Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze e Tecnologie Agrarie, Ambientali e Alimentari

Ciclo XXVII

Settore Concorsuale di afferenza: 07/B2

Settore Scientifico disciplinare: AGR/03

Influence of plant structure, cultural practices and environmental conditions on the development of the bacterial canker of kiwifruits

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Esame finale anno 2015

ABSTRACT

Italy has a preeminent rank in kiwifruit industry, being the first exporter and the second largest producer after China. However, in the last years kiwifruit yields and the total cultivated area considerably decreased, due to the pandemic spread of the bacterial canker caused by *Pseudomonas syringae pv. actinidiae* (Psa). Several climatic conditions and cultural practices affect the development of the bacterial canker. This research work focused on the impact of agricultural practices and microclimate conditions on the incidence and epidemiology of Psa in the orchard. Therefore, the effect of fertilization, irrigation, use of bio-regulators, rootstock, training system and pruning were examined. The effect of different tunnel systems was analyzed as well, to study the plant-pathogen interaction. Considering the importance of insects as vectors in other pathosystems, the role of *Metcalfa pruinosa* in the spread of the bacterial canker was investigated in controlled conditions. In addition, quality and storage properties of fruits from infected plants were assessed. The study of all these aspects of the agronomic practices is useful to define a strategy to limit the bacterial diffusion in the orchard. Overall, excess nitrogen fertilization, water stress, stagnant water supplies, pruning before summer and the high number of *Metcalfa pruinosa* increased the Psa incidence. In contrast, tunnel covers may be useful for the control of the disease, with special attention to the kind of material.

Key words: *Pseudomonas syringae pv.actinidiae*, Actinidia, kiwifruit, environmental condition, cultural practices*, Metcalfa pruinosa* Say (1980), fertilization, irrigation, bio-regulators, rootstock, training system and pruning, light, tunnel.

ACKNOWLEDGEMENTS

First thing want to express my gratitude to the Alma Mater Studiorum, University of Bologna and to my advisor Prof. Guglielmo Costa for gave me the opportunity to develop my thesis, and to my co-advisor Dr. Francesco Spinelli for the supervision and advise.

Thanks to all the colleagues that helped me in the field, laboratory and computer work.

Finally, want to special thanks to Ufficio tecnico Agrintesa, for the support in the activity in the orchards in Faenza.

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INTRODUCTION

The genus *Actinidia* Lindl (order *Theale*s, family *Actinidiaceae*) comprises over 50 species, mostly originated in Southwest China (Ferguson, 1990); the two most cultivated species are *A. chinensis* and *A. deliciosa*. The fruit of *Actinidia* species is known worldwide as kiwifruit, and is appreciated for its sweet, slightly acidic flesh and high nutritional value, especially due to its high content in vitamin C (Ferguson *et al.,* 1991). Italy has a preeminent rank in kiwifruit industry, being the first exporter and the second largest producer (415000 tonnes per year) after China, and followed by New Zealand and Chile (Palmieri *et al.,* 2014). In recent years, kiwifruit cultivation faced a crisis due to the spread of bacterial canker in the production areas. In 2010 Italy resorted to the eradication of a large number of orchards, especially in Lazio and Piemonte. Between 2010 and 2012, in Italy, kiwifruit cultivation area was reduced by about 2000 hectares, with production losses of 10–50% per hectare (Donati *et al.,* 2014).

The canker is caused by the gram-negative bacterium *Pseudomonas syringae pv. actinidiae* (Psa, *Proteobacteria,* gamma subdivision; Order *Pseudomonadales*; Family *Pseudomonadaceae*; Genus *Pseudomonas*; *Pseudomonas syringae* species complex, genomospecies 8; pathovar *actinidiae*). This bacterium is aerobic, motile, and rod-shaped, with polar flagella, oxidase-negative, arginine dihydrolase-negative and represents the most serious disease that affected *Actinidia* species since their introduction in the Italian territory (Donati *et al.,* 2014). This bacterium was observed for the first time in Japan in 1984 (Serizawa *et al.*, 1989) and, later, it was firstly isolated in Lazio (central Italy) in 1992 (Scortichini, 1994); however, starting from 2007, the disease assumed pandemic characteristics and posed a severe plant health issue (Balestra *et al.,* 2009). The pandemic strains of Psa were isolated in Latina, and later spreaded to the whole Italian territory (Testolin, 2012). Genomic analyses allowed to associate the pandemic strains in one homogeneous group, named biovar 3, genetically distinct from early isolates (biovar 1) and from isolates responsible of the 1990s outburst (biovar 2) (Scrotichini *et al.*, 2012). Biovar 3 strains are genetically characterized by the presence of pathogenesis-related sequences (integrative conjugative elements, ICEs), horizontally acquired from other *P. syringae* pathovars (Butler *et al*., 2013).

Pseudomonas syringae pv. actinidiae can effectively colonized the kiwifruit plants (yellow and green fleshed) throughout the year. The plants affected by the bacterium after the winter can

show withering of buds and young branches, due to the infections occurred in winter. In this time the bacterium can disperse a large amount of inoculum within and between orchards due to the production of exudates. In the spring, the environmental conditions (12-18°C and humidity) for the multiplication of the bacterium are most frequent. In this phase, bacterial colonization of the plant may occur by of penetration through the stomata and the lenticels. Psa can spread systematically in the plant and move from the leaf to the young shoots, causing leaf spots, flower necrosis and fruits collapse with production loss. High temperatures in the summer can reduce the multiplication and spread of the bacterium in the orchard (Scrotichini *et al.*, 2012). However, in this season, heavily infected plants may wilt and die.

In field conditions, the bacterium survives epiphytically on the surface of plant, in water films enriched with nutrients secreted from plant hosts; it may also survive for long periods in the litter and in waste of pruning (Donati et al., 2014; Spinelli *et al.,* 2012). Mild temperatures (12-18 ° C) in autumn and spring promote the multiplication of Psa; high humidity and rains play an important role in its spread, inducing the exctretion of exudates from cankers (Gullino *et al.,* 2012). Moreover, wounds facilitate the penetration of the bacterium (Scortichini *et al.,* 2012). The scars by falling of leaves and pruning cuts provide access to the pathogen for several days after their formation (Spinelli *et al.,* 2012). Psa is also able to enter the plant through natural openings, such as the stomata and the lenticels. A critical phase of the life cycle of Psa is the ability to endophytically migrate from the leaves to shoots and canes via apoplast (Donati *et al.,* 2014). This systemic invasion of the plant may determine the rapid death of plants (Spinelli *et al.,* 2012).

The chemical means adopted in the fight against bacterial canker are preventive and aim to the reduction of inoculum and risk of infection.In this sense, chemical treatments mostly rely on cupric coverage formulates, coupled to measures of good hygiene in the orchard. In contrast, the use of antibiotics, allowed in Asia and New Zealand, is forbidden in Italy, and several streptomycin-resistant strains of Psa have been isolated (Donati *et al.*, 2014). Some good results in the control of the disease were obtained with the plant resistance inducer, acibenzolar-S-methyl (Cellini et al., 2014).

AIMS OF THE THESIS

The various climatic conditions and cultural practices can affect the development of Psa, by modifying the environmental parameters required for Psa multiplication and spread, or the host's

ability to react to the invading pathogen. This research work focused on the impact of agricultural practices and the microclimate conditions on the incidence and severity of the disease in the orchard. For this purpose the influence of fertilization on disease development was investigated, in particular: the effect of nitrogen form, dosages and mode of application in controlled conditions and the effect of micronutrients deficiency or excess in vitro and controlled conditions. Kiwifruit cultures during the vegetative - productive cycle need high water supplies, for this reason the effect of the irrigation rate and the influence of the water source on the spread of the disease in field conditions were analyzed. The importance of specific micro-climatic conditions in determining Psa virulence was investigated, by testing whether the different types of training system or different pruning systems could influence the microclimate inside the orchard and affect the effectiveness of coverage leaf treatment carried by the farmers. Knowing the importance of insects as vectors in other pathosystems, we investigated whether insects with sucking-stinging mouthparts could act as Psa vectors. The effect of commonly used bio-regulator on the development of the disease was also assessed. In addition, the influence of the disease on fruit production, quality and storability was studied. For this purpose, fruits from diseased plants were analysed for the main quality parameters at harvest and during storage. In recent years, the use of plastic tunnels to protect kiwi-plants from Psa spread has become increasingly common. For this reason we analyzed if the tunnel can minimize the risk of infection by modulating the microenvironmental conditions. The study of all these aspects of the agronomic practices will be useful to define which ones are significant in limiting the bacterium diffusion in the orchard thus allowing an integration of different practices in order to disadvantage the virulence of the bacterium.

This thesis includes three chapters that were structured as scientific papers, with a relative Title, Introduction, Materials and Methods, Results and Discussion, References, Figures and Tables. Chapters 1 focuses on the “Optimization of cultural practices to reduce the development of *Pseudomonas syringae pv. actinidiae*”. Chapters 2 focuses on the “Evidences of the role of *Metcalfa pruinosa* (Say 1830) as a vector of *Pseudomonas syringae pv. actinidiae*”. Chapter 3 concerns “The effect of the use of tunnel on spread control of Pseudomonas syringae pv. actinidiae in the orchard”. Finally, General Conclusions are reported.

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Optimization of cultural practices to reduce the development of *Pseudomonas syringae pv. actinidiae*

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ABSTRACT

The bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae*, is considered one of the most severe diseases affecting several cultivated *Actinidia* species, including *A. chinensis* and *A. deliciosa*. Kiwifruits have always been considered a fruit with an high intrinsic quality due to the strong nutraceutical value, but also to the absence of contaminants such as pesticide residues. With the emergence of this devastating disease, the use of pesticides rapidly increased and therefore the kiwifruit quality can be maintained only by ensuring agricultural practices that reduce the need of toxic xenobiotic compounds. The aim of this study was to provide an in-depth understating of the influence of agricultural practices on disease development and spread. Therefore, the role of fertilization, irrigation, use of bio-regulators, rootstock, training system and pruning on the incidence and epidemiology of PSA were examined. Nitrogen fertilization had a direct effect on the pathogen's endophytic growth. Furthermore, the depletion of some micronutrients, such as iron, increased the disease. The water stress consequent to a reduced irrigation resulted in higher symptoms. Concerning pruning, the open cuts remains a possible entry point for more than 30 days. In addition, the pruning performed in late season resulted more risk for infection. To test curative pruning, preliminary data were collected on the migration rate of Psa inside the different plant organs. Among the different training system evaluated in this study, the gender double curtain the allowed a more efficient penetration of the phytosanitary treatments that may increase the disease control. However, no differences on disease incidence were observed. Concerning the use of bioregulators, synthetic gibberellin, such as forchlorfenuron, reduced in controlled condition both the disease incidence and severity.

Synthetic auxins, on the other hand, showed a detrimental effect with higher symptomatology. In field conditions, none of the used bioregulators showed any effect on the disease incidence and development. Several species were tested for their susceptibility to Psa in order to develop a tolerant rootstock. *Arguta* spp. resulted the most tolerant to the disease and further experiments are need to test its possible exploitation as a commercial rootstock. Finally, the influence of the disease on fruit yield, quality and storability was evaluated.

Keywords: *Pseudomonas syringae pv. actinidiae*, fertilization, irrigation, bioregulators , microelements, nitrogen, pruning, training system, microclimate.

INTRODUCTION

The bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), is considered the most dangerous adversity of kiwifruit (Donati *et al.*, 2014), affecting all the cultivated varieties of *Actinidia deliciosa* and *A. chinensis*. The pathogen can penetrate through natural openings (such as flowers, stomata and lenticels) and wounds, and spreads rapidly in the host's tissues, causing the plant death and severe crop losses. The development of the disease is related to permissive climatic and environmental conditions, mostly occurring in spring and autumn: for instance, a temperature between 10 and 20°C, water availability, with rain and wind facilitating the pathogen dispersal (Vanneste *et al*., 2011). Nutritional imbalances, affected by fertilization and leaf/fruit demography, may also promote pathogen growth or depress plant defences (Bangerth, 1979). Therefore, a combination of cultural practices, such as different training and pruning systems, fertilization and irrigation rates and the control of leaves/fruits ratio might produce negative conditions for the bacterial colonization of the plant (Testolin, 2012).

Furthermore, all of the cultural management practices, affecting the vine vegetative and reproductive performances, are exploited to force productivity, at the expense of other physiological processes. Therefore, the resulting imbalance can adversely affect the plant's ability

to react to abiotic or biotic stress, making it even more susceptible to disease.

The study of the role of cultural practices on the Psa incidence and development will be useful to define the proper orchard management to which reduce the use of pesticides to control the disease. The aim of this study was to provide an in-depth understating of the influence of agricultural practices on disease development and spread. Therefore, the role of fertilization, irrigation, use of bio-regulators, rootstock, training system and pruning on the incidence and epidemiology of PSA were examined.

Finally, the influence of the disease on fruit production, quality and storability was studied. For this purpose, fruits from diseased plants were analysed for the main quality parameter at harvest and during storage.

MATERIALS AND METHODS

All the experiments on the role of the different cultural practices were performed on *A. deliciosa* (cv. Hayward) plants. The experiments in controlled condition were performed on potted plants 30 cm tall. As growing medium obtained by mixing 1:1 (v/v) peat and sand, with standard drip irrigation (1.33 $lday^{-1}plant^{-1}$). The peat mineral concentration declared by the manufacturer was: NH^{4+} 25gm^{-3}, NO^{3-} 35gm-^{3}; P_2O_5 104 gm^{-3}, K_2O 120gm^{-3}; MgO 12gm^{-3}; micronutrients 25 gm^{-3}. The plants were kept at 60 % RH, 22° C and with a light-dark cycle of 16:8 hours.

If not differently specified, in the experiments in controlled conditions, each treatment was divided in 4 biological replicates of 6 plants each.

If not differently specified, the field trials were performed on *A. deliciosa* (cv. Hayward) plants in commercial orchards approximately 10 years old. Standard fertirrigation was applied during the whole season.

Influence of fertilization on disease development

To assess the role of fertilization different trials were performed. In the first one, the influence of nitrogen form and amount was investigated.

In the second trial, the role of radical or foliar administration of N was investigated. Finally the role of micronutrients on the disease development was studied. For this experiment, both the micronutrients deficiency or excess was tested.

Effect of nitrogen form, dosages and mode of application in controlled conditions

I. *Nitrogen form*

Seedlings of *A. deliciosa* (six replicates per treatment) were transplanted on sand, with no nitrogen fertilization for one month. The following nitrogen fertilizers were tested at the same concentration (150 Kg N ha^{-1}, equivalent to 0.15 Kg $plant^{-1}$): ammonium nitrate (NH_4NO_3), ammonium sulphate [$(NH_4)_2SO_4$] and NPK applied as Poly-Feed [Fertica SA (16N-8P-32K). The same amount of P and K provided with the NPK fertilization was also supplied to the plant treated with ammonium nitrate or ammonium sulphate each time ¼ of the final N amount was administrated.
All fertilizers were applied monthly for 4 times and N was performed 4 months after the beginning of fertilization strategy. Biometric and physiological parameters were monitored monthly. Whereas, symptoms were monitored at 15, 30 e 40 days after inoculation.

II. Nitrogen dosage

Seedlings of *A. deliciosa* (six replicates per treatment) were transplanted on sand, with no nitrogen fertilization for one month. Subsequently, they were treated twice a week with 0, 0.286 or 2.86 mg NH_4NO_3 per plant, plus 6.75 g K_3PO_4 in each plant. These amounts were calculated to correspond to 0%, 10% and 100% of 80 kg N ha^{-1} (equivalent to 0.08 Kg $plant^{-1}$) in open field, which represent a standard fertilization strategy.

III. Mode of nitrogen application

The radical or foliar applications of N were compared. 2.86 mg NH_4NO_3 per plant were dissolved in 2 ml of water and the full volume of solution was provided either by root drench or by foliar application.

Effect of micronutrients deficiency or excess in vitro and controlled conditions

The first experiment aimed to verify whether a microelement deficiency influence the disease development. Micropropagated *A. deliciosa* plants (six replicates per thesis) were grown on agarized medium containing Murashige and Skoog (M&S) inorganic salts, except one of the following: Ca, Fe, Mn, B, Zn. Control plants were provided with all M&S inorganic salts.

The second experiment aimed to investigate whether an excess of the same microelements may have effects on the development of the disease. Seedlings of *A. deliciosa* grown in greenhouse conditions with a standard NPK fertilization were supplied twice a week for two months with: $CaSO_4$ (60 µmol), $MnSO_4$ (2 µmol), $ZnSO_4$ (2 µmol), FeEDTA (50 µmol) or H_3BO_3 (25 µmol). Control plants did not receive any mineral supplement.

Influence of irrigation on disease development

I. Effect of the irrigation rate in controlled conditions

The water field capacity was determined by drying at 110° C an aliquot of soil till a complete evaporation of the water. Thereafter, water was applied till percolation and the minimal water volume needed for percolation was successively used to irrigate the same amount of soil (100% field capacity). The 50 and 25% of the field capacity was calculated accordingly to the water volume needed to reach the 100% field capacity. The weight of each pot was recorded a water was supplied to maintain constant the recorded weight. Inoculation was performed 3 months after the beginning of the differential irrigation strategy.

II. Influence of the source of the water supply on the spread of the disease in field conditions

Thirty infected commercial orchards located in Faenza area were divided according to the source of water supply. Three different water sources were identified: artificial reservoirs inside the orchards, artificial dwell (Canale Emiliano Romagnolo ramification Senio-Lamone (Faenza)) and natural rivers (Marzeno, Senio, Lamone). On each commercial orchard, disease symptoms and spread were monitored monthly for 3 years. The disease presence was correlated with the different sources of water supply.

Inoculation

A week after adding the first dose of nitrogen, the seedlings were spray-inoculated with a PsaGFPuv suspension (10^9 $CFUml^{-1}$). This strain expresses the green fluorescent protein GFPuv (Spinelli et al 2011). Prior inoculation, Psa was grown on Luria Bertani plates incubated at 27°C for 48 hours. In the first 48 hours after inoculation, the relative humidity was raised to 100%.

In the experiments on micronutrients, the inoculation was performed 2 week 2 after the micronutrients depletion or 2 week after the first micronutrient application. Inoculation was performed by dipping each plant Psa CFB7286GFPuv suspension (10^8 $CFUml^{-1}$).

Symptoms developments were monitored weekly. One month after inoculation, the endophytic population of the pathogen inside each plant was measure. For this purpose, plants were surface-sterilized by washing them firstly in ethanol (70%) and successively in NaOCL (1%) and finally rinsed in sterile water for two times. Each plant was ground in 5 ml of sterile 10 mM $MgSO_4$ and serially diluted 1:10; 3 drops of 10 µl for each dilution were plated on Luria Bertani agarized medium in order to quantify Psa presence as Colony Forming Unit (CFU) per gram of wood tissue.

Biometric parameters.

In all the experiments, the following biometric parameters were monitored: plant weight, number of new leaves and chlorophyll fluorescence (measured with the PSI equipment, Photon Systems Instruments, Czech Republic). On micropropagated plants also carbon exchange was measured by means of the EGM-4 equipment (PP System international, Amesbury, USA).

Role of pruning in the disease cycle and curative pruning

The experiments aimed to investigate: I. the role of pruning cuts as possible entry points for Psa and the time need by the plant to heal the cuts to avoid infection, II. the influence of the different commercial pruning strategies on the disease occurrence, III. the migration rate of Psa inside the plant organs in order to steer curative pruning.

I. Pruning cut as possible entry points

The experiment amied to determines the time needed in field conditions by plant to completely heal the pruning cut till they become sealed to Psa invasion. The trial was performed on *A. delciosa* and *A. chinensis* adult plants in the experimental orchard of Sant'Anna (Cadriano – 44°33'0.54''N; 11°23'8.77''E). In January 2011 and 2012, the plants were pruned and at 0, 1, 2, 3, 4, 25, 26, 29, 31 and 32 days after the pruning, the branches were detached and brought to glasshouse for the inoculation (60% RH, 22° C). Inoculation was performed by placing a cotton plug soaked in a bacterial suspension of Psa CFB7286GFPuv suspension (108 CFUml-1). After inoculation all the branches were kept at 100% RH for 2 days. Ten days after inoculation, the distal part of the branches was surface disinfected by washing it twice in ethanol (70%) for 3 minutes and twice in NaClO (1%) for other 3 minutes. Finally, all samples were washed in sterile water 3 times. After sterilization, a portion of 3 cm from the cut was excised from the branches to determining Psa endophytic population as previously described.

II. Pruning strategy

The first trial aimed to determine the best period to perform pruning in order to minimise the risk of Psa infection. The trials were performed from 2011 till 2013 in a commercial *A. deliciosa* (cv. Hayward) orchard located in Faenza (Italy, 44°15'13.00"N & 11°52'49.53"E). The orchard was located in highly infected area. Standard prevention treatments were applied: Bordeaux mixture (equivalent to 20 g ha^{-1} of active copper) every 15 days from November to February and from April to September, Bion® at beginning and full blooming (100 g ha^{-1}) (Syngenta, Basel, Switzerland). Pruning was performed approximatively each 15 days starting from November to March. The different pruning treatments as reported in table 1. After each pruning event, the orchard was sprayed with Bordeaux mixture. The symptomatology was recorded in the following March, April, May, June and October by two independent observers following the visual scale reported in table 2.

