatives constituted the trigger to expand on topics concerning the study and to research potential natural antagonists. These appear to be very interesting in the control of bacterial diseases, however, further investigation is required, especially regarding the control of *Pseudomonas syringae* pv. *actinidiae* (Psa).

To understand and evaluate the effectiveness of a natural antagonist, a biocontrol agent, characterized by the necessary safety features for its use in relation to human health and to the environment, was tested.

In compliance with the above requirements and legal restrictions the chosen biocontrol agent was the *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 (Bap), which is, to date, the only BCA registered in Europe for plants of *Actinidia* spp. against Psa and that can be used during the flowering period .

The open field experiment with Bap showed that the best results in controlling Psa (disease incidence) were obtained in the theses in which Bap was used alone or in combination with copper hydroxide.

The use of copper hydroxide did not have any negative effects on Bap's *fitness* and effectiveness in controlling the disease for the whole growing season (Solioz *et al*., 2010). The effects of this combination were appreciated both in 2012 and in 2013, especially from the end of July onwards, when the control theses (untreated and Cu^{++}) showed a significant increase of disease.

No symptoms of phytotoxicity from copper hydroxide and no negative effect on beneficial organisms (e.g. Bees) were observed in any of the tests carried out.

The experiments showed, a considerable increase in disease index for the theses that did not receive any preventive treatment during the flowering period (untreated and Cu^{++}).

Conversely, where the BCA was utilised the development of the disease, after flowering and in the following phases of the season was significantly controlled.

Despite the fact that Bap was isolated from soil, it showed an excellent capacity to survive on the phylloplane, ensuring a prolonged activity against Psa.

In consideration of the fact that the effectiveness of a biocontrol agent is measured, not only by its ability to produce antibiotic substances, but also by its degree of competition for nutrient sources and space, the isolation of BCAs in the same ecological niche of Psa seemed to be the best choice (Völksch & May, 2001).

The isolated BCAs, 3 of *Bacillus* genus and 3 of *Pseudomonas* genus, by screening tests *in vitro*, proved to be particularly effective in inhibiting the growth of Psa.

After the selection, it was decided to evaluate their effectiveness *in planta* and their ability to survive in epiphytic manner on the *Actinidia*'s phyllosphere.

After the BCAs were sprayed on the plants their antagonistic capacity has significantly affected the population of Psa, causing its remarkable reduction. From these experiments emerged that *P. s.* pv. *actinidiae* is not really an epiphytic bacteria pathogen. After 7 days it showed a drastic reduction of its population. The same behaviour was seen in the presence of the BCAs, however for each entry CFU/cm^2 of Psa were always statistically lower than for the control. During all experiments, all BCAs maintained their population values stable.

All the BCAs maintained the same trend of survival, proving to be resident epiphytes and that the presence or absence of Psa did not affect their population dynamics.

Among the Gram+ bacteria, BAR showed the best inhibitory ability towards 7286 CFPB; while among Gram- bacteria, PfI was the strain with the best performances, however these were inferior to BAR.

The percentage reduction of Psa CFU/cm^2 level was around 20% (BAY and PfII), Psp showed slightly lower values , while Bs activity was lower (11%).

Considering the worldwide spread of the Kiwifruit Bacterial Canker and taking into account predictive models about Psa spread (Narouei Khandan *et al*., 2013; Narouei Khandan *et al*., 2014), it was important to test the BCAs (BAR, BAY, Bs, PfI, PfII and Psp) in respect of Psa strains isolated in different Kiwifruit areas of the world.

In order to achieve this, the tests have been carried out also on Psa strains isolated in China, during the abroad study and research period, in addition to the Psa strains already present in the DAFNE's collection, in this way all geographic areas of the world affected by Psa have been represent.

The results of *in vitro* essays indicate that the best inhibitors of the 28 Psa strains tested were PfI and PfII BCAs, with an average zone of inhibition of about 6 mm,

while, among Gram+ bacteria, the best performances were obtained by BAY and BAR BCAs with a lower level of Psa inhibition halo (5 mm). Psp and Bs showed a medium-high inhibition halo, but were not effective against all Psa strains. PfI and PfII BCAs, despite being the best performers *in vitro* tests, did not show the best result *in planta*, maintaining however medium-high performances as competitors; the opposite is true for BAY and BAR.

After demonstrating their ability as biocontrol agents, attention has been focused to the understanding of BCAs' competitive capabilities.

As emerged from the literature (see §8.1), bacteria on the phyllosphere are able to compete through mechanisms that are activated by some genes only in consequence of *quorum sensing*, therefore it was decided to evaluate for BCAs and the selected Psa strains their biofilm and biosurfactants production and their ability to change the pH.

To understand if the active substance produced by BCAs vs. Psa strains acted through pH variation, an essay was conducted to estimate the medium pH resulting from their growth. The pH did not appear to be involved in the antagonistic activity because both pathogens and antagonists shifted the pH substrate to the alkalinity (average pH 8.5).

Following the *in planta* tests, the evaluation of biofilm production was performed to assess how the 6 BCAs and 28 Psa strains were able to sustain the abiotic stress that normally affect the phyllosphere (UV, dehydration, wash-out, etc.) and, consequently, their ability to colonize the organs' surface.

This study suggests that the 6 BCAs are all strong biofilm producers, while all 28 Psa strains are weak biofilm producers.

BCAs proved to be typical epiphytes able to colonize the leaf surfaces (competition for space) and, therefore, to be less exposed to agents that may compromise their *fitness*. Otherwise, if Psa strains can not penetrate in the host, they die after a short lapse of time.

To validate the potential agronomic applications of BCAs, the production of biosurfactants was evaluated. As emerged from E24 essay, all 6 BCAs were proficient producers of these surfactants.

As emerged from the literature (see §8.2), biosurfactants can improve the superficial adherence of solid-liquid interface, solubilise water-insoluble substances, thus increase their accessibility, increase the availability of the nutrients, have antibiotic faculties, be elicitors of induced systemic resistance (ISR), vary the pH toward

alkalinity (confirming the results of pH test) and facilitate the production of biofilm (AHL diffusion) because they are one of the constituents of its matrix.

All these characteristics demonstrate that the 6 BCAs are able to grow and multiply independently, colonizing the niches in which the pathogen could grow, and antagonising it through antibiosis and competition for space and nutrients.

To investigate the nutritional competition, a characterization of the metabolic profiles was conducted through the Biolog System.

Because different organisms can use the same sources (genetic redundancy) (Campbell *et al*., 1997), the Biolog system does not provide absolute certainty in the identification of the species, but can provide an exact metabolic profile that can be subsequently attributed to a specific ecological niche.

The alternative use of the Biolog System to obtain the "metabolic" characterization of selected bacterial strains showed that both pathogens and BCAs may prefer and utilize the same carbonaceous and nitrogenous sources.

This confirms that the BCAs isolated in *Actinidia* ecosystems affected by Psa, not only occupy the same ecological niche, but compete for the same sources. Considering that carbonaceous sources for bacterial survival are limited on the phyllosphere, their utilization by these BCAs strongly limits the possibility of Psa's success.

From the results it emerged that the metabolic differences among Psa's strains are not only due to their area of origin, but they may also vary in relation to environmental conditions, which exert selective pressure.

The 6 BCAs that were selected proved to be efficient in the biocontrol of Psa strains isolated in different geographic areas, through antibiosis and competition for space and nutrients. The results indicated that the selected BCAs hold promising potentials also for other agri-environmental uses, for which they need to be tested in open field and be possibly registered, as in the case of *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747 that has been registered to protect Actinidia plants from Psa during the blossom time.

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