The second trial aimed to verify if a differential pruning in the most common training system used in Italy may influence the disease development. Since pergola vine training is the most widespread in Italy, different pruning were tested in this training system:

- Standard - standard business practice of pergola, with canes that reach to the ground;
- Short - with cutting the lower part of the canes of the plants;

- Zero leaves - with spring pruning was cut the vigorous shoot of the third leaf over the last fruit of the shoot.

The experiment was carried out in a commercial orchard of *A. deliciosa*, located in Faenza (Italy, 44°15'13.00"N & 11°52'49.53"E). Symptom development was monitored monthly for 1 year. During the experiment, also microclimatic data inside the canopy were recorded at hourly intervals. Finally, the penetration of phytosanitary treatments in the different managed canopies was estimated.

To assess the penetration of the phytosanitary treatments, the canopy managed with the three different types of pruning were treated by using a sprayer BW-DVR with ground speed of 3.9 km/h at 3 atm with plate nozzle from 1-2 mm. The trees were sprayed from both sides. The effectiveness of spraying operations was tested by placing water-sensitive papers (WSP) on the upper side and on the lower side of leaves. WPS is coated with a yellow surface, which is stained dark blue by imprinting aqueous droplets (Salyani *et al.*, 1999). The drop spots were analyzed with the software MacBiophotonics ImageJ 1.48 (MacBiophotonics, McMaster University, Hamilton, ON, Canada) to derive the size of the droplets and to calculate the Volume Median Diameter (VMD or d50). Drops were classified according to the index adopted by the British Crop Protection Counciland the American Society of Agricultural & Biological Engineers (Baldoin, 2012, tab. 4)

III. Effectiveness of curative pruning

a. Rate of bacterial migration in controlled condition and inside grafted and self-rooted plants in orchard conditions.

In order to verify whether curative pruning can reduce the disease impact in the orchard the first step is to evaluate the rate of migration of Psa inside the plant. The experiment was performed on the most susceptible host plant (*A. chinensis*). Six potted plant were inoculated by wounding a shoot at 50% of its total length with a blade dipped in PsaCFB7286GFPuv suspension (10^4 $CFUml^{-1}$). The inoculation point was labelled and 14, 21 and 120 days after inoculation, section of 1 cm were excised every 10 cm above and below the infection site from the plant tip till roots. The scheme of the sampling in reported in figure 19. A Special attention was paid to the branching node to verify if the pathogen migration is influenced by these anatomical parts.

b. Testing the coppicing as a possible curative system for compromised plants

The experiment aimed to verify if the coppicing of infected trees could be used as curative system for highly infected plants. Coppicing could be effective only if the bacterial migration does not progress beyond the grafting point and no bacterial colonization is found inside the root system. The experiment was performed in a commercial orchard of *A. chinensis (cv.* Jintao) located in Castel del Rio (Italy, 44°13'21.76"N & 11°30'23.47"E). All the trees were uprooted. In this orchard, samples were collected from 5 grafted plants and 5 self-rooted plants. Wood samples of the plant were taken above and below the grafting point, and from roots (>1 cm diameter) and radicles (<1 cm diameter). Psa endophytic population inside the wood was determined as previously described.

c. Testing if commercial pruning play a role in the spread of the disease inside the orchard.

The experiments were conducted in 3 commercial orchards located in Faenza one each for *A. deliciosa* cv. Hayward (44°15’12.85’’N; 11°50’51.94’’E), *A. chinensis* cv. Hort16A (44°14’10.24’’N; 11°49’35.26’’E), and *A. chinensis* cv. Jintao (44°20'1.31"N; 11°49'45.15"E). Each of the orchards presented approximately 60% of infected plants. In three years of observation (2012-2014), the pruning practices were monitored, in order to verify the possible existence of a correlation between the diseased plants in relation to their position on the row. Data were analysed by k function of Ripley (K (ds), with SPPA 2.0 software) to check if there was a spatial correlation between dead plant and the pruned plants, as a function of their arrangement on the row. Ripley’s K (ds) is s function describing special homogeneity, by measuring the Euclidean distances between pairs of points-events, and indicates the probability of randomly obtaining a given special distribution.

Effect of the training system on disease incidence

A commercial field of *A. deliciosa* located in Castel Bolognese (Italy, 44°20'16.24"N & 11°48'58.30"E) was divided in two parcels, where the vines were trained according with the two typical training systems in Emilia-Romagna (‘pergola’ and ‘gender double curtain’, GDC) (fig.21). Symptom development was monitored monthly for 1 year. During the experiment, also microclimatic data inside the canopy were recorded at hourly intervals. Finally, the penetration of

phytosanitary treatments in the different managed canopies was estimated. Two data logger sensors (EasyLog-UBS-1) were placed in each parcel, at the level of the leader and on the outer side in mid-length of the canes. With this data logger were collected hourly data of temperature and humidity for six months (June-November). The data obtained from the sensors were expressed as an average of 10-day cumulates. Finally, the spray penetration inside the canopy was evaluated as previously described.

Influence of bioregulators on disease development

The experiments were performed both in controlled and field conditions.

In commercial production, both synthetic auxin and citokinins are used. Our experiments aimed to verify whether they may influence plant susceptibility to the disease. Both commercial products and the pure active principles were tested. The different treatments, dosages and time of application are reported in table 10.

Plants were inoculated as previously described. Symptoms were evaluates at 15 and 30 days after inoculation. Furthermore, also the Psa endophytic population was measured as previously described. All the experiments were repeated twice.

In field conditions, the experiments were conducted in 4 commercial orchards of *A. deliciosa cv.* Hayward. The orchards were located at Faenza belonging to Consorzio Agrario (44°19’11.10’’N, 11°53’51.46’’E) Terremerse (44°16’03.35’’N, 11°50’05.43’’E), Granfrutta Zani (44°16’02.14’’N, 11°55’01.98’’E) e Agrintesa (44°15'13.00"N, 11°52'49.53"E) cooperatives.

Standard fertirrigation and crop managment practices were applied. Bordeaux mixture (equivalent to 20 gha^{-1} of active copper) was applied as standard prevention treatment every 15 days from November to February and from April to September. Sitofex was applied 3 weeks after full blooming (1l/ha Sitofex®, 1000l/ha water, experimental launches atomizer). Maxim was applied 2 weeks after petal fall (2 cp/ha Maxim®, 1000l/ha water, experimental launches atomizer). Both products were applied according to the indication (1-1.3l Sitofex® in 500-1000l/ha water, 1-2 cp/ha Maxim®, 800-1200l/ha water) reported on the commercial label. The experimental design consisted in randomized blocks of 7 plants on the same row. Each block was repeated on 6 rows. The treated rows were separated by an untreated row to prevent contamination by drift. Symptoms developmen was monitored monthly. Since the bioregulators may influence fruit production, at the end of the experiment, yield and the main fruit quality parameters were

recorded as described in previous work (Noferini *et al.*, 2013). At harvest, production data (fruits number and production of 3 or more plants) were recorded in several repetitions per treatment. The qualitative analyzes were performed on a sample of 20 fruit. The qualitative data were subjected to statistical analysis one-way ANOVA with Duncan test (P <5%).

Screening of the *Actinidia* genus to select resistant rootstock

Influence of cultivar on the bacterial migration within the plant - The experiment was carried out on self-rooted seedlings of *A. chinensis, A. arguta and A. deliciosa* in controlled conditions. The seedlings were inoculated by spraying with Psa strain 7286 expressing the Green fluorescent protein GFPuv (PsaGFPuv, Spinelli *et al.*, 2011) at the concentration of 10^7 $CFUml^{-1}$. For each species, 6 replicates of 14 seedlings each were considered. At 7, 14 and 21 days after inoculation, bacterial canker symptoms were recorded as described in previous work (Cellini *et al.*, 2014). Furthermore, at the end of the experiments, the Psa endophytic population was assessed as previously described. Finally, since the pathogen migration inside the plant has been demonstrated to negatively correlate with plant resistance (Montefiori, 2014), this parameter was evaluated in independent experiments. For this experiment, potted plants belonging to the following species were wound inoculated as previously described: *A. deliciosa, A. chinensis, A. arguta cv. Issai, A. arguta cv. Missionario C., A. arguta cv. Weiki, A. arguta cv. Ananasnaya, A. arguta cv. Cornell, A. arguta cv. Jumbo green.* At 7, 14 and 21 days post inoculation, the plant was cut in 1 cm sections starting from the inoculation point. The sections were ground in 1 ml of sterile 10 mM $MgSO_4$ to quantify the bacterial population, as previously described.

Effect of Psa infection on fruit production, quality and storability

Quality and storage life of fruits from infected plants - In 2013, 100 fruits were collected from symptomatic and asymptomatic plants in commercial orchards of A. deliciosa cv. Hayward (44°15’12.85’’N, 11°50’51.94’’E), A. chinensis cv. Hort16A (44°14’10.24’’N, 11°49’35.26’’E), and A. chinensis cv. Jintao (44°20'1.31"N, 11°49'45.15"E) located in Faenza. The fruits were analyzed in three different periods: harvesting, two month after cold storage and end cold storage.

In 2014, only A. deliciosa cv. Hayward (44°15’12.85’’N, 11°50’51.94’’E) fruits were collected from asymptomatic and symptomatic plants. From the latter ones, fruit samples were also taken specifically from canes where symptoms were present. Analyses were performed at the harvesting, after cold storage and after a week of shelf life after storage.

The following parameters were measured: fruit weight, DA Index (IAD), flesh firmness, ethylene production, flesh colour, soluble solid contents, dry matter and tritable acidity. IAD is the index of absorbance which is measured with the "kiwi-meter"(TR, Forli, Noferini *et al.*, 2013). The DA index was measured both on the outer wall of the fruits ("external DA Index"), and at 1mm in *A. deliciosa* and 2 mm *A. chinensis* depth ("Internal DA index"). The flesh firmness was measured by penetrometer (Fruit Texture Analyzer, Güss) with 8 mm tip on two orthogonal faces of the fruit, after peeling. Ethylene was measured by gas chromatography (Dani 86.10 HT gas-cromatograph) after two days of closure of vessels of 1.7l, containing two fruits each. Flesh colour was determined by a Minolta colorimeter (CR-400, Konica Minolta, Italy) after removing 1 mm (for *A. deliciosa*) or 2 mm (for *A. chinensis*) of peel on two portions of the fruit placed orthogonally. The different thickness of the removal is in agreement with the common analytical procedures, which are based on the different colouring of the layers of the fruit pulp. The data obtained are expressed in coordinated layers the index °HUE. Soluble solid content was measured by a digital refractometer Atago (Optolab, Modena, Italy), by cutting the two end caps of the fruit, squeezing the juice and by averaging the values obtained by each party. Dry matter was determined by drying a 2-mm thick fruit slice at 60 ° C for 48 hours. Acidity was measured on 10 ml of each fruit juice obtained by squeezing the pulp, diluted in 30 ml of distilled H_2O. The prepared sample was titrated with NaOH (0.25 N) using an automatic titrator (Compact Titrator Crison).

Statistical Analysis - Significance of correlations was assessed with Fisher's exact test, assuming a confidence level of 0.1 or 0.05. The STATISTICA ver. 5software (StatSoft Inc, Tulsa, USA) was used for calculation.The software SAPP 2.0 was used for geostatic analysis of the data.

RESULTS

Influence of fertilization on disease development

Effect of nitrogen form, dosages and mode of application in controlled conditions

Nitrogen form - The application of different sources of N influenced the disease development (fig. 1). The plant receiving NPK fertilization were the ones with lower incidence, whereas the ones fertilized with ammonium nitrate showed the highest. However, due to the high variability of response inside each of the fertilization treatment, none of the observed differences

was statistically significant. All the recorded biometric parameters (production of new leaves, plant weight and quantum yield) were not influenced by the form of nitrogen applied to the plants (data not shown).

Nitrogen dosage - The amount of nitrogen provided to the plants influence the incidence and the severity of the symptom development with the plants receiving the highest N dose showing the mildest symptomatology (fig. 2). On the other hand, the symptoms development did not clearly correlate with the Psa endophytic population inside the plant differentially fertilized (fig. 3). Indeed, the plant receiving the full N dose, which showed less symptoms, harboured the highest bacterial population (fig. 3). Concerning the biometric and physiological parameters, the different dosages of N did not influence the potential photosynthetic efficacy (QY) (fig. 4), but they had an effect on the ratio between fresh and dry matter and on the production of new leaves (fig. 5 and 6, respectively). More in details, the full nitrogen dose decreased the dry matter content in leaves (fig. 5) and also caused a significant leaf drop (fig. 6).

Mode of nitrogen application - The comparison between root or foliar application of the full nitrogen dose did not show any difference neither in the symptom development, nor in the bacterial endophytic population (fig. 2 and 3, respectively).

Effect of micronutrients deficiency or excess in vitro and controlled conditions

The depletion of any single microelement, among those tested, did not cause statistically significant differences compared to control in symptoms, although the deficiency of specific micronutrients, such as Ca and Mn, increased symptoms severity (fig. 7). On the other hand, the lack of Fe resulted in a milder symptomatology. The depletion of Ca, Fe and Mn generally caused an increase in Psa endophytic population (fig. 8). The symptoms development did not clearly correlate with Psa endophytic population, in particular the lack of Fe, which has brought an increase of 28% of the bacterial population than the control also showed the mildest symptomatology (fig. 8). The excess of micronutrients did not statistically influence neither symptoms development, nor Psa endophytic population (fig. 9 and 10, respectively). However, the increase of B and Ca slightly increased symptom severity and Ca also increased Psa endophytic population.

The lack of specific micronutrients did not statistically influenced leaf expansion, dry-to-fresh matter ratio, quantum yield and CO_2 exchange (data not shown). The excess of trace elements did not induce significant differences compared to the control in all the morphological parameters

monitored (data not shown). Only QY was significantly reduced by the increase of micronutrients being the excess of B, Mn and Ca showing the greatest effect (fig. 11).

Influence of irrigation on disease development

Effect of the irrigation rate in controlled conditions - The reduction of irrigation inversely correlated with the increase in disease incidence and symptomatology (fig. 12). Furthermore, the effect of the reduced irrigation was more prominent on symptom development than on disease incidence, being the differences observed in the latter not statistically significant.

Influence of the source of the water supply on the spread of the disease in field conditions - The source of the water supply used for irrigation also influence the disease incidence (fig. 13). Indeed, in all the 3 years of experiments, the irrigation with water from artificial ponds collecting the rain from the watershed where the orchards are located increased the disease incidence both in *A. chinensis* and *A. deliciosa* when compared with orchards irrigated by using running water coming wither from natural river or artificial channel (fig. 13).

Role of pruning in the disease cycle and curative pruning

Pruning cut as possible entry points - The wound caused by the pruning cut represented a possible entry point for Psa for longer than 1 month both in *A. deliciosa* and *A. chinensis* (fig. 14). Since the last monitoring of Psa endophytic population was performed in both species at 32 days after the cut, the experiments do not allow to determine the minimal time needed by the plant to completely seal the wounds. Furthermore, the experiments was carried out in late winter, when the pruning is usually performed, and, therefore, no information were collected of the time needed by the plant to heal a wound in the other seasons the plant needs more than 30 days.

Pruning strategy - The experiments aimed to verify which pruning time and strategy may reduce the risk of Psa infection. Pruning per-formed when the plant is completely dormant resulted in the lowest Psa incidence and severity, while late pruning, per med at the beginning of the vegetative season, were the interventions with the highest incidence and severity (table 3).

Regarding the most appropriate strategy, three different pruning types were tested on the Pergola training system. Standard (standard business practice of pergola, with canes that reach the ground), short (the lower part of the canes of the plants are cut away) and zero leaves (with spring pruning the vigorous shoot of the third leaf over the last fruit of the shoot is cut away) were compared (fig. 15). In none of the trials performed in two consecutive years, any disease

incidence was recorded. However, the experiments allowed to collect an useful set of data concerning the factors influencing Psa epiphytic growth, such as microclimatic parameters in canopy and the penetration of phytosanitary treatments.

As far as the microclimatic conditions are concerned, temperature and leaf wetness, collected within the canopies showed no significant differences in any part of the canopy between three different types of pruning and between different parts of the plant (Fig. 16, 17, 18).The microclimatic data were expressed as an average of 10-day cumulates. In the leader zone (fig. 16a), short pruning showed lower temperatures compared to the standard pruning, throughout the period of observation (average of 1.6 ° C below the standard pruning). In the start canes zone (fig. 16b), the short pruning showed lower temperatures compared to the other two type of pruning and the standard pruning showed the highest temperatures in this area of the canopy (average of 9.6°C over the short pruning). In the middle canes zone (fig. 16c), the standard pruning showed lower temperatures compared to the other two type of pruning.

The effectiveness of spraying operations was tested by placing water-sensitive papers (WSP) on the leaves. The drop spots obtained on WSP were analyzed to calculate the Volume Median Diameter (VMD). The analysis of drop spots obtained with WSP no showed significant differences in the different types of pruning at early stage of leaves development. Instead, at full leaf development the thesis short recorded more effective water sprays penetration; in fact the drops were of medium size in the leaves uppers. In the other two theses there has been a decrease in the efficiency of penetration, in fact, the drops were fine- or very fine (tab. 5).

Effectiveness of curative pruning:

Rate of bacterial migration in controlled condition and inside grafted and self-rooted plants in orchard conditions - In order to perform an effective curative pruning, it is necessary to understand the path and speed of Psa migration inside the host plant. The experiment carried out aimed to evaluate the rate of migration of Psa during time. At 7, 14, 21 and 120 days after inoculation, plants were processed to verify the maximal length of migration (tab. 7). In 21 days, the maximal length of migration was 3 cm root ward from the infection point and only 2 cm upward. At the maximal distance of migration, Psa population was generally comparable to the one at the infection point. The migration correlated, at least partially, with the host susceptibility being faster in *A. chinensis* in comparison with *A. deliciosa*. In 4 months time, the maximal migration upward reached 14.5 cm (till the apical bud), whereas backward, it reached 138 cm (tab.

7). Furthermore, Psa completely colonized also the roots reaching also the most distant (142.5 cm) ones from infection point, passing the graft. The experiment also allowed to verify if Psa may migrate through the branching points (nodes) in the different directions. The results show that Psa may migrate equally in all the different branches from a central node (fig. 19).

Testing the coppicing as a possible curative system for compromised plants - The analyses on plants sampled from the uprooted infected *A. chinensis (cv. Jintao)* orchard confirmed that Psa is able to reach the roots, regardless of whether the plant is grafted or self-rooted. In fact, the roots are infected if the aerial part of the plant is infected with $4.27x10^{\wedge 8} \pm 1.11x10^{\wedge 8}$ $cfug^{-1}$ in grafted plants and $1.15Ex10^{\wedge 8} \pm 5.17x10^{\wedge 7}$ $cfug^{-1}$ in self-rooted plants. The radicles have showed a bacterial population higher than the aerial part (tab. 8). Therefore, performing coppicing in order to graft a new scion on the existing rootstock does not seem an effective strategy to re-grow new productive plants.

Testing if commercial pruning play a role in the spread of the disease inside the orchard – In order to verity the possible existence of correlation between the disease plants in function of their position on the row,the distribution of plants uprooted after severe infection was monitored in 3 infected commercial orchard over three years. Knowing that the distance between the plants is less than 5 meters, geostatistical analysis of the data did not show a significant influence between the cut plants placed nearby (fig. 20). This data show that the manual pruning operations along the row do not favour the disease spread.

Effect of the training system on disease incidence - In this experiment, the influence of the different training on the disease development was tested. In Emilia-Romagna *Actinidia* is farmed with two types of training system: pergola and Gender Double Curtain (GDC) (fig. 21). In the whole duration of the experiment (six months), no disease occurrence was observed. However, the influence of these two training system on the microclimatic conditions inside the plant or the effectiveness of water sprays was assessed. VMD analysis of treatments showed no differences in the early stages of development of the leaves. On the other hand, treatments operated a month before the harvesting, allowing a full leaf development, showed the most effective results on the upper leaves in the GDC vine training (tab. 10). Meteorological data collected within the canopies showed no significant differences between two different training systems (fig. 22). The data were expressed as an average of 10-day cumulates. Knowing that the disease produce symptoms only a mild temperatures (approx. below 25°C), the evolution of temperature analyzed between the different parts of the two training system. Differences in temperature between GDC and pergola

(fig.23) showed that inner and outer canopies trained as GDC are, on average, 17.2 °C and 5 °C warmer, respectively, compared to the pergola. This date were also reflected in the difference of the moisture, in fact pergola has on average increased humidity of 31.2% compared to the GDC in the interior and 51.6% on the outside (fig. 23).

Influence of bioregulators on disease development

The experiments were conducted both in controlled and orchard conditions. When applied 15 days prior inoculation, the syntetic Forchlorfenuron (Sitofex) did not influence the diseases incidence (fig. 24 a), but reduced both the symptom severity and Psa endophytic population (fig. 20 b and c, respectively). The syntetic auxin 3,5,6-TPA (Maxim) increased disease incidence and severity and Psa endophytic population (fig. 24). More in details, the lowest dosage increase disease incidence and severity, but not the bacterial population, whereas, the highest one increase the severity and the bacterial population, but not the incidence.

When applied 30 days prior inoculation, the highest dose of sitofex (3ml L^{-1}) significantly reduce the disease incidence, severity and the pathogen endophytic population (fig. 25). None of the other treatments affected the disease incidence and the endophytic population of Psa.

In a second set of experiment, also the pure active principles where tested. In this case, all the treatments were applied at 7 days before the inoculation and symptoms were recorded at 10 and 30 days after inoculation. Furthermore, at 30 days from the inoculation also disease severity and Psa endophytic population were recorded (fig. 26-27 c).The use of the pure active principle confirmed the results obtained with the commercial compounds. Also in this case the synthetic cytokine reduced the disease incidende (fig. 26a), whereas, auxin increased it (fig. 27a). None of the texted compounds significantly affected the disease severity, even though auxinic compounds generally incremented it. Regarding, Psa endophytic population, also in this case, the synthetic cytokines reduced it, while the auxin increased it (fig. 26-27c).

The field trials allowed to investigate the effect of synthetic bioregulators both on the disease development and on the vegetative and productive performances of the plants. The experiments were conducted in 2013 on 4 different commercial orchards. Both cytokine and auxin incremented the disease incidence when applied to increase fruit production (tab. 11). Concerning the effect of the two bioregulators on fruit production and size, the compounds generally cause a moderate increase in the yield plant and in the fruit size (tab. 12). None of the results were statistically

significant. Furthermore, none of the monitored qualitative parameters (sugar content, dry matter, flesh firmness, titrable acidity, flesh colour) was significantly influence by the treatments (data not shown).

Screening of the Actinidia genus to select resistant rootstock

Different genotypes belonging to *A. arguta* than *A. chinensis* and *A. deliciosa* were tested for their susceptibility for Psa. The aim of the experiment was to screen for resistant varieties to be possibly used as rootstock for the commercial varieties. In the experiment performed by stab inoculation all the accessions belonging to *A. arguta* showed to be the most resistant to the disease showing the lowest Psa endophytic population and migration inside the tissues (tab. 13). In this species, the cultivar *A.arguta* cv. Ananasnaya and *A. argura cv.* Jumbo green showed the lowest migration, instead, the cultivars *A. arguta* cv. Missionario C. and *A. arguta* cv. Weiki presented a greater migration reaching 2 cm from the infection site (tab. 13).

In the experiments performed by spray inoculation, *A. deliciosa* generally harboured the lowest Psa epiphytic population (fig. 28). On the other hand, no differences among Psa endophytic population were observed in the three considered species. *A. arguta* showed less severe symptoms than the other two species, especially three weeks after inoculation (fig. 29).

Effect of Psa infection on fruit production, quality and storability

The analysis conducted in the two years of experimentation on the fruits from symptomatic and asymptomatic plants showed that the quality parameters are influenced by the presence of the disease and the changes in fruit quality can be observed both at harvest and after cold storage. Fruits harvested from symptomatic plants have a lower weight, particularly in *A. chinensis* cultivars (tab. 14). Flesh colour at harvest is also affected by the disease, being enhanced in *A. chinensis* cultivars, and clearer in *A. deliciosa* cv. Hayward (tab. 14).

In the second year of experiments, the fruits were grouped in 3 categories: the fruits harvested form healthy plants, the one from symptomatic plants and, finally, the one harvested from a symptomatic branch. After 2 months of storage and 1 week of shelf life, fruits from diseased plants produce significantly more ethylene than healthy ones (fig. 28). More in details, the fruits harvested from symptomatic branches produced the highest amount of ethylene (0.1386ppm/g l

hour), followed by the ones harvested on symptomatic plants and by the one from healthy plants (0.0031ppm/g l hour) (fig.28).

DISCUSSION

Due to the influence of different environmental conditions (Donati *et al.*, 2014) on Psa and bacterial canker, the role of agronomic practices commonly carried out by farmers (such as training system, pruning, sanitation pruning, irrigation, fertilization) in the incidence and severity of the disease was explored. Moreover it was analysed if the presence of the bacterium in the plant could change the fruits quality and storage life.

Influence of fertilization on disease development

Our findings showed that nitrogen fertilization promotes bacterial growth and disease development. The increase in nitrogen fertilization may favour the disease since new succulent and more susceptible shoots are produced. Furthermore, nitrogen fertilization results in more dense canopy which may provide favourable microclimatic conditions for Psa epiphytic growth. Finally, the increase in shoot growth will lead to the need of more pruning intervention, thus augmenting the risk of infection through pruning wounds.The effect of nitrogen on Psa did not depend on its administration mode (root or spray). On the other hand, the application of NPK fertilizers, which also provide a balance amount of the other macronutrients, seems to decrease the plant susceptibility to Psa in comparison with fertilisers providing exclusively nitrogen. In conclusion, we suggest a reduction in the use of nitrogen fertilization.

Several environmental factors and physiological conditions may influence the absorption and the transport of the mineral elements within the plant (Ferguson et al., 2003). This imbalance in the mineral elements absorption, can cause stress in the plant and make it more susceptible to pathogens attack. For this reason, we tested whether Psa growth or host plant defences could be affected by an excess or a deficiency of microelements, or by different rates of nitrogen. In general, micronutrient deficiency promotes bacterial growth, especially in the case of iron. In literature, it is reported that iron is a key nutrient for *Pseudomonas* spp. (Cornelis, 2010). We have found that also the deficiency promotes the growth of the bacterium in planta, possibly due to the fact that the iron deficiency might induce an early senescence, facilitating Psa growth by the

inactivation of plant defences and the demolition of nutrient storages. The excess of microelements caused no significant differences in Psa growth.

Influence of irrigation on disease development

The water availability seems to influence the disease development both directly and indirectly. The direct effect of relative humidity on bacterial growth and spread is well known (Vanneste *et al.,* 2011). Indeed, leaf wetness is crucial to allow Psa epiphytic growth. In our experiments, an indirect effect of water was observed. In fact, the plant treated with a reduce irrigation showed a higher symptomatology. This observation may be explained by the stress caused by water scarcity and by the colonization of vascular tissues by Psa. The colonization of the xylem vessels by the pathogen and it accumulation in the branching nodes, as shown by our experiments on the bacterial migration, may reduce the conductivity of the vessel and worsen the drought stress in the leaves which results in more diffused symptoms. Indeed, in drought stressed plants, Psa symptoms results in large desiccated areas harbouring a consistent bacterial population, which differ from the most common angular leaf spots. Finally, in presence of water scarcity, the plant produces ethylene that has been shown to contribute to Psa virulence (Cellini et al., 2014). The spread of *Pseudomonas syringae syrinagae* has been demonstrated to be to the water cycle (Morry *et al.*, 2008). In addition, these bacteria colonise a wide range of aquatic environment and the phytopatogenic strains may survive for long in water. Psa has been found to survive for more than a week in sterile water (Vanneste *et al.,* 2013), but, so far, Psa presence in irrigation water or in the water shed where infected orchards are located has not been demonstrated (Cindy Morris, personal communication). Our research showed a positive correlation between the disease incidence and the use of irrigation water pumped from artificial basins collecting the rain water from the infected orchards, thus suggesting a possible role of irrigation water in the local spread of the disease. However, further experiments are needed to fully elucidate the role of irrigation water in vectoring Psa. For example, in the area where the experiments have been performed, the only irrigation method used is drop irrigation to the root system and there is no evidence of Psa infecting the plants *via* the root system. A tentative explanation could be that irrigation by water containing Psa may increase the pathogen population in the litter where it can survive, even in harsh conditions, for months (Tyson *et al.,* 2014). The increased environmental Psa population in the orchard may facilitate the spread of the pathogen to susceptible host parts such as leaves and flowers.

Role of pruning in the disease cycle and curative pruning

Pruning is a key activity in the management of kiwifruit vineyard at it contribute to maintain the vegetative and productive balance of the plants, thus allowing to obtain high fruit production and high quality. In our research the different aspect related to pruning from the role of cuts as possible entry points to the influence of the training systems on the disease development.

Our results show that pruning cuts are a risky entry points for Psa and, in the period when pruning is usually performed (late winter) they need more than a month to heal. Therefore, treatments able to seal the pruning cuts or the spray protective compounds able to reduce Psa population need to be promptly applied after pruning. Further experiments are needed to evaluate the most effective and efficient methods to protect the pruning cut. In addition, in order to reduce phytosanitary treatments, the pruning activity must be reduced as much as possible and the different intervention must be concentrated at a short time distance. Finally, our results point out that the less risk period to perform pruning is when the plant is completely dormant. On the other hand, pruning interventions performed at the restart of the vegetative activity result the riskiest ones.

The comparison of different training systems most widely used in our region, Gender Double Curtain – (GDC) and pergola showed that the two systems are characterized by similar microclimatic conditions inside the canopy, but they allow a differential penetration of phytosanitary treatments. More in details, the GDC showed a better accessibility of the canopy to water sprays, due to a greater openness of the canopy at the top next to the leader. Psa is more aggressive at mild temperature (approx. below 25° C), thus the relative difference was analysed between the two training system. The obtained microclimatic data show that the GDC training system allows higher temperatures and lower humidity compared to the pergola one, due to the increased air flow into the canopy. In GDC training system the temperature in leader zone are higher than the pergola-trained ones, because this trained vine receives sunlight even in this zone of canopy. The increase of temperature may play a role in reducing the disease development. Indeed, Psa shows the highest pathogenicity between 12-18 ° C, while its virulence is inhibited by temperatures above 25 °C (Scortichini *et al.*, 2012). The more efficient penetration of phytosanitary spray treatments, a higher light penetration and ventilation which, on one side rise temperature and, on the other, reduces the humidity and the time of wetting the leaves, thus

disadvantaging the bacterium epiphytic phase, suggest that GDC system may be the more suitable one to reduce the disease development.

Nonetheless, the tradition Pergola system may be implemented with specific pruning interventions to be more suitable to decrease Psa infection risk. For these purpose 3 different pruning strategies were tested on the Pergola system (standard, short and zero leaves). On complete expansion of the leaf surface, the standard pruning resulted in the most closed canopy, while the short pruning allowed the best accessibility of canopies to water sprays. In fact, the drops on the water-sensitive papers (WSP) were of medium size on the upper leaves. In the other two thesis (standard and zero leaves) a decrease in the spray penetration was observed. In these treatments, in fact, the drops were fine- or very fine.

In comparison with the other two pruning systems, the standard pruning was characterised by the higher temperatures in the canopy near the leader, due to the increased density of the canopy and lower air circulation. In the canes area the standard pruning showed lower temperatures compared to the other thesis, probably, because of the less solar radiation due to the increased leaves presence. In the zero leaves system, higher temperature values were observed in canes area, probably due to the greater penetration of light inside the canopy. These data suggest that the short and leaves zero pruning forms allow better aeration and irradiation of the foliage. According to collected data, in pruning practice, the canopy thinning should not be excessive to avoid high rates of foliar evapotranspiration, associated to stomata opening and the risk of Psa penetration in plant.

Furthermore, the effectiveness of curative pruning a coppicing was tested. Our results showed that Psa can move both acropetically and basipetally. The migration speed seems to be limited in the first 3 weeks after infection, but in 4 months time the bacterium is able to colonize the whole plant from the apical bud till the fine roots. Therefore a prompt pruning of the infected limps may prevent the systemic migration of the pathogen. Nonetheless, when symptoms (i.e. cankers) are evident on the cane, the infection occurred months before and, likely, the bacteria has already move systemically. The branching nodes and the grafting point do not constitute an impediment for bacterial migration. Therefore, coppicing and re-grafting is not a suitable practice to reconstitute a productive plant.

Finally, the research also aimed to evaluate whether the current pruning practices may contribute to spread the disease. In order to contain the disease spread in open field, a prompt sanitary pruning and eradication of whole infected plants are crucial practices. The geospatial

analysis of the orchard demonstrated that, in the early stages of infection, the diseased plants mostly aggregated along the row rather than among adjacent rows. Thus, mechanical pruning performed along the row or, in case of hand pruning, the lack of an effective disinfection of tools may be regarded as a major issue for the bacterial spread.

Influence of bioregulators on disease development

In controlled conditions, the synthetic cytokine (Forchlorferon), applied up to one month prior inoculation, may induce a modearte resistance against Psa. On the other hand, the auxinic compound 3,5,6-TPA incresed the disease development. Observation on treatments with synthetic auxins and cytokinins agree with previous observations, where auxin and cytokinins showed, respectively, antagonistic and synergistic effects on SA signalling (Pieterse *et al.,* 2012, and references therein). In addition, the application of 3,5,6-TPA in young plants caused phytotoxic effects including deformation of the shoot an hyperthrophic growth of the shoot tips which also resulted in diffuse micro and meso-lesions of the plant surface. These lesions may facilitate Psa infection and spread.

In field conditions, the use of bioregulators slightly increased Psa disease incidence. However, the disease incidence in all the 4 orchards considered for the experiments was very low. This, further experiments are needed to fully elucidate the influence of bioregulators in field conditions

Screening of the Actinidia genus to select resistant rootstock

Different species were tested for their susceptibility to Psa in order to select possible candidates for new resistant rootstocks. The *A. arguta* genotypes resulted the most resistant, being the one with the mildest symptomatology and the minor Psa migration inside the tissues. However, further studies are necessary to verify whether *A. arguta* may be used as a rootstock for *A. deliciosa* and *A. chinensis*. Firstly, its compatibility with the commercial species must be tested. In addition, in case of compatibility, the effect on fruit production and quality must be evaluated. Moreover, the type of resistance and the possible induction of tolerance in the scions must be evaluated. Finally, it must be verified whether Psa may migrate from the scion to a resistant rootstock. In fact, if the pathogen can not move beyond the grafting point, then chopping can be used to obtain a faster reconstitution of highly infected orchards.

Effect of Psa infection on fruit production, quality and storability

In order to verify whether fruit quality, besides crop yield, was affected by the bacterial canker, fruits were separately harvested from healthy, symptomatic plants or from symptomatic canes and the quality parameters and the storage of these fruits were monitored. In general, fruits from symptomatic plants were smaller and with shorter storability. The lower fruits storability may be due to a reduced allocation of nutrients to the fruits in the plants affected by the disease. Indeed, the plant, during the infection may allocate a substantial part of the available resources in resistance mechanisms in order to survive to the infection. Although not a typical climacteric fruit, kiwifruit produces ethylene in its post-harvest life (Sfakiotakis et al., 1999). In this work, a higher ethylene production was recorded by fruits from the symptomatic plants, and in particular by those from the most infected parts, compared to fruits from asymptomatic plants.

Our experiments showed that training systems and pruning intervention allowing a better light penetration may contribute to the reduction of the disease development. An other positive aspects of these pruning is linked with fruit quality and storability and the suggested systems may reduce the negative influence that the disease has on those characteristics of the fruits. Indeed, the radiant energy influences the fruit growth and the berries exposed to high light intensity have a high quality and can be stored for long time, thank to an increase in dry matter and carbohydrate storage (Tombesi et al., 1993).

In conclusion, this work provided an overview of the influence of the different agricultural practices on the Psa disease development. Our findings provide new insight in the role of fertilization, irrigation, pruning and use of bioregulators in influencing Psa spread and symptoms occurrence. A reduced fertilization, a balance irrigation, and the limitation of the use of bioregulators and pruning interventions may contribute to reduce Psa disease severity.

In addition, less dense training systems, such as GDC, which allow a better light and phytosanitary spray penetration, and contribute to increase canopy temperature and reduce leaf wetness should be adopted in new kiwifruit plantation.

The obtained results suggests that further investigations are needed to harmonise the agricultural practices with the most effective and efficient control strategies of the disease in order to developed and integrated disease management system.

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TABLES AND FIGURES

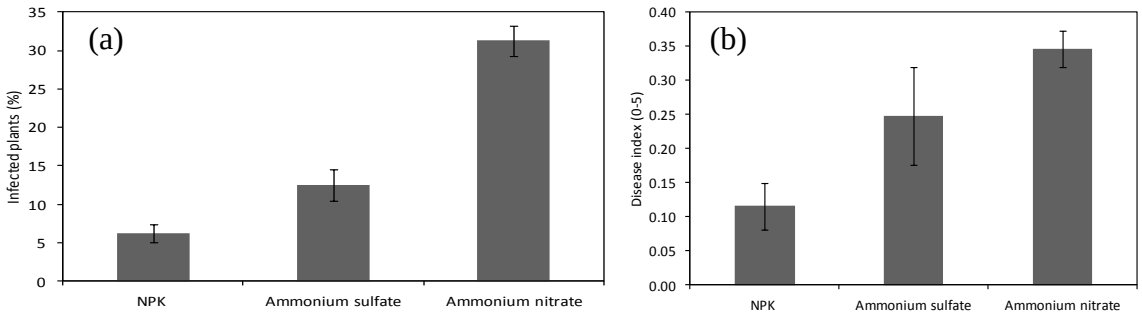


Fig.1- Influence of the nitrogen form used on the *Pseudomonas syringae pv. actinidiae* development: (a) infected plants percentage, 40 day after inoculation with *Pseudomonas syrigae pv. actinidiae* suspension (1x10^10 cfuml^{-1}). (b) Symptoms, severity scale: 0 – healthy leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spots, few coalescent spots; 3 – 4% of the leaf area affected, spost start to coalesc; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

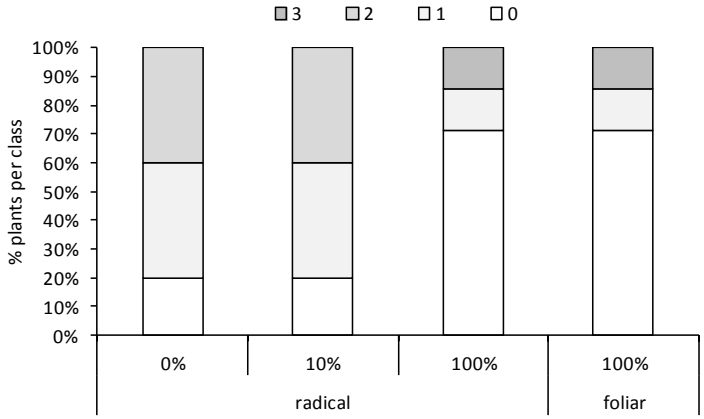


Fig.2 - Relative distribution of foliar symptoms one month after spray inoculation (mean, n=6) of *A. deliciosa* seedlings treated with different nitrogen dosage and mode of application. Scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

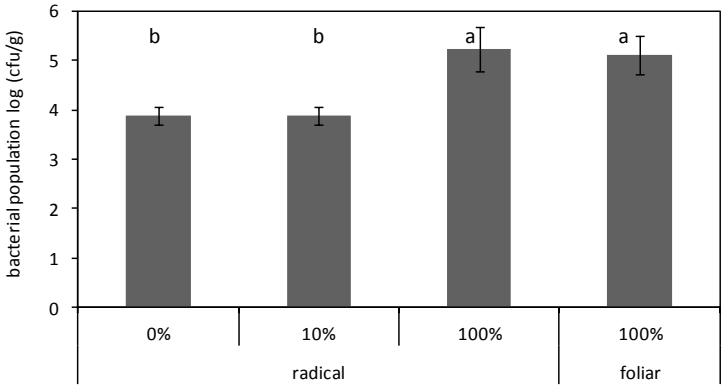


Fig.3 - Endophytic *Pseudomonas syringae pv. actinidiae* populations in *A. deliciosa* seedlings treated with different nitrogen dosage and mode of application (mean ± SE, n=6). Values marked with different letters are significantly different according to Fisher's LSD test (P<0.05).

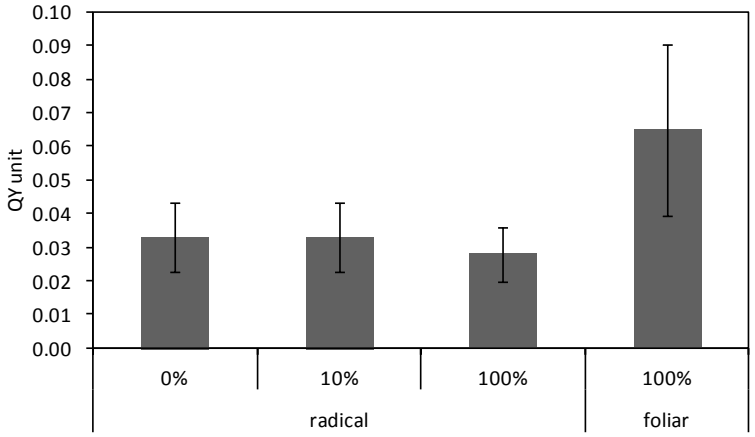


Fig.4 - Quantum yield in *A. deliciosa* seedlings treated with nitrogen dosage and mode of application from beginning to end of treatment (mean ± SE, n=6). The data show no significant differences according to Fisher's LSD test (P<0.05).

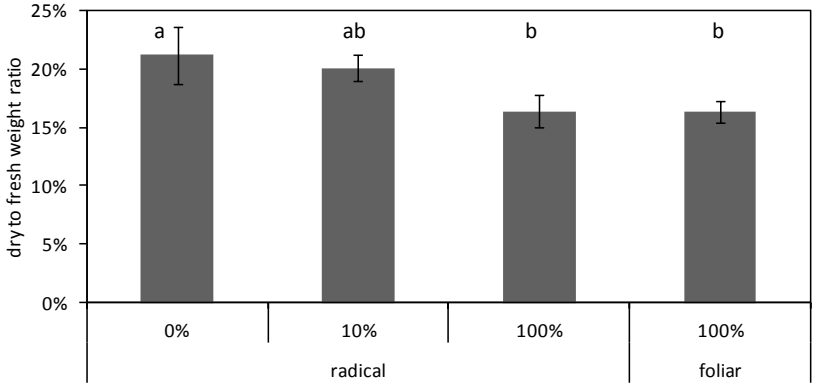


Fig.5 – Dry matter calculated as the percentage of dry/fresh weight ratio in *A. deliciosa* seedlings treated with different nitrogen dosage and mode of application (mean ± SE, n=6) Values with different letters are significantly different according to Fisher's LSD test (P<0.05).

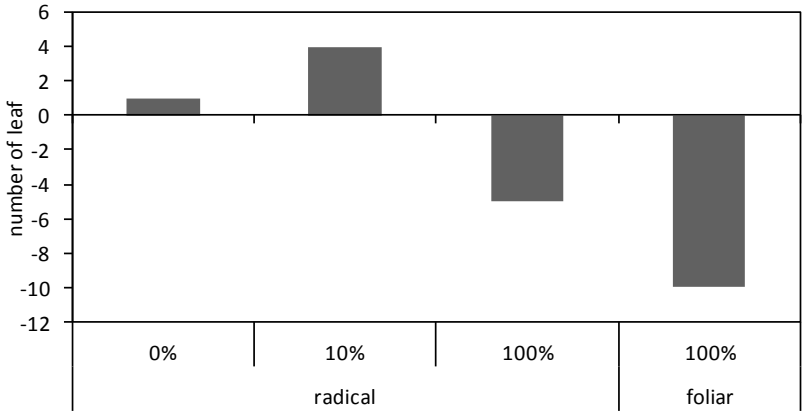


Fig.6 – Difference in the number of leaves present at start and end of the experiment in *A. deliciosa* seedlings treated with different nitrogen dosage and mode of application (n=6).

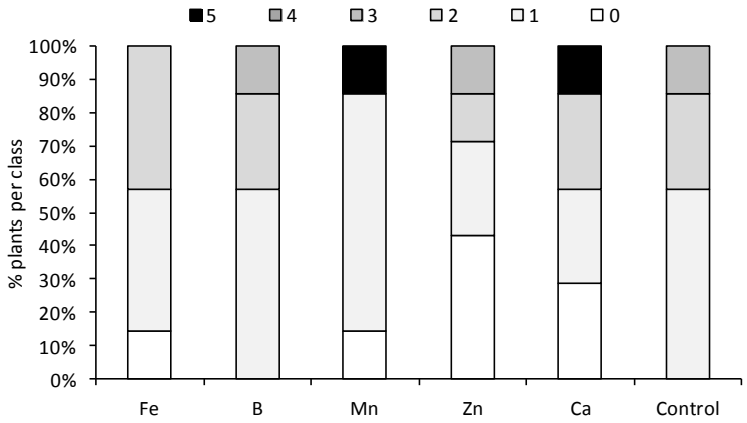


Fig.7- Relative distribution of foliar symptoms one month after inoculation in *A. deliciosa* micropropagated plant grown on media depleted of single microelement (n=6). Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

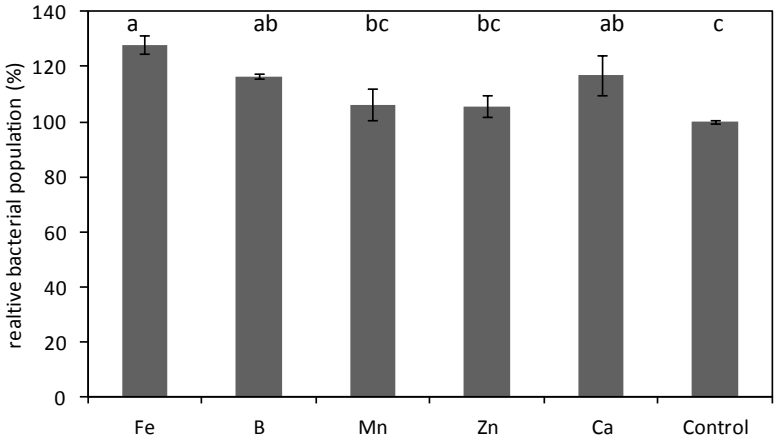


Fig.8 -Endophytic *Pseudomonas syringae pv. actinidiae* populations one month after inoculation in *A. deliciosa* micropropagated plant grown on media depleted of single microelement. The data are expressed in percentage increase compared to control (mean ± SE, n=6). Values with different letters are significant differently by Fisher's LSD test (P<0.05).

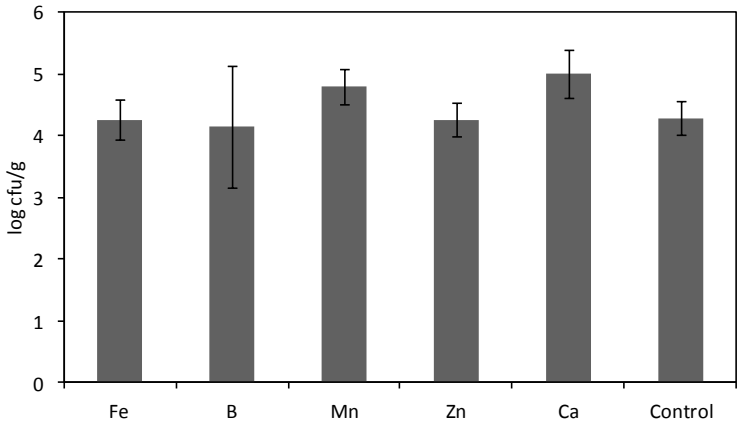


Fig.9- Endophytic *Pseudomonas syringae pv. actinidiae* populations one month after spray inoculation in *A. deliciosa* seedlings grown on media with an excess of single microelement (mean ± SE, n=6). The data show no significant differences according to Fisher's LSD test (P<0.05).

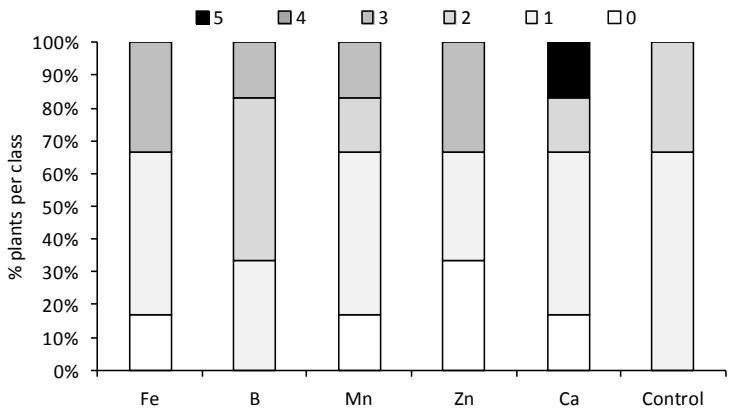


Fig.10 – Relative distribution of foliar symptoms one month after spray inoculation in *A. deliciosa* seedlings grown on media excess of single microelement (n=6). Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

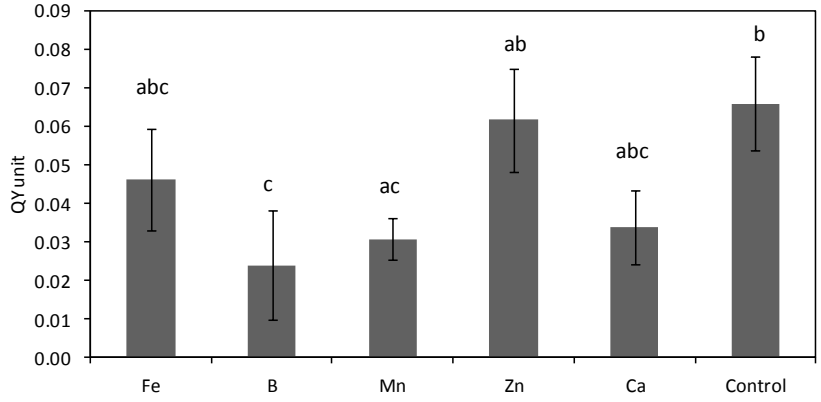


Fig.11 – Quantum yield in *A. deliciosa* seedlings grown on media excess of single microelement from beginning to end of treatment, expressed in PSI units (mean ± SE, n=6) Values with different letters are significantly different according to Fisher's LSD test (P<0.05).

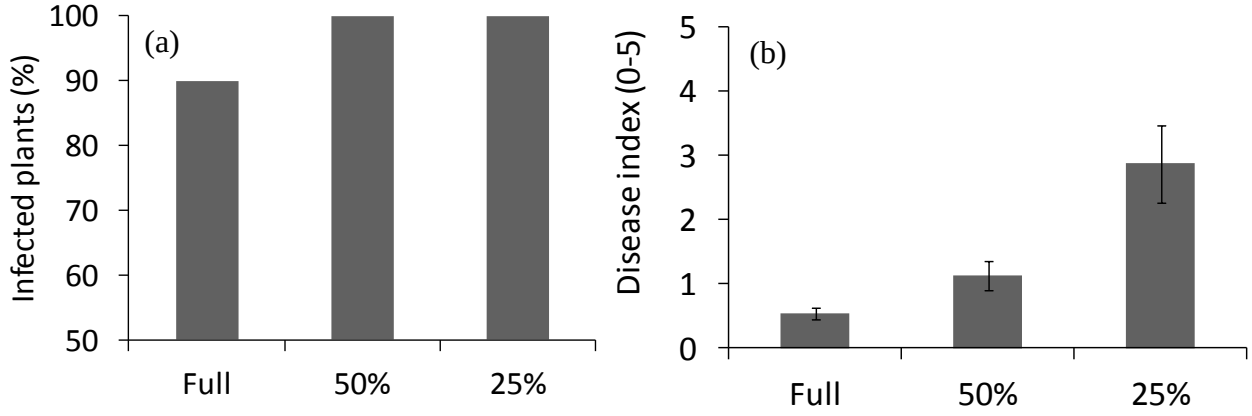


Fig.12 –Influence of plant water status on the development of *Pseudomonas syringae pv. actinidiae:* (a) percentage of infected plants, (b) plant symptoms. Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

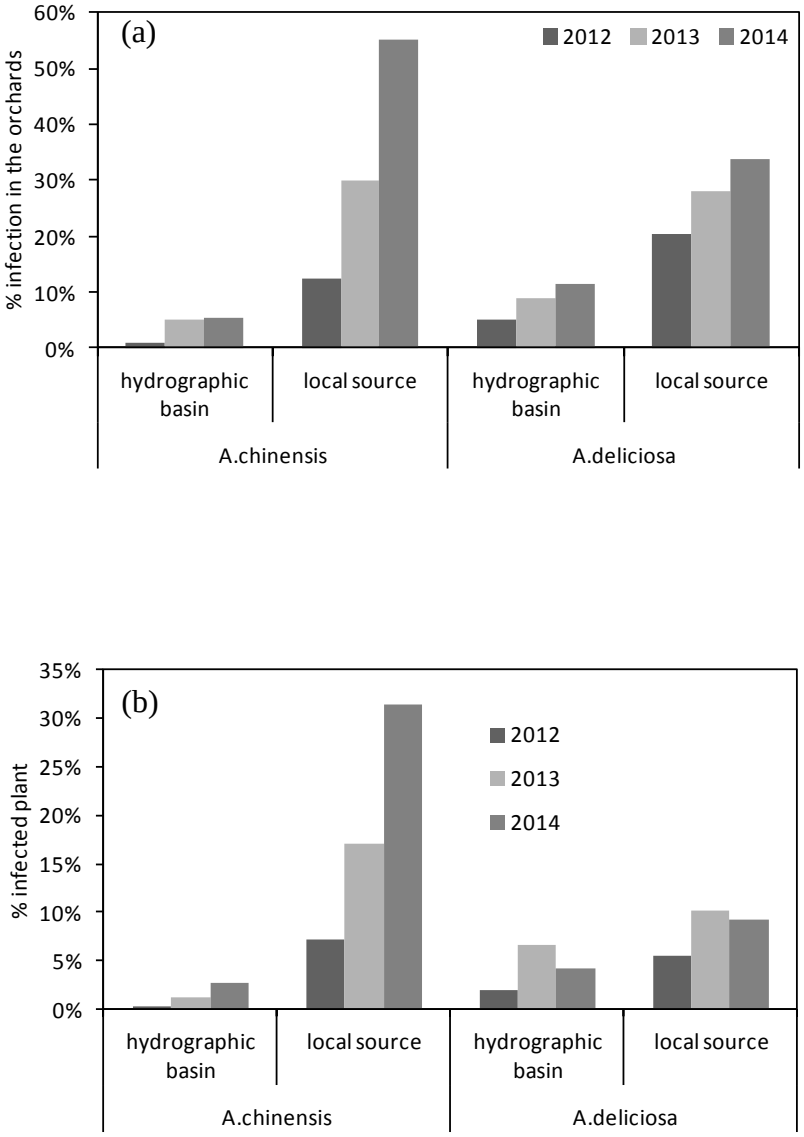


Fig.13 – Influence of the source of the water supply on the disease spread in kiwifruit commercial orchards in Faenza: hydrographic basin (running water coming either from natural river or artificial channel), local source (artificial ponds collecting the rain). (a) percentage infection in the orchard; (b) percentage of infected plant in the areal examined (n= 30 farms, within 28 Km)

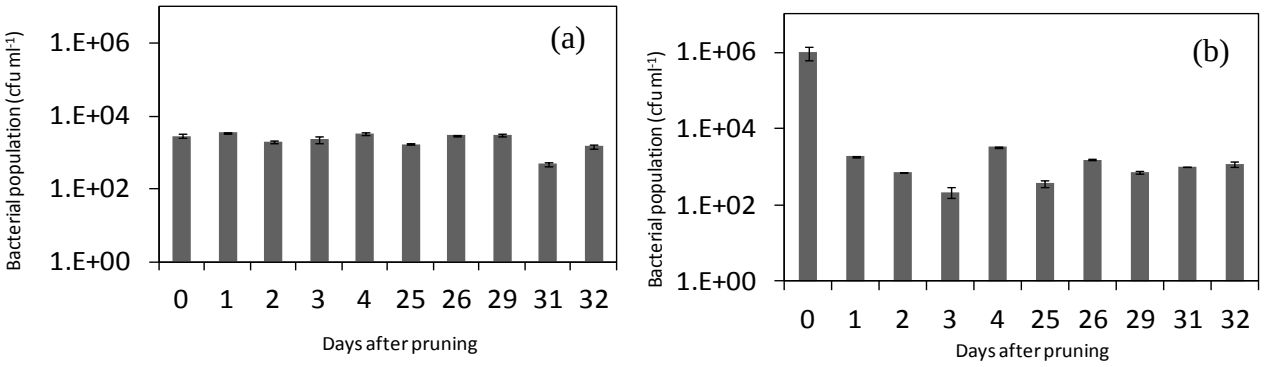


Fig.14 – Bacterium penetration through the pruning cuts made in the course of a month during the winter period (10 days inoculation titre= $8x10^{\wedge 8}cfuml^{-1}$): (a) *Actinidia deliciosa*; (b) *Actinidia* chinensis (mean ± SE).

Treatment label	Date	Phenological stage
1	Beginning of November	5% leaf fall
2	End of November	40-50% leaf fall
3	Beginning of Dicember	95% leaf fall
4	End of Dicember	100 % leaf fall
5	January	Dormant wood
6	Beginning of March	Bleeding sap

Tab.1 – Different pruning treatments of the first trial aimed to determine the best period to perform pruning in order to minimise the risk of *Pseudomonas syringae pv. actinidiae* infection.

Rank	Symptoms
0	none
1	Leaf spots in 1-2% of the leaves
2	Leaf spots in 3-5% of the leaves
3	Cankers and 1 year old wood
	Shoot dieback
4	Cankers on 3-4 year old leader and cut off of the infected leaders
5	Cankers on the trunk and plant cut off at the rootstock level
	Plant dead and pulled out

Tab.2 - The visual scale of plants of the first trial aimed to determine the best period to perform pruning in order to minimize the risk of *Pseudomonas syringae pv.actinidiae* infection.

Treatment label	Date	Phenological stage	Incidence (%)	Symptoms
1	Beginning of November	5% leaf fall	4.4 ± 0.02	1.7
2	End of November	40-50% leaf fall	6.7 ± 0.04	2
3	Beginning of Dicember	95% leaf fall	4.4 ± 0.02	0.7
4	End of Dicember	100 % leaf fall	11.1 ± 0.08	1.4
5	January	Dormant wood	2.2 ± 0.02	1
6	Beginning of March	Bleeding sap	13.3 ± 0.04	3

Tab.3 – Disease incidence in relation to the period of pruning *in A. deliciosa i*nfected commercial orchard (mean ± SE).

Category	Symbol	Color code	Approximate VMD (µm)
Very Fine	VF	Red	< 145
Fine	F	Orange	145 – 225
Medium	M	Yellow	225 – 325
Coarse	C	Blue	325 – 400
Very Coarse	VC	Green	400 – 500
Extremely Coarse	XC	White	> 500

Tab.4 – Drop classification in according to the index adopted by the British Crop Protection Counciland the American Society of Agricultural & Biological Engineers.

Fig.15 – Three types of pruning: standard (standard business practice of pergola, with canes that reach the ground), short (the lower part of the canes are pruned out) and zero leaves (with spring pruning, the vigorous shoot of the third leaf over the last fruit of the shoot are cut) were compared.

month	Types of pruning	upper side of leaves VMD	lower side of leaves VMD
	standard	XC	XC
mar	short	XC	XC
	standard	M	VF
apr	short	VF	VF
	standard	F	M
may	short	F	F
	standard	F	F
	short	M	F
jun	Zero leaves	F	F
	standard	F (a)	F
	short	M (b)	F
sep	Zero leaves	VF (a)	F

Tab.5 - Effect of the pruning system (control/short/zero leaves) on canopy accessibility to water sprays on the upper and lower side of the canopy. Data are expressed according to the VMD (Volume Median Diameter of water drops) index, following the classification BCPC and ASABE: (VF) x<145µm; (F) 145<x<225µm, (M) 225<x<325µm, (C) 325<x<400µm, (VC) 400<x<500µm, (XC) x>500µm. Values with different letter are significant different by Fischer's LSD test (P<0.05), applied on the data expressed in µm.

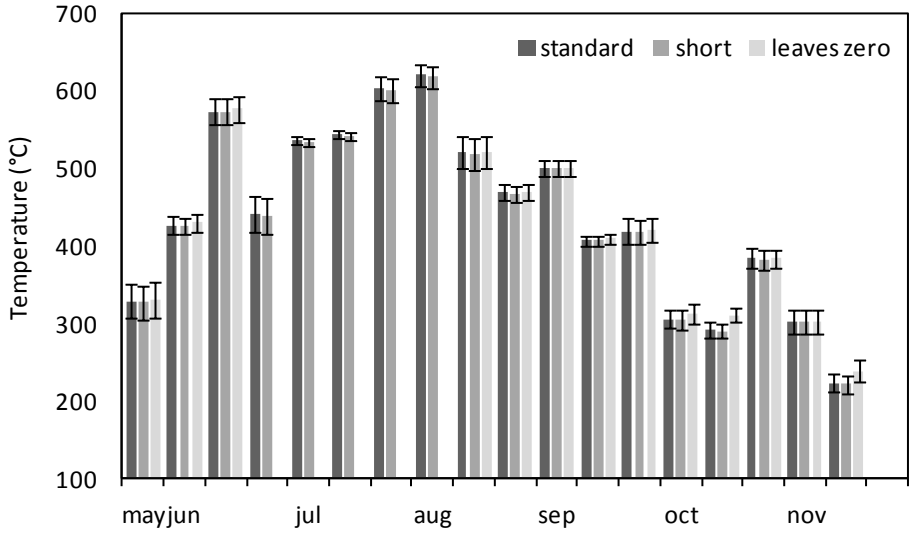


Fig.16 – Temperature in the canopies according to the pruning system: standard, short, zero leaves. Data are reported as the average of 10 days of daily cumulate (mean ± SE). The data show no significant differences according to Fisher's LSD test (P<0.05). Missing data are due to technical issues.

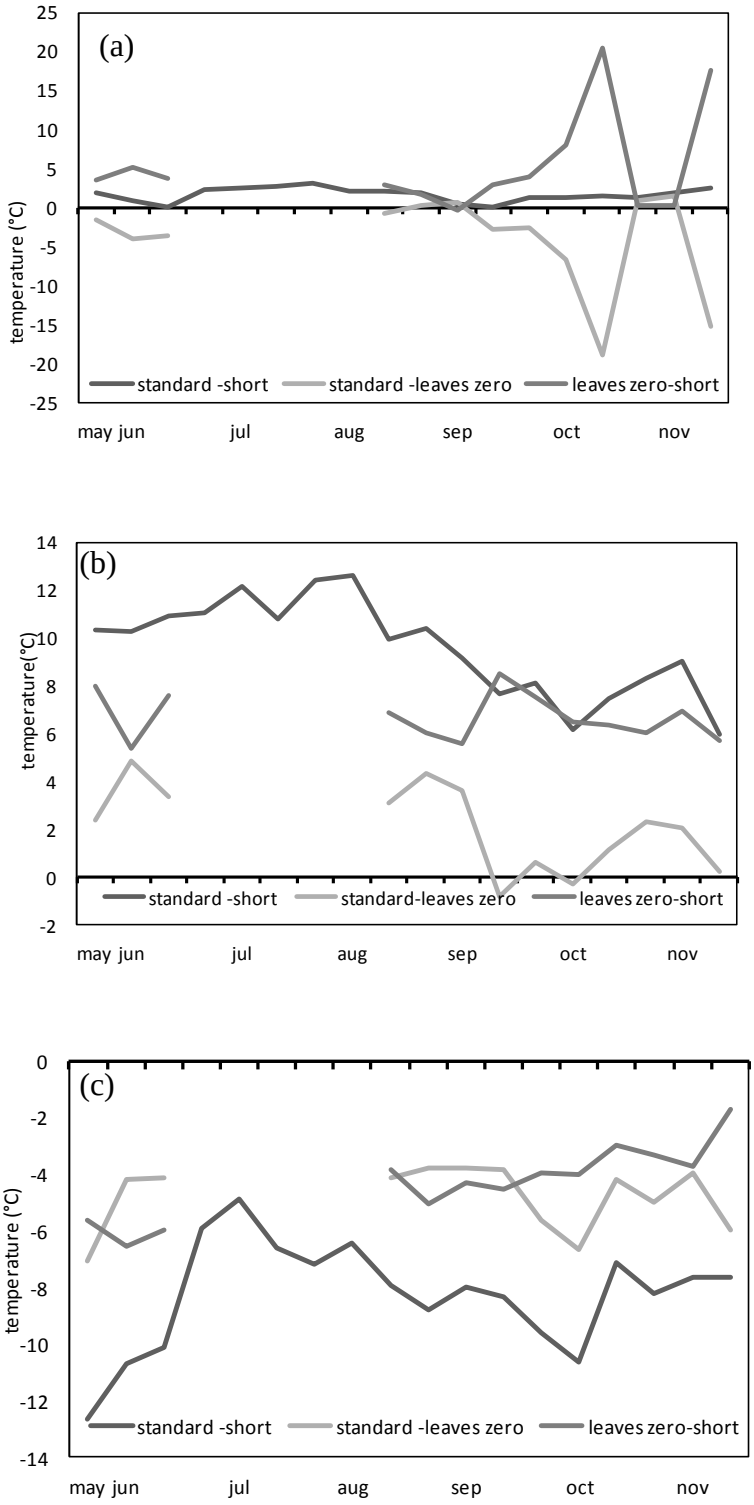


Fig.17 – Difference between the temperatures in the three zones of the canopy (a – leader; b - start canes, c - middle canes) between the three theses (standard- short; standard- leaves zero, leaves zero-short). Data are reported as the mean of 10 days of daily cumulated. Missing data are due to technical issues.

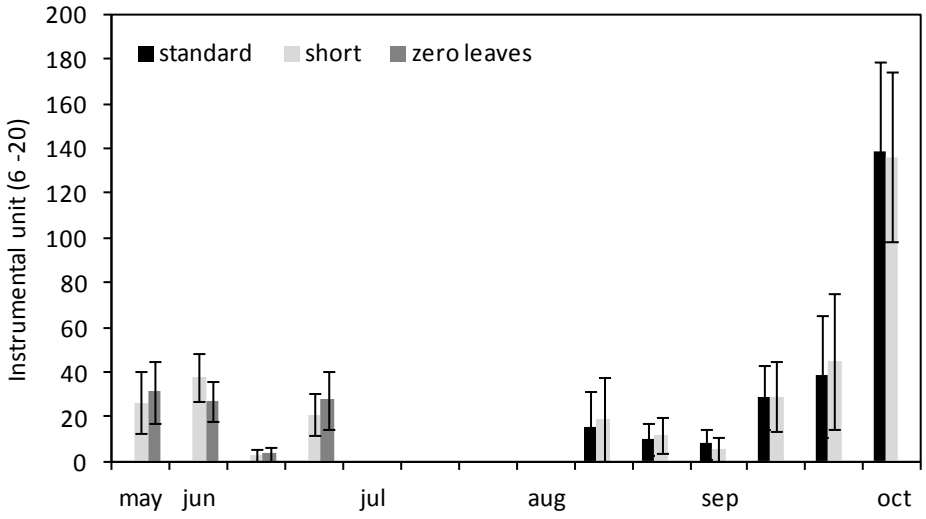


Fig.18 – Leaf wetness are reported as the mean of 10 days of daily cumulated (mean ± SE). The measurement unit is given by the scale of the sensor. The data show no significant differences with three types of pruning, in according to T-test (P<0.05). Missing data are due to technical issues.

Thesis	Incidence (%)	Symptoms
testing farm (harvesting + bordeaux mixture)	2.2 ± 0.02	1.3
thinning + bordeaux mixture	2.2 ± 0.02	1.3

Tab.6 – Training effect of post-harvest on the disease development *in A. deliciosa i*nfected commercial orchard (mean ± SE).

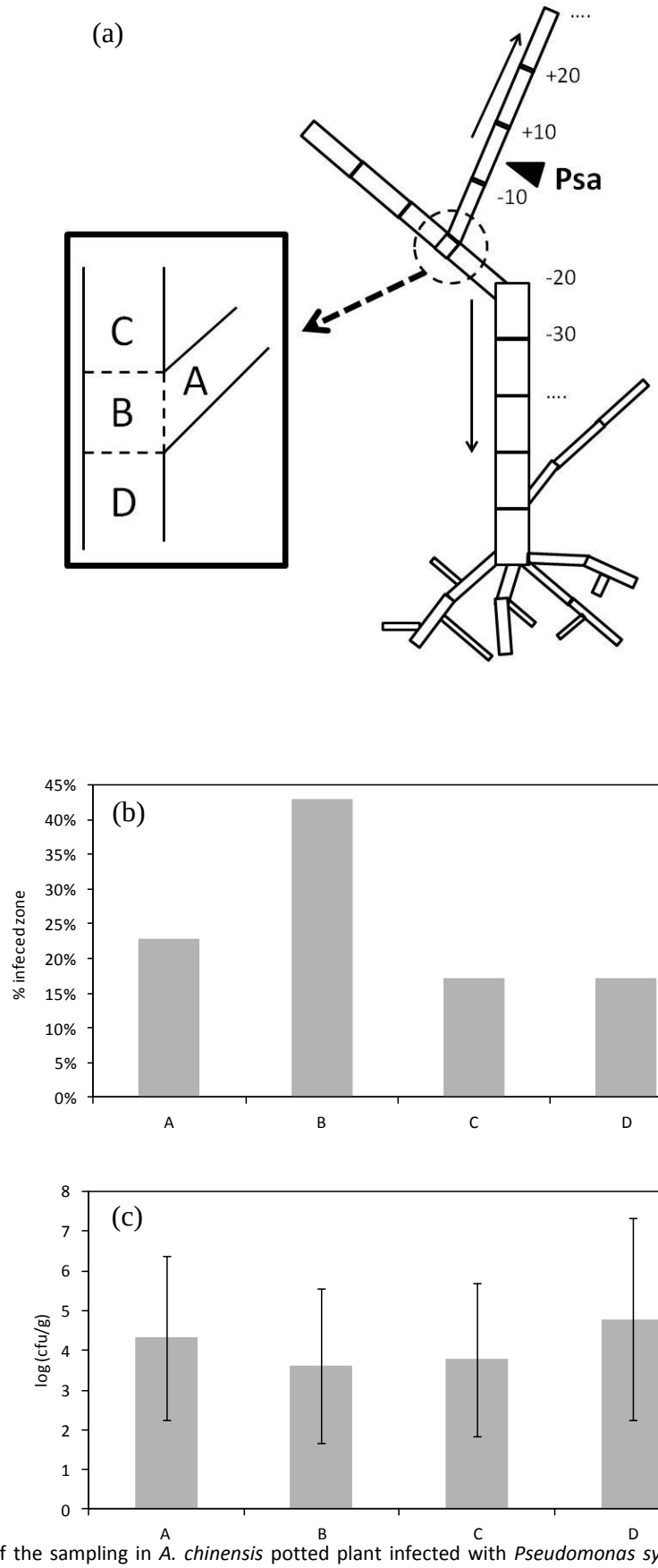


Fig.19 – (a) Scheme of the sampling in *A. chinensis* potted plant infected with *Pseudomonas syringae pv. actinidiae*, in order to evaluate the rate of migration of bacteria in the plant; (b) percentage of infected zone of the branching node; (c) bacterial population in infected zone of the branching node, after 4 months of infection.

		Time after Psa inoculation			
		7 day	14 day	21 day	4 months
Apical migration	cm	0	2	2	14.5
	Psa log (cfu/g)	6.41	5.81	5.57	8.86
Basal migration	cm	0	2	3	138
	Psa log (cfu/g)	6.41	5.57	5.34	7.85
Roots	cm				142.5
	Psa log (cfu/g)				6.23

Tab7 – Maximum migration of *Pseudomonas syringae pv. actinidiae* at 7, 14, 21 and 120 days after inoculation in *A. chinensis* potted plants (mean, n=4).

Zone	Grafted plant		Self-rooted plant	
	Infected part (%)	CFU (cuf/g)	Infected part (%)	CFU (cuf/g)
Scion/ plant	80%	3.03E+08 ± 8.12E+07	80%	2.63E+08 ± 7.88E+07 ab
Rootstock	80%	2.23E+08 ± 7.76E+07		
Root (> 1 cm)	100%	4.27E+08 ± 1.11E+08 A	80%	1.15E+08 ± 5.17E+07 Ba
Radicle (< 1 cm)	60%	4.05E+08 ± 3.69E+07	40%	4.90E+08 ± 1.00E+07 b

Tab.8 – Incidence and population of *Pseudomonas syringae pv. actinidiae* in heavily contaminated, self-rooted and grafted plants sampled from *A. chinsensis* (cv.Jintao) infected commercial orchard (mean ± SE, n=5). Values marked with different lower-case letters are significantly different according to Fisher's LSD test ($P<0.05$) for different part of the single type of plant; different capital letters mark significant differences according to the Student' T-test between grafted and self-rooted plant.

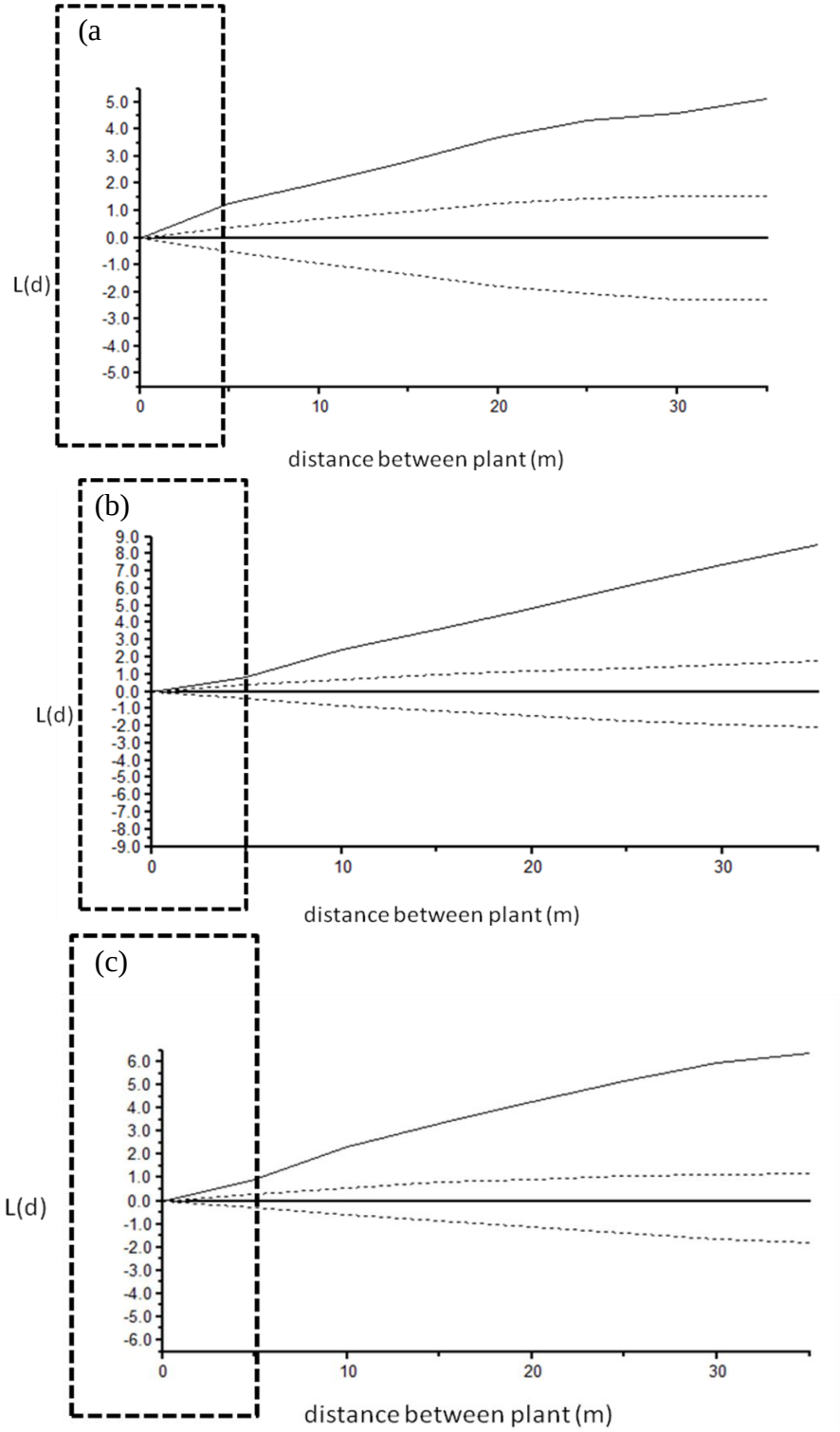


Fig.20 – Ripley's K distribution in three infected commercial orchard of (a) *A.chinensis* cv. Jintao, (b) *A.chinendis* cv. Hort16A, (b) *A. deliciosa* cv. Hayward, showing the positive association among pruned plant Dashed lines show the confidence interval (P<5%).

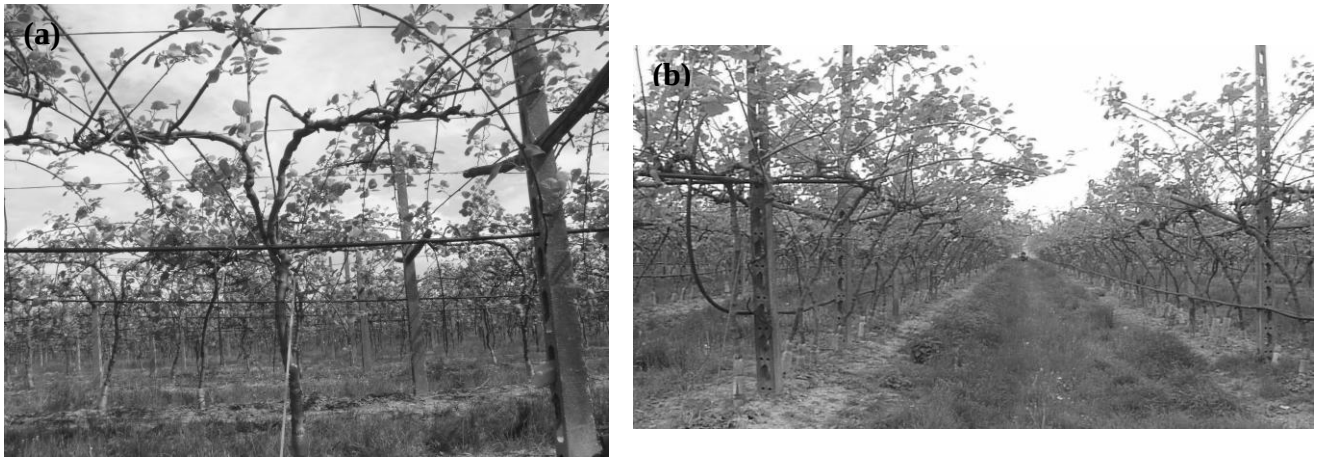


Fig.21– Two training system in *A. deliciosa* cv. Hayward: (a) pergola training system; (b) GDC (Gender Double Curtain) training system.

month	training system	upper side of leaves VMD	lower side of leaves VMD
apr	GDC	F	F
	pergola	VF	F
may	GDC	M	VF
	pergola	F	F
sep	GDC	F (*)	VF
	pergola	VF (*)	VF

Tab. 9 – Effect of the training system (GDC/pergola) on canopy accessibility to water sprays, on the upper and lower side of the canopy. Data are expressed according to the VMD (Volume Median Diameter of water drops) index, following the classification BCPC and ASABE: (VF) x<145µm; (F) 145<x<225µm, (M) 225<x<325µm, (C) 325<x<400µm, (VC) 400<x<500µm, (XC) x>500µm. Data marked with an asterisk (*) were significantly different according to the Student's T-test , with P<0.05, applied on the data expressed in µm.

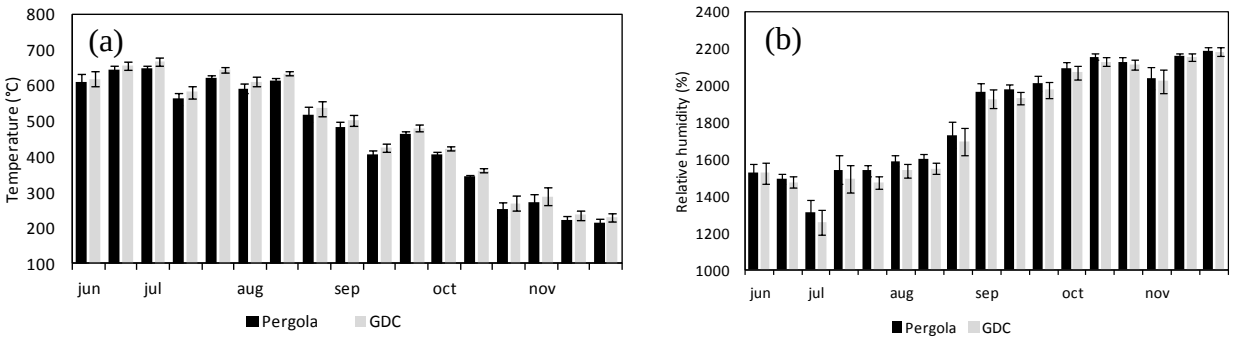


Fig. 22 – (a) Temperature and (b) humidity of the two types of training system: pergola and GDC in commercial orchard of *A. deliciosa*. Data are reported as the average of 10 days of daily cumulate (mean ± SE). The data show no significant differences according to T-test (P<0.05).

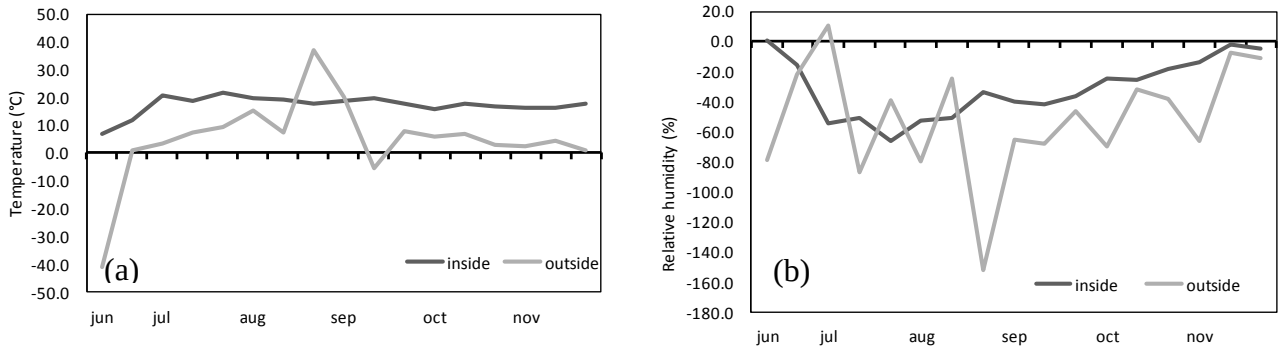


Fig.23– Degrees between the average of 10 days of daily cumulate of GDC and pergola system. (a) temperature and (b) humidity on the inside and outside of the canopy of *A. chinensis*.

Experiment I								Experiment II							
Auxins				Cytokinins				Auxins				Cytokinins			
applied commercial product	applied active principles	Times	Dose	applied commercial product	applied pure active principles	Times	Dose	applied commercial product	applied pure active principles	Times	Dose	applied commercial product	applied pure active principles	Times	Dose
Maxim® (L.Gabbi)		15-30 day after Psa inoculation	20 mg/L	Sitofex® (AlzChem Trostberg GmbH)		15-30 day after Psa inoculation	1 ml/L	Maxim® (L.Gabbi)		7 day before Psa inoculation	20 mg/L	Sitofex® (AlzChem Trostberg GmbH)		7 day before Psa inoculation	1 ml/L
Maxim® (L.Gabbi)		15-30 day after Psa inoculation	40 mg/L	Sitofex® (AlzChem Trostberg GmbH)		15-30 day after Psa inoculation	3 ml/L	Maxim® (L.Gabbi)		7 day before Psa inoculation	40 mg/L	Sitofex® (AlzChem Trostberg GmbH)		7 day before Psa inoculation	3 ml/L
water				water					3,5,6-TPA	7 day before Psa inoculation	20 mg/L		Forchlorfenuron	7 day before Psa inoculation	1 ml/L
									3,5,6-TPA	7 day before Psa inoculation	40 mg/L		Forchlorfenuron	7 day before Psa inoculation	3 ml/L
								water				water			

Tab. 10 - The different treatments, dosages and time of application of bioregulators (auxins, cytokinins) in controlled and field conditions.

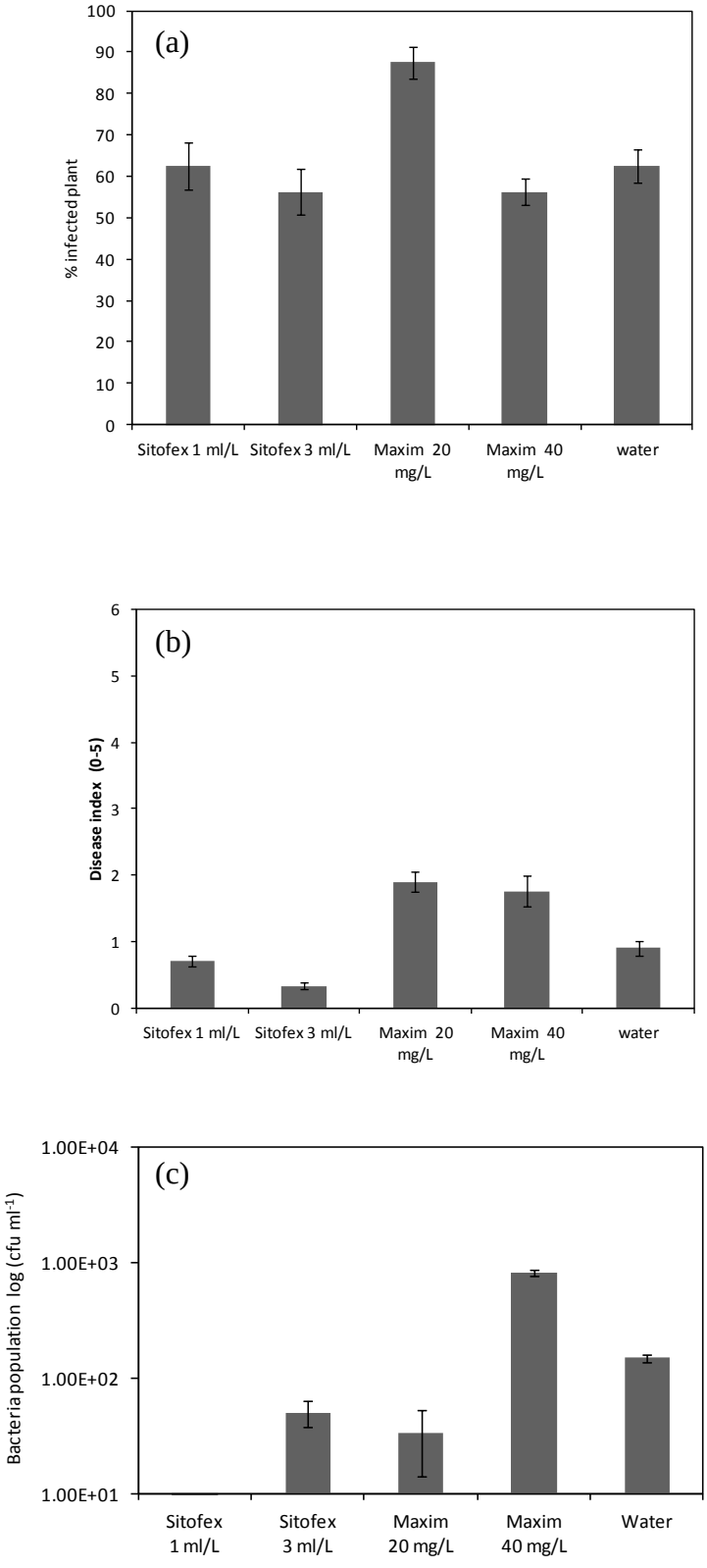


Fig.24– Experiment I – Influence of bioregulator (auxin, citokinins) on disease development with spray treatment 15 day after *Pseudomonas syringae pv. actinidiae* inoculation. The data show: (a) percentage of infected plant, (b) symptoms, (c) bacteria population. Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

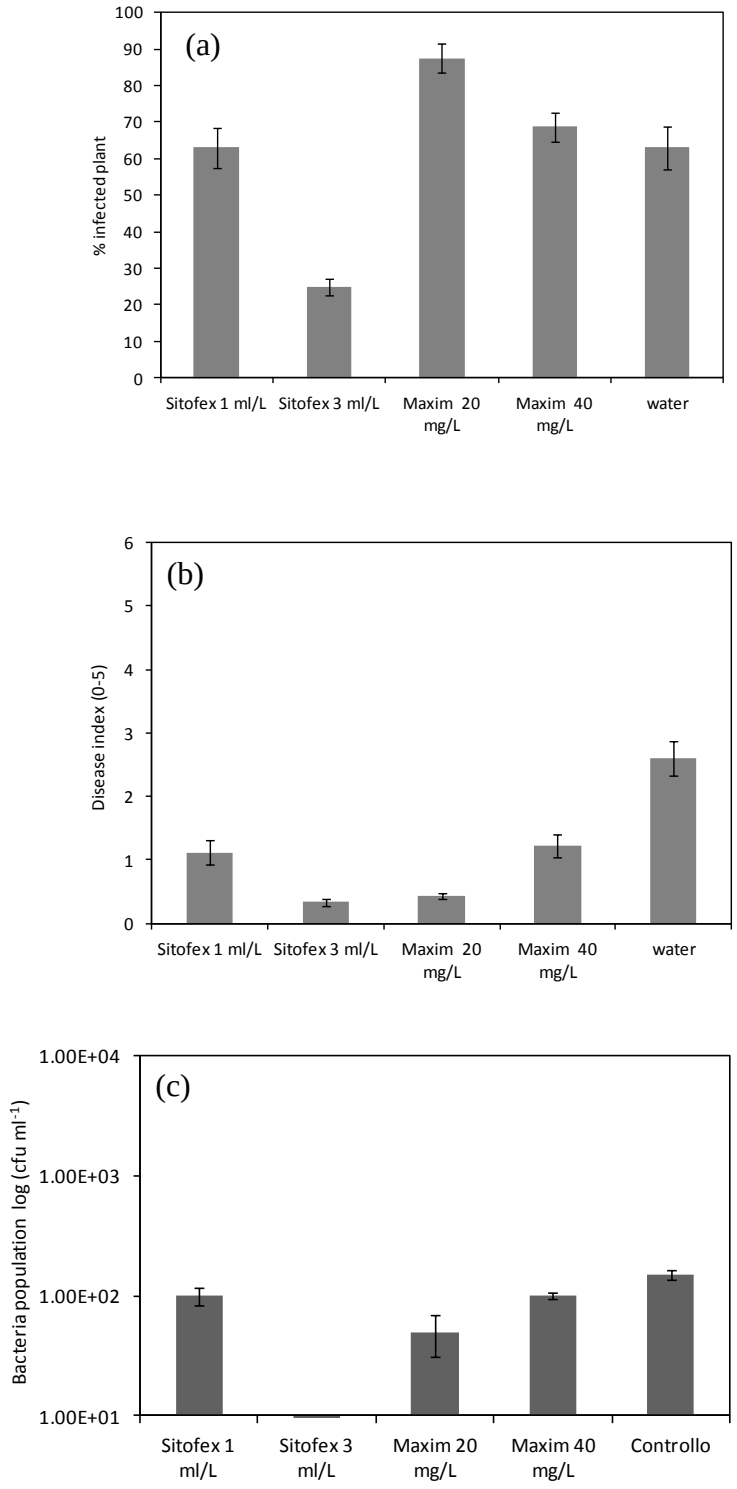
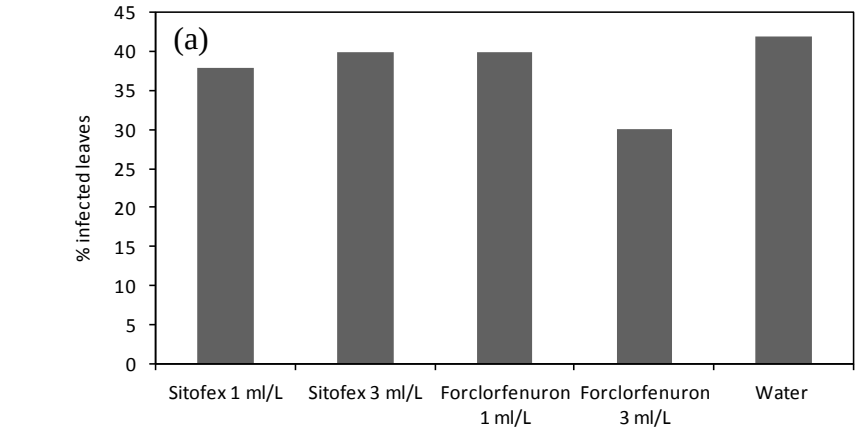
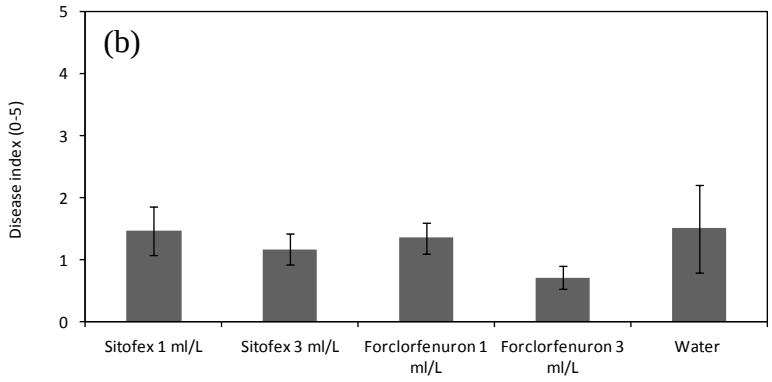


Fig.24– Experiment I – Influence of bioregulator (auxin, citokinins) on disease development with spray treatment 30 day after *Pseudomonas syringae pv. actinidiae* inoculation. The data show: (a) percentage of infected plant, (b) symptoms, (c) bacteria population. Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.





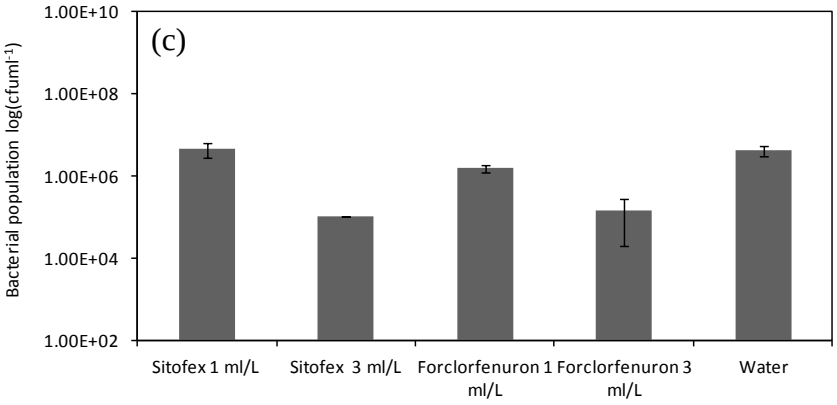


Fig.26– Experiment II – Influence of citokinins bioregulator on disease development with spray treatment 7 day before *Pseudomonas syringae pv. actinidiae* inoculation. The data show: (a) percentage of infected plant, (b) symptoms, (c) bacteria population. Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

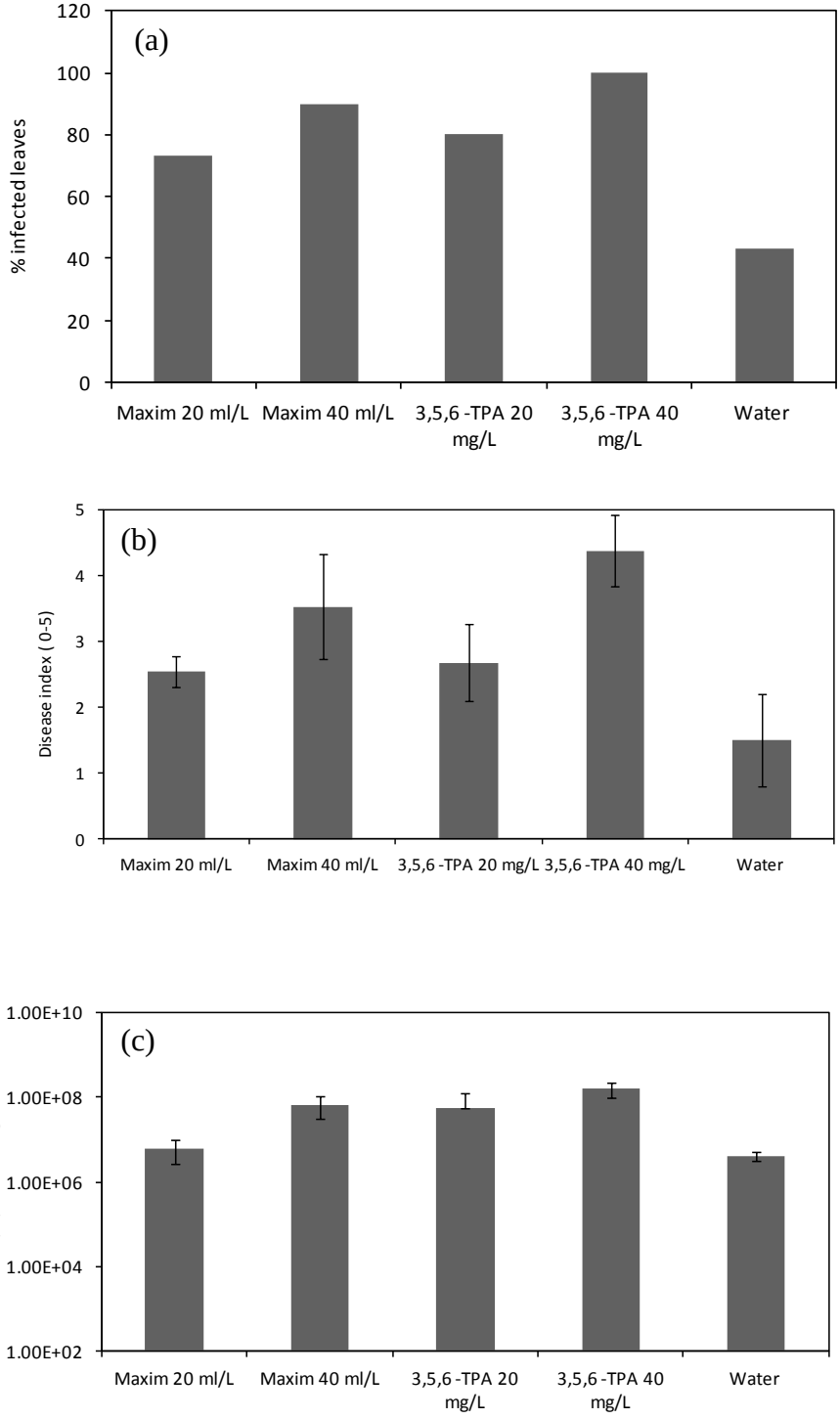


Fig.27– Experiment II – Influence of auxin bioregulator on disease development with spray treatment 7 day before *Pseudomonas syringae pv. actinidiae* inoculation. The data show: (a) percentage of infected plant, (b) symptoms, (c) bacteria population. Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

	number symptomatic plants		
date	control	Sitofex®	Maxim®
31/01/2012	0	0	0
01/03/2012	0	0	0
12/03/2012	0	0	0
30/04/2012	0	0	0
07/06/2012	2	2	3
25/06/2012	2	2	3
06/08/2012	3	7	7
03/10/2012	4	8	8
17/10/2012	4	8	8

Tab. 11 – Number of symptomatic plant in Gran frutta Zani infected commercial orchard. The plants were treated with Sitofex® (1l/ha) and Maxim® (2 cp/ha).

	Terremerse		Consorzio Agrario		Gran frutta Zani		Agrintesa	
Theses	kg/plant	average weight (g)	kg/plant	average weight (g)	kg/plant	average weight (g)	kg/plant	average weight (g)
Control	273.4	82.4	24.2	91.4	13.5	104.1		94.1
Sitofex	242	83.5	27.1	90.5	14.1	108.8		113.8
Maxim	282.8	67.6	25.8	73.7	16.6	113.8		112.2

Tab.12 – Effect of bioregulators in 4 orchard of *A. deliciosa* in Faenza: the products applied were Sitofex® (1l/ha) and Maxim® (2 cp/ha).

Genotype	Time	Point infection log (cfu/g)	Apical migration (cm)	Psa log (cfu/g)	Basal migration (cm)	Psa log (cfu/g)
A. arguta cv. Issai	7 days after inoculation	5.06				
A. arguta cv. Missionario C.		4.81				
A. arguta cv. Weiki		5.10				
A. arguta cv. Ananasnaya		5.25				
A. arguta cv. Cornell		4.84				
A. arguta cv. Jumbo green		5.26				
A. deliciosa		5.39				
A. chinensis		6.41				
A. arguta cv. Issai	14 days after inoculation	5.44				
A. arguta cv. Missionario C.		6.10				
A. arguta cv. Weiki		5.50				
A. arguta cv. Ananasnaya		6.50				
A. arguta cv. Cornell		5.93				
A. arguta cv. Jumbo green		5.35				
A. deliciosa		6.91				
A. chinensis		7.38	2 (whole plant)	5.81	2	5.57
A. arguta cv. Issai	21 days after inoculation	4.91	2	5.28		
A. arguta cv. Missionario C.		4.66	2	4.52	2	4.00
A. arguta cv. Weiki		5.71	2	4.90	2	3.74
A. arguta cv. Ananasnaya		5.21				
A. arguta cv. Cornell		5.42	2	4.28		
A. arguta cv. Jumbo green		4.71				
A. deliciosa		5.60	2 (whole plant)	6.15	3	4.79
A. chinensis		6.37	2 (whole plant)	5.01	3	5.34

Tab.13 – Bacterial populations after stab inoculation in stem tissues of seedlings risen from *A.chinensis, A.deliciosa* and *A.arguta* (mean ± SE, n=3), according to distance from the point of inoculation.

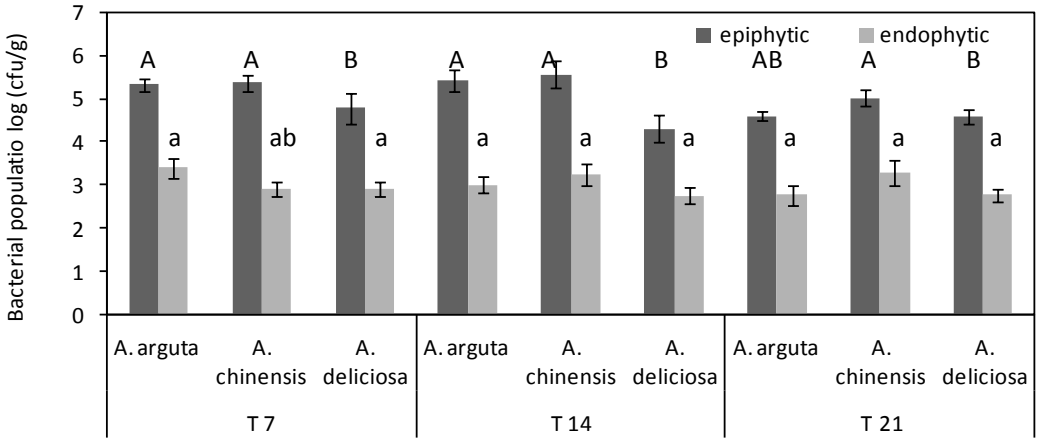


Fig.28 – Endophytic and epiphytic populations of Psa (log cfu/g fresh weight) on seedlings of *A. arguta, A. chinensis* and *A. deliciosa*, after 7, 14or 21 days from spray inoculation (mean ± SE, n=14). Values with different letter are significantly different accotding to Fisher's LSD test (P<0.05).

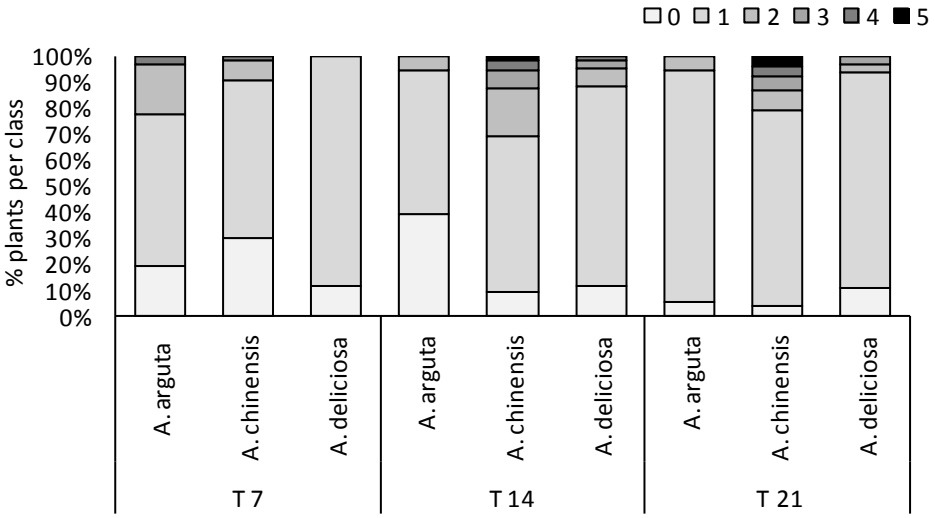


Fig.29– Relative distribution of foliar symptoms 7, 14 or 21 days after spray inoculation (mean, n=14). Severity scale: 0 – healthily leaf; 1- <1% of the leaf area affected; 2 – 1-2% of the leaf area affected, single spot, few coalescent spots; 3 – 4% of the leaf area affected, spot start to coalescent; 4 – 5-9% of the leaf area affected, coalescent spot covering vine and increase size; 5 > 10% of the leaf area affected.

		weight (g)		skin DA (DA index)		flesh DA (DA index)		Hue angle (°)	
harvest	Hayward sym	115.32 ± 16.09		1.27 ± 0.12		0.41 ± 0.07		115.18 ± 0.46	*
	Hayward asy	116.65 ± 21.63		1.25 ± 0.09		0.43 ± 0.10		103.50 ± 1.65	
	Hort 16A sym	103.27 ± 14.08	*	1.31 ± 0.14		0.09 ± 0.10	*	102.60 ± 2.23	*
	Hort 16A asy	113.11 ± 18.83		1.26 ± 0.16		-0.03 ± 0.10		107.29 ± 7.37	
	Jintao sym	66.45 ± 15.93	*	1.11 ± 0.19		0.10 ± 0.12	*	104.01 ± 1.99	*
	Jintao asy	89.24 ± 16.69		1.02 ± 0.15		0.03 ± 0.08		106.02 ± 2.66	
2 months storage	Hayward sym	111.54 ± 14.34		1.36 ± 0.23		0.26 ± 0.06	*	115.78 ± 0.45	
	Hayward asy	109.29 ± 23.06		1.42 ± 0.23		0.42 ± 0.07		115.61 ± 0.53	
	Hort 16A sym	102.07 ± 14.16	*	1.40 ± 0.12	*	-0.03 ± 0.03	*	104.23 ± 1.95	*
	Hort 16A asy	111.62 ± 20.16		1.22 ± 0.19		-0.09 ± 0.03		102.20 ± 1.07	
	Jintao sym	82.03 ± 14.16		1.08 ± 0.20		0.06 ± 0.09		104.84 ± 1.75	
	Jintao asy	75.90 ± 9.34		1.08 ± 0.16		0.07 ± 0.08		104.92 ± 1.42	
4 months stirage	Hayward sym	113.82 ± 15.88	*	1.24 ± 0.25		0.09 ± 0.07	*	115.46 ± 0.85	
	Hayward asy	104.28 ± 17.49		1.16 ± 0.28		0.12 ± 0.06		115.52 ± 0.71	
	Jintao sym	73.42 ± 14.74	*	1.09 ± 0.24		0.02 ± 0.10	*	103.77 ± 2.07	*
	Jintao asy	82.99 ± 14.15		1.19 ± 0.27		-0.07 ± 0.05		101.90 ± 1.81	
		firmness (kg)		**sugar content (°Brix)**		**dry matter (% of fresh)**		**acidity (g/l)**	
harvest	Hayward sym	7.72 ± 0.77		5.60 ± 0.26		0.15 ± 0.01		19.65 ± 0.55	
	Hayward asy	7.67 ± 0.76		5.52 ± 0.21		0.15 ± 0.01		19.30 ± 1.67	
	Hort 16A sym	5.76 ± 1.07		9.50 ± 0.99	*	0.18 ± 0.01	*	15.61 ± 1.28	*
	Hort 16A asy	5.36 ± 1.34		11.42 ± 1.93		0.19 ± 0.01		16.71 ± 0.59	
	Jintao sym	5.67 ± 0.46	*	10.51 ± 0.72		0.22 ± 0.06	*	12.90 ± 0.59	
	Jintao asy	5.38 ± 0.52		10.88 ± 1.26		0.17 ± 0.01		13.20 ± 0.47	
2 months storage	Hayward sym	6.83 ± 0.54		9.59 ± 0.60		0.15 ± 0.01		15.55 ± 0.83	
	Hayward asy	7.05 ± 0.67		9.73 ± 0.37		0.15 ± 0.01		16.74 ± 0.55	
	Hort 16A sym	6.08 ± 0.88		16.33 ± 0.65		0.19 ± 0.01		11.15 ± 0.99	*
	Hort 16A asy	6.50 ± 0.74		16.99 ± 0.86		0.20 ± 0.01		5.48 ± 0.82	
	Jintao sym	5.39 ± 0.53		12.85 ± 0.41	*	0.17 ± 0.00		13.37 ± 0.35	
	Jintao asy	4.99 ± 0.46		13.22 ± 0.33		0.17 ± 0.00		13.21 ± 0.70	
4 months stirage	Hayward sym	3.26 ± 0.85	*	11.43 ± 0.43	*	0.37 ± 0.03		11.86 ± 1.00	
	Hayward asy	4.39 ± 1.31		10.66 ± 0.61		0.36 ± 0.03		13.28 ± 0.72	
	Jintao sym	2.34 ± 0.46		13.88 ± 0.63	*	0.41 ± 0.04		9.77 ± 0.49	
	Jintao asy	2.00 ± 0.62		14.70 ± 0.55		0.44 ± 0.03		9.61 ± 0.61	

Tab.14 - Qualitative analysis of kiwi fruits taken from *A. deliciosa* (cv. Hayward) or *A. chinensis* (cv. Hort 16A or Jin Tao) symptomatic (sym) or asymptomatic (asy) plants: weight, sikn I_{DA}, flesh I_{DA}, Hue angle, firmness, sugar content, dry matter and titratable acidity, measured at harvest, after 2 months of cold storage, after 4 months of cold storage. Values are expressed as mean ± SE, n=30; those marked with an asterisk (*) are significantly different according to Student's T-test, with $P<0.05$.

		weight (g)			skin DA (DA index)			flesh DA (DA index)			Hue angle (°)		
	asymptomatic	114.39	± 25.51	a	1.36	± 0.09	a	0.43	± 0.12	a	115.09	± 0.47	a
	symptomatic	113.26	± 26.77	a	1.35	± 0.12	a	0.36	± 0.11	b	115.03	± 0.58	a
harvest	diseased zone	101.60	± 26.96	b	1.28	± 0.15	b	0.40	± 0.13	ab	115.20	± 0.45	a
	asymptomatic	125.16	± 19.46	a	1.31	± 0.08	a	0.21	± 0.18	a	100.98	± 2.18	a
2 months	symptomatic	124.38	± 26.63	a	1.35	± 0.09	a	0.21	± 0.09	a	103.36	± 2.09	b
storage	diseased zone	99.14	± 25.43	b	1.22	± 0.16	b	0.18	± 0.10	a	115.00	± 0.47	c
	asymptomatic	111.64	± 26.93	a	1.41	± 0.08	a	0.09	± 0.09	a	115.00	± 0.47	a
	symptomatic	113.76	± 26.47	a	1.37	± 0.12	a	0.13	± 0.11	ab	115.34	± 0.61	a
shelf life	diseased zone	103.84	± 26.87	a	1.29	± 0.12	b	0.15	± 0.11	b	115.29	± 1.07	a
		firmness (kg)			**sugar content (°Brix)**			**dry matter (% of fresh)**			**acidity (g/l)**		
	asymptomatic	7.11	± 0.84	a	5.78	± 0.49	a	0.37	± 0.05	a	21.08	± 2.00	a
	symptomatic	6.97	± 0.79	ab	6.21	± 1.02	b	0.37	± 0.05	a	20.05	± 0.75	a
harvest	diseased zone	6.65	± 0.75	b	5.87	± 0.81	ac	0.39	± 0.05	b	21.50	± 0.69	b
	asymptomatic	4.24	± 0.79	a	10.96	± 0.87	a	0.39	± 0.05	a	14.05	± 0.46	a
2 months	symptomatic	4.66	± 0.74	a	11.69	± 1.25	b	0.40	± 0.05	a	13.17	± 0.54	ab
storage	diseased zone	4.26	± 1.31	a	11.05	± 1.62	b	0.39	± 0.06	a	12.57	± 0.35	b
	asymptomatic	1.46	± 0.66	a	13.61	± 0.77	a	0.31	± 0.04	a	11.67	± 0.02	a
	symptomatic	1.85	± 1.16	a	13.35	± 1.38	a	0.29	± 0.03	b	9.79	± 0.05	ab
shelf life	diseased zone	1.49	± 0.88	a	12.14	± 1.51	b	0.29	± 0.03	b	8.03	± 0.05	b

Tab.15 – Qualitative analysis of kiwi fruits taken from *A. deliciosa* (cv. Hayward) asymptomatic plants, symptomatic plants, and vines specifically presenting symptoms presenting symptoms : weight, sikn I_{DA}, flesh I_{DA}, Hue angle, firmness, sugar content, dry matter and titratable acidity, measured at harvest, after 2 months of cold storage, shelf life. Values are expressed as mean ± SE,n=30; for each parameter and time point, data marked with different letters are significantly different according to Fisher's multiple range test (P<0.05).

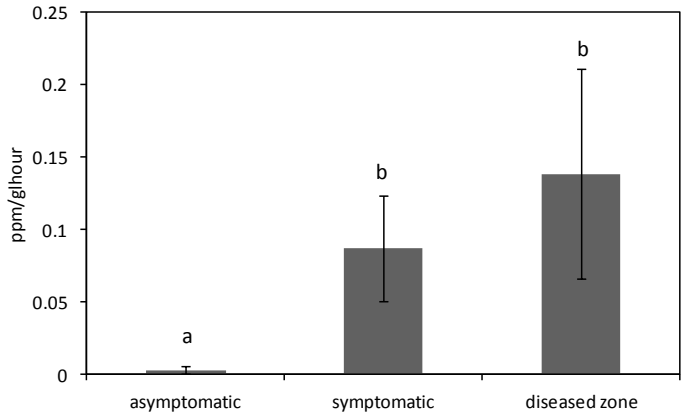


Fig.30 – Ethylene production from *A. deliciosa* (cv. Hayward) fruits after a week of shelf life (n=30). Measures were taken on samples of two fruits closed in a 1.7 l vessel for 2 days. Different letters indicate significant differences according to Fisher's multiple range test (P<0.05).

Evidences of the role of *Metcalfa pruinosa (Say 1830)* as a vector of *Pseudomonas syringae pv. actinidiae*

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ABSTRACT

Over the past 20 years, the area devoted to kiwifruit cultivation has steadily increased. Italy exports almost 70% of its production to other countries of the European Union, Russia, North America, Far East and Brazil. However, in the last few years, kiwifruit yields and the total cultivated area considerably decreased , due to the pandemic spread of the bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae*. The bacterium is able to infect host plants *via* natural opening or wounds. In other bacterial diseases of fruit trees, the wounds caused by sucking insects represent risk points for the invasion of the host plant(Nakato *et al.,* 2014). Moreover, the role of sucking insects as vector of bacterial pathogens, such as, for example, phytoplasma, is widely known. However, there is not yet any evidence of the role of insect-related wounds as entry point or of sucking insect as vector of *Pseudomonas syringae* pv. *actinidiae. Metcalfa pruinosa* Say (1830) is the most common sucking insect affecting kiwifruit vines in the regions where *Pseudomonas syringae* pv. *actinidiae* is present. Therefore, the possible role of *M. pruinosa* in the spread of the bacterial canker was investigated in controlled conditions. This study demonstrates the ability of *M. pruinosa* to act as a vector of *Pseudomonas syringae* pv. *actinidiae*. The data obtained in laboratory studies will be confirmed by analyzing the insect in the real orchard conditions, to verify the possible influence of environmental conditions in the insect - plant interactions.

Key words: *Metcalfa pruinosa* (Say, 1830), insect-mediated disease transmission, *Pseudomonas syringae pv actinidiae*, host plants.

INTRODUCTION

Over the past 20 years, the area dedicated to kiwi cultivation remarkably increased. Italy, is the second largest kiwifruit producer after China, with a production of 430 tones, equal 24% of the total world production (Kiwi dossier 2012, 2013). However, from 2008, severe reductions in yields and in the area devoted to kiwifruit cultivation were registered, due to the spread of the bacterial canker of kiwifruit. The first outbreak of the disease was registered in 1992, while the current pandemic outbreak occurred from 2008 and it is still spreading (Vanneste *et al.,* 2011). The disease is caused by *Pseudomonas syringae pv. actinidiae* (Psa), a rod-shaped, Gram-negative, strictly aerobic and mobile bacterium (Takikawa *et al.,* 1989), which affects both *Actinidia chinensis* and *Actinidia deliciosa* (Donati *et al.,* 2014*)*. Psa is able to penetrate through wounds or natural openings, such as stomata or lenticels (Spinelli *et al.,* 2011). Once inside the apoplast, it is able to move in the plant vascular system, spreading systemically, and it can cause rapidly the plant death (Spinelli *et al.,* 2012). Psa can spread inside the orchard by rain, wind, insects, animals and humans (Spinelli *et al.,* 2012).

It is well established that many pathogenic microorganisms such as fungi, bacteria, viruses and phytoplasmas are transmitted accidentally by insects or by a specific vector insect from one plant to another (Alexander *et al,.* 2005). Insect and pathogens may also develop a close symbiotic relationship that contributes to the transmission of bacteria from tree to tree. A well know example of this interaction is represented by the olive tree pathogen *Pseudomonas savastanoi* that is vectored by the olive fly (*Bactrocera oleae*). It was esteemed that 30-40% of the damage and yield was due to the direct or indirect effects of pathogens transmission by insects (Agrios, 2004). Insect can vector pathogen also on the long distances form one cultivated area to the adjacent ones (Brown *et al.*, 2000). *Metcalfa pruinosa* Say (1830) (*Hemiptera Flatidae*) is considered a very important invasive species in Europe, due the strong gregarious behavior, the rapid multiplication and spread and to its wide range of host plants including *Actinidia spp*. (Grozea *et al.,* 2011). It was introduced in Europe accidentally from North America (Wilson *et al.,* 1981). The insect is widespread in the whole Italian territory thanks to its strong polyphagia and the absence of natural enemies (Gervasini *et al.,* 1999). *M. pruinosa* (M.p.) presents a univoltine life cycle with wintering in the egg stage deposited in the crevices of the bark from August onwards. Hatching takes place in May-July; and the insect live on the plants until late summer (Zangheri *et al.,* 1980; Tremblay *et al.,* 1994). *M. pruinosa* feeds by sucking plant saps at all post-

embryonic stage. Neanids preferably feed on the undersides of the leaves. Nymphs move from the leaves to the new branches and then return on the leaves for metamorphosis. *M. pruinosa* can contribute to spread a number of plant viruses such as GFLV ("*Grapevine Fanleaf Virus*") and GLRaV-3 ("*Grapevine Leafroll - associated Virus" 3*) (Gervasini *et al.,* 1999) and phytoplasma (Olmi, 2003). *M. pruinosa* produce a substantial amount of honeydew that may represent a growing substrate for bacterial pathogens and my also attract bees. Therefore, the latter may act as secondary vectors of the bacterial pathogen further increasing the disease pressure inside the orchard.

This study investigated the possible role of *M. pruinosa* to vector *Pseudomonas syringae pv. actinidiae* in controlled conditions.

MATERIALS AND METHODS

Insect sampling in kiwifruit vineyard – *M. pruinosa* insects were captured from a kiwifruit orchard located near Bologna, Italy (44°33'00.72''N; 11°23'07.67"E). The insects were sampled from mid-May to early July. Insect collection was repeated for two consecutive seasons.

Artificial feeding of *M. pruinosa* - Insect chambers were made by cutting the bottom of 15 ml completely transparent tubes. The opening created by the cut was wrapped in anti-aphid net to allow gas exchange. After sterilization, the internal volume of the cap was filled with sterilized feeding solution (buffer TE 10mM Tris, 1 mM EDTA, pH 8, amended with 50 g L^{-1} sucrose). The feeding solution contained 3×10^8 cfu mL^{-1} of Psa (strain CFBP7286-GFPuv, which expresses the Green fluorescent protein GFPuv) (Spinelli *et al.,* 2011). The open surface of the cap was closed by a layer of Parafilm to prevent the direct contact between the insect and the feeding solution. On the distal part of each cap was a green film to mimic the foliar surface and attract the insect for feeding. Each tube contained one adult or one neanid of *M. pruinosa* and a total of 50 insects were used for these experiments. The tubes were incubated at 22° C, 70% RH and a light-dark cycle of 16:8 hours. The insects were kept in the feeding tubes for 2, 4, 5, 6 or 9 days. Immediately after the feeding period, each insect was individually frozen in liquid nitrogen and stored at -80 ° C for subsequent analysis.

Microscopic *visualization of Pseudomonas syringae pv. actinidiae on* Metcalfa pruinosa – A sample of nine insects, both adults and nymphs, coming from the artificial feeding *of M. pruinosa* experiment (three insect for 2, 4, 5 day of feeding) were killed in liquid nitrogen and frozen at -80°C. After two weeks, they were sectioned longitudinally and observed under a binocular microscope to verify the presence of the bacterium through the observation of its florescence on the insect under a UV source (excitation wavelength of GFP-B: 460-500 nm, emission wavelength: 510–560 nm). These analyses were performed using a Nikon SMZ25 fluorescence microscope (Nikon Instruments Corporation, Tokyo, JAPAN), with an optical system provides zoom ratio of 25:1 (zoom range 0.63 x - 15.75 x), LED DIA light intensity control and epifluorescence filter cube selection.

Transmission of Psa by *Metcalfa pruinosa* – The experiment was divided in two steps. During the first one, we tested whether *M. pruinosa* adults feed on artificial solutions containing Psa were able to transmit the disease to healthy plants. In the second step, *M. pruinosa* adults feeding on disease plants were used to infect healthy plants. Seedlings resulting from *Actinidia deliciosa* cv. Hayward were used as plant hosts. *M. pruinosa* adults were fed for 7 days as previously described in the experiments on artificial feeding. In this case, the feeding solution consisted in an extract of *Actinidia deliciosa* cv. Hayward leaves containing 10^6 cfu mL^{-1} Psa 7286 GFPuv. After the artificial feeding, each insect was individually transferred in a chamber fixed on the adaxial surface of the kiwifruit leaves. The chamber consisted in a cylinder of 3 cm diameter and 2 cm high, opened on both sides. The distal side was closed by an anti-aphid net, while the proximal one was closed by the leaf surface. Insects were kept in the chamber for 2 weeks. During the experiment, the plants were kept at 22° C, 70% RH and a light-dark cycle of 16:8 hours of natural light.

Thereafter, each exposed leaf and insect were surface sterilized by washing them firstly in ethanol (70%) and successively in NaClO (1%) and finally rinsed in sterile water for two times. The leaves or insects were ground in 5 or 1.5 ml, respectively, of sterile 10 mM $MgSO_4$. The homogenate was serially diluted 1:10, and 3 drops of 10 µl for each dilution were placed on agarized Luria-Bertani medium in order to quantify Psa populations present inside the leaves or the insects.

To verify whether insects feeding on diseased plants can spread the pathogen to healthy ones, fifteen seedlings obtained from *Actinidia deliciosa* cv. Hayward were infected by spray inoculation with a Psa CFB7286GFPuv suspension (4.2×10^5 cfu mL^{-1}). The inoculated plants were

caged in an anti-aphid net to retain the insects. Two days after infection, on each plant, 6 adults of *M. pruinosa* were placed on the plant and allowed to feed on it for 7 days. Thereafter, each group of 6 insects was transferred on healthy kiwifruit plant and allowed to feed on it for 7 days. The fifteen healthy plants receiving each 6 *M. pruinosa* adults were kept for 1 month to monitor the symptoms development.

Psa population was quantified, as previously described, on experimentally inoculated plants (1 week after inoculation), in the insects (14 days after the feeding on inoculated plants) and in the plants receiving the supposedly infected insects (30 days after the feeding).

Statistical analysis - Significance of correlations was assessed with Fisher's exact test, assuming a confidence level of 0.1 or 0.05. The STATISTICA ver. 5software (StatSoft Inc, Tulsa, USA) was used for calculation.

RESULTS

In the artificial feeding experiments, the percentage of infect insects reached the 67%, but it negatively correlated with the duration of artificial feeding (Fig. 2a). The infected individuals hosted a high Psa population, which decreased according to the increase of the feeding time on infected solutions (Fig. 2b). The apparent increase observed after 9 days of feeding is linked to the increase of insect weigh due to its growth (Fig. 2b, 3). In fact, the bacterial population positively correlated with the insects' weight (Fig. 3). The percentage of infected neanids (33 %) was lower than the one of adults (49%).

Also the insects feeding on infected plants resulted contaminated with Psa, and the average percentage of infected insects (46%) was the same as the one from artificial feeding (46%). Moreover, in the case of feeding from infected plants, the insects generally harbored a high Psa population, which did not clearly correlate with the pathogen population inside the plants where the insect fed (Table 1).

The microscopy localization of Psa on *M. pruinosa* showed a clear bacterial contamination on the mouth parts, legs and the abdominal metameres (Fig. 4). The sectioning of insects showed that the bacteria concentrated also in the inner parts of the mouth and abdomen showing the spread of the pathogen in the digestive apparatus (Fig. 4).

Concerning the ability of vectoring the pathogen, *M. pruinosa* adults fed on infected solution were able to transfer the diseased to the 33% of plants and, one month after the insect-mediated disease infection, the plants harbored an average of 2.92 × cfu g^{-1} of plant fresh weight. Furthermore, also the insects fed on infected plants were able to vector the disease to healthy plants. In fact, after the visit of the insects from diseased plants, 13 plants out of 15 were positive for Psa (Tab. 1). A positive correlation was also noted between the number of infected vector insects feeding on a single acceptor plant, and the bacterial titre in the same plant (Fig. 5).

DISCUSSION

Psa was found in 46% of the insect samples in artificial feeding experiments and in an average of 46% of insects feeding on infected plants. The quantification of Psa inside the insect and the microscopical visualization confirmed the bacterium presence in the insect's guts. In addition, the pathogen can be transmitted by contaminated insects to healthy plants. All together, these data demonstrate that the insects could vector the bacterium from plant to plant. Furthermore, the number of infected insects feeding on a plant, rather than their bacterial load, is determinant for the disease transmission. Therefore, low transmission efficiency may be hypothesized from *M. pruinosa*, while a high number of feeding events would increase the chance of infection. This is particularly important in the light of *M. pruinosa*'s gregarious behavior.

Notably, the transmission of Psa via puncturing insects is not influenced by the number and status of generally acknowledged infection routes, such as stomata, flowers and wounds.

The insects' developmental stage could also be hypothesized to affect their bacterial load, in agreement with other studies on bees (Gätschenbergar *et al*., 2013). It was noted that the amount of bacteria found in the insect is dependent on the stage of insect development (whith neanids showing a lower percentage of infection), its weight and the ability to suck the food.

The negative correlation between infected insect percentage and days of feedings may be due to the low viability of the bacteria in artificial solution or to the activity of insect immune system (Vilmos *et al.,* 1998), which may produce several antimicrobial compounds, as shown for bees (Gätschenbergar *et al.,* 2013).

One of the symptoms of the bacterial canker of kiwifruit consists in droplets or masses of sticky exudate (ooze) on the surface of host tissues (Donati et al, 2014) which could stick on the limbs and bodies of visiting insects. In natural conditions, the sugars in the bacterial exudate may attract *M. pruinosa* and other insects as a food source, making them possible vectors of the bacteria contained in the same exudates (Capinera, 2008). In addition, *M. pruinosa* itself a produces sugary honeydew that may smear the plant surface and attract other insects. If Psa could be detected in this secretion, it may contribute further to Psa dispersion. Besides its feeding behavior, *M. pruinosa* could carry pathogens externally on their bodies after the contact with infected plant tissue or bacterial ooze as shown in our research and in previous studies (Shetlar, 2003). In the biological cycle of *M. pruinosa*, another crucial stage is oviposition, consisting in the mechanical injection of the eggs in host tissues. Since oviposition takes place from September to October, when the weather conditions are still favorable for the development of the bacterial canker, it may significantly contribute to the spread of Psa.

In conclusion, our results showed the capacity of *M. pruinosa* to spread the disease in controlled conditions. However, the capability and the importance of *M. pruinosa* as a vector of Psa in field conditions has not yet been evaluated and further experiments are needed to fully understand the contribution of *M. pruinosa* to Psa disease spread. For example, the possible production of infected honeydew, the attraction of *M. pruinosa* by bacterial exudates and the survival of the pathogen inside and outside *M. pruinosa* in different climatic conditions need to be precisely evaluated. Nonetheless, the results of this study show that attention should be paid to *M. pruinosa* and possibly to other insects, in order to limit Psa spreading.

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TABLES AND FIGURES

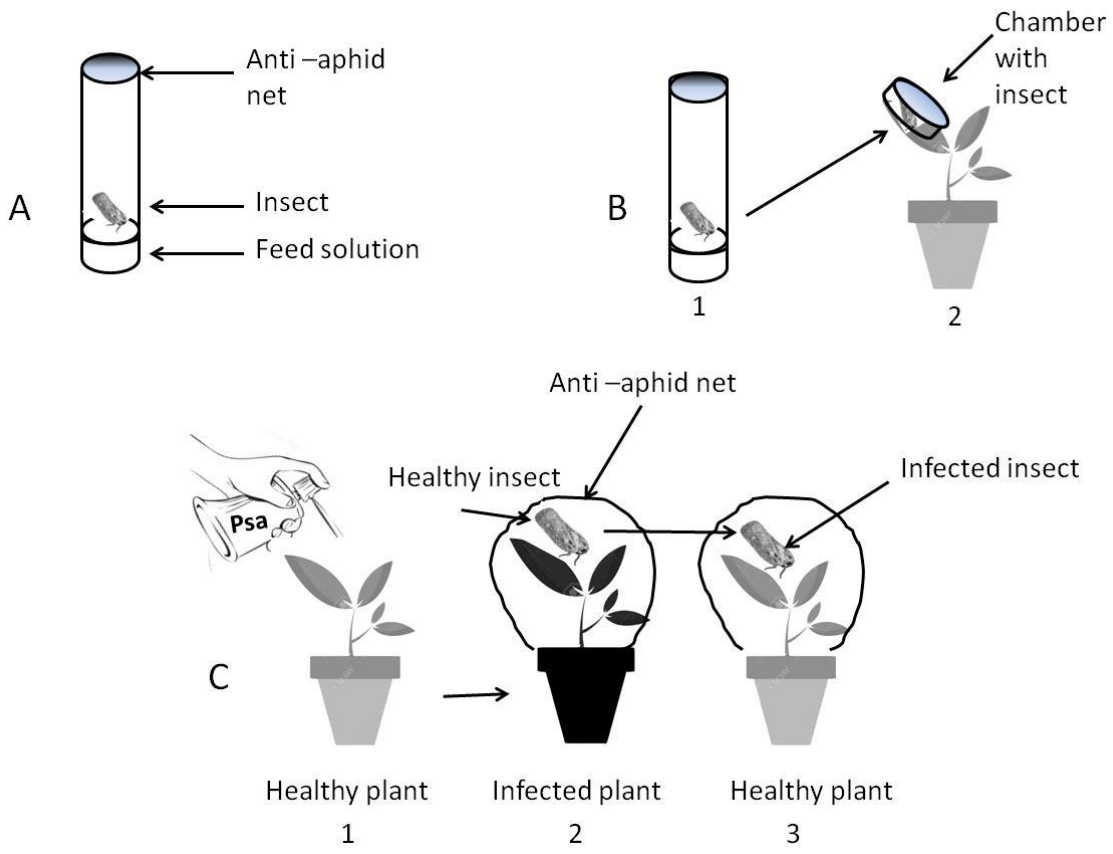

Fig. 1 – Experiment set up: (A) Artificial feeding of *Metcalfa pruinosa* - Insect chambers: transparent tubes (15ml), with feeding solution in the cap and anti-aphid net on the opposite site; (B) Transmission of *Pseudomonas syringae pv. actinidiae* by *Metcalfa pruinosa*: first step: adult fed on artificial solution containing *Pseudomonas syringae pv. actinidiae*, second step: the insect was transferred in a chamber fixed on the adaxial surface of the kiwifruit leaves; (C) Transmission of *Pseudomonas syringae pv. actinidiae* by *Metcalfa pruinosa*: first step: spread inoculation of healthy plants with *Pseudomonas syringae pv. actinidiae*, second step: inoculated plants were caged in an anti-aphid net to retain 6 insects; third step: each group of 6 insects was transferred on a healthy kiwifruit plant and allowed to feed on it for 7 days.

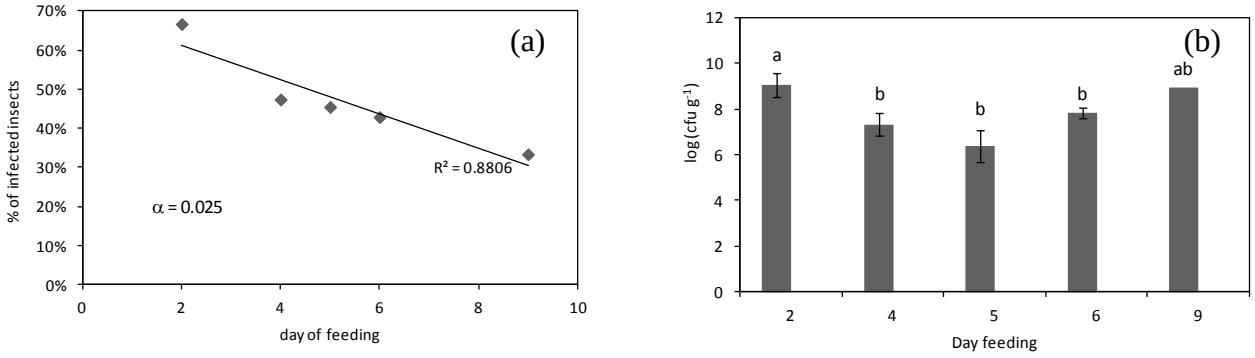

Fig. 2– (a) The relationship between the percentages of infected insects and days of artificial feeding. The correlation was significant according to the Fisher exact test with a confidence level of 0.0025 (b) Population of *Pseudomonas syringae pv. actinidiae* expressed as colony Forming Unit per ml (cfu/ml) in the insect in according to the days of artificial feeding. Value with different letters are significantly different according to the Fischer's LSD test (P <0.05).

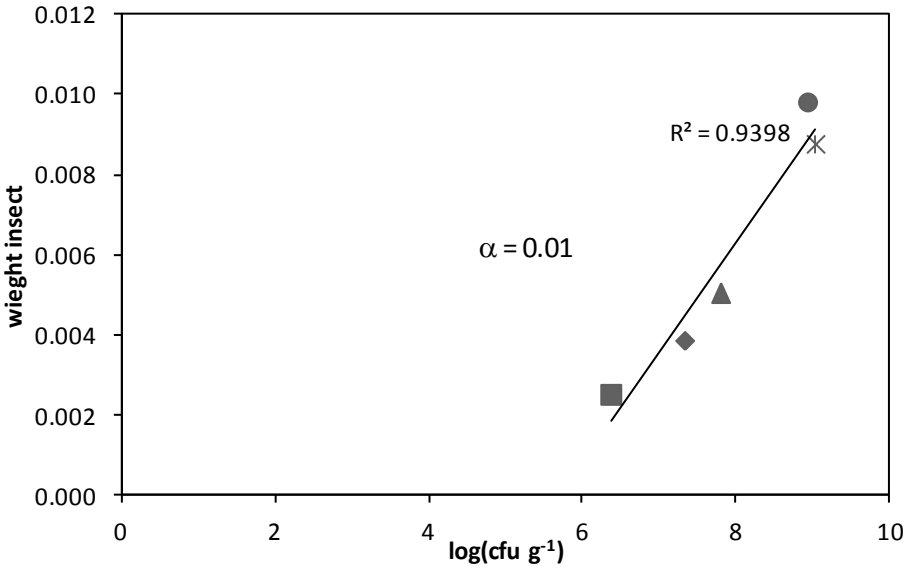

Fig.3 - The relationship between the weight insect and amount of *Pseudomonas syringae pv. actinidiae* in the insect (cfu/ml). Day of artificial feeding: ▪ - 5 days; ◊ - 4 days, Δ – 6 days; ✳- 2 days and • - 9 days. The correlation was significant according to the Fisher exact test with a confidence level of 0.01.

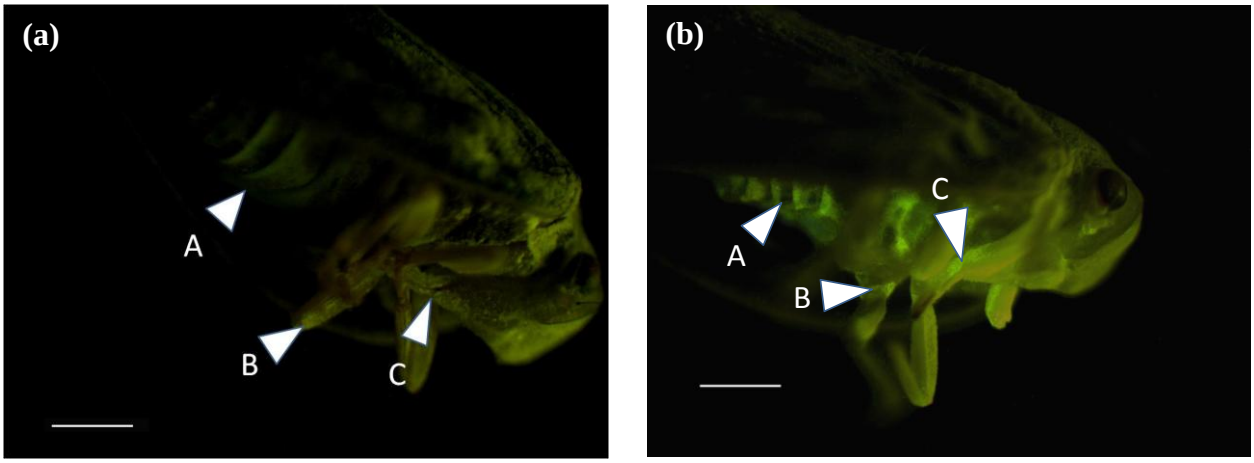

Fig. 4 – Micrograph of the insect with zoom magnification of 12x (bar measurement = 0.83 mm). (a) Healthy *Metcalfa pruinosa* under natural light: A – 2°- 3° sternites; B - limb; C – mouthparts. (b) Infected *Metcalfa pruinosa* under GFP-B (ex 460-500, em 510–560) light: A-presence of *Pseudomonas syringae pv. actinidiae* on the 2° - 4° sternites; B – presence of *Pseudomonas syringae pv. actinidiae* on the limb; C - presence of *Pseudomonas syringae pv. actinidiae* on the mouthparts.

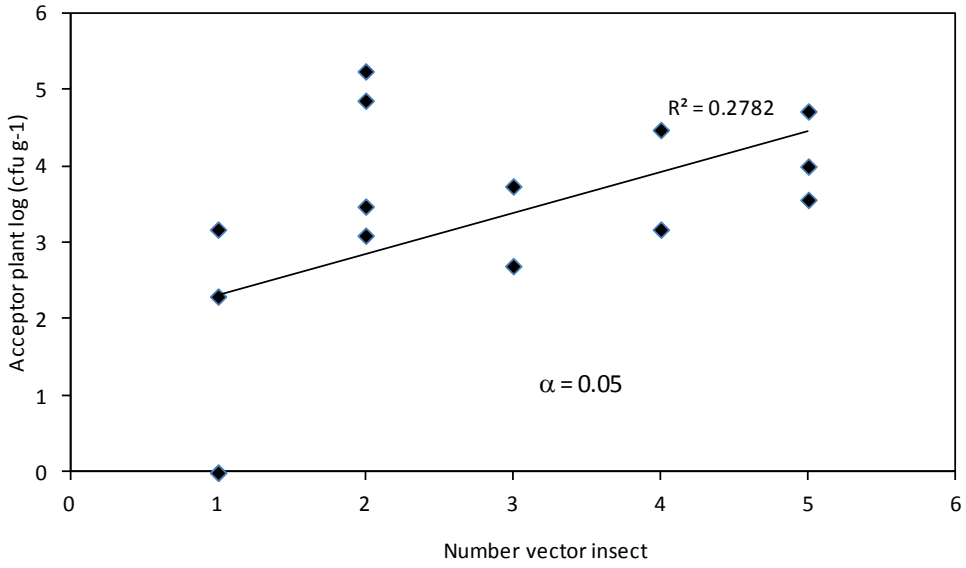

Fig. 5 – The relationship between the presence of colony forming units per ml and the number of insect feeding on them. The correlation was significant according to the Fisher exact test with a confidence level of 0.05.

Infected plant (cfu g^{-1})	Insect (cfu g^{-1})	% of infected insect	Healthy plant (cfu g^{-1})
7.25E+04 ± 1.25E+04	7.68E+00 ± 8.52E-01	33%	1.25E+03 ± 2.50E+02
7.25E+04 ± 1.25E+04	8.88E+00 ± 1.36E-02	50%	5.00E+02 ± 2.89E+02
1.23E+05 ± 2.66E+04	6.67E+00 ± 2.20E+00	17%	0.00E+00 ± 0.00E+00
1.23E+05 ± 2.66E+04	6.88E+00 ± 2.72E+00	50%	5.50E+03 ± 5.00E+02
6.75E+04 ± 9.46E+03	7.80E+00 ± 7.67E-01	83%	5.25E+04 ± 1.38E+04
6.75E+04 ± 9.47E+03	4.51E+00 ± 4.47E-02	33%	3.00E+03 ± 1.47E+03
6.50E+05 ± 1.19E+05	4.77E+00 ± 4.45E+00	17%	2.00E+02 ± 2.00E+02
8.00E+05 ± 1.47E+05	9.15E+00 ± 8.46E+00	17%	0.00E+00 ± 0.00E+00
9.00E+04 ± 1.00E+04	7.23E+00 ± 1.17E+00	67%	1.50E+03 ± 1.19E+03
7.25E+04 ± 9.46E+03	9.14E+00 ± 5.27E-02	33%	7.25E+04 ± 4.79E+03
5.00E+05 ± 1.08E+05	7.28E+00 ± 9.20E-01	83%	1.00E+04 ± 7.07E+03
9.25E+05 ± 4.79E+04	8.66E+00 ± 3.86E-01	33%	1.75E+05 ± 7.50E+04
1.20E+06 ± 1.22E+05	8.94E-01 ± 7.24E+00	17%	1.50E+03 ± 8.66E+02
3.00E+06 ± 4.08E+05	7.54E+00 ± 1.05E+00	83%	3.67E+03 ± 2.19E+03
2.00E+06 ± 4.08E+05	6.57E+00 ± 2.31E+00	67%	3.00E+04 ± 7.07E+03

Tab 1 – Experiment of transmission of bacterium. Quantity of *Pseudomonas syringae pv. actinidiae* present as Colony Forming Units per ml (cfu/ml) in the infected plant, in the vector insect and in the healthy plant respectively.

The effect of the use of tunnel on spread control of *Pseudomonas syringae pv. actinidiae* in the orchard

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ABSTRACT

The bacterial canker of kiwifruit, caused by *Pseudomonas syringae pv. actinidiae* (Psa), is a severe disease affecting all the cultivated *Actinidia* spp. The micro-climatic conditions in orchards area are critical factors in determining the local severity and extent of disease. Among them, the light intensity and quality is crucial for plant development, but it may also affect movement, survival and virulence of Psa. Since plastic covers modify the micro-climatic conditions in the orchard, the use of tunnels could be a new practice to control the disease. Therefore, in this study the use of permanently closed tunnel and seasonal closed tunnel were tested for the disease control in two infected commercial *A. chinensis* orchards. The use of permanently closed tunnel showed a positive result in the control of the disease by the reduction of the leaf wetness. Since commercial covers might modify light intensity and composition, trials were performed in laboratory conditions to improve the effect of different plastic covers on the bacterium pathogenicity and plant defenses.

Key words: light, blue, red, intensity, quality, *Pseudomonas syringae pv actinidiae*, host plants, disease, tunnel.

INTRODUCTION

The bacterial canker of kiwifruit, caused by *Pseudomonas syringae pv. actinidiae* (Psa), is the most severe plant health concern for all the cultivated *Actinidia* species. In Italy the first case was recorded in 1992 in Lazio (Scortichini, 1994), and Psa is now distributed in the all Italian productive regions. Psa is a Gram-negative bacterium able to penetrate in plants through natural openings, such as stomata and wounds. Once the host is colonized, symptoms such as leaf spots, production of exudates and wilting of branches may appear, progressively driving to the death of the plant. The micro-climate in orchards is critical in determining the local severity and extent of disease (Young, 2012). Among the main environmental factors affecting primary infection, moisture, temperature and light affect stomata opening, thus providing a possible route of entry for the bacterium into the plant (Spinelli *et al.,* 2012). To control the environmental moisture the use of plastic tunnels could be an effective practice. Plastic covers are currently applied to several fruit tree species, to influence some physiological parameters and to limit the leaf wetness (Grandi *et al.,* 2014). In fact, water films may wash foliar treatments out, and promote the proliferation of pathogens, including Psa. As well, light may also influence bacterial pathogenicity by regulating bacterial movement: in fact, motility on aerial plant surfaces was enhanced by red light during dark and low-light periods in *P. syringae* strains, while blue light inhibits bacterial motility, but promotes virulence when coupled with white light (Wu *et al.,* 2013). On the other hand, the exposure of the plant to UV-B radiation increases plant resistance to pests and pathogenic microorganisms, and may also influence the microbial phyllosphere composition (Ballaré *et al.,* 2012) and other physiological processes of adaptation to the environment. The objective of this work was to evaluate the effect of the use of different tunnels on the control of Psa spread. Thus, two trials were carried out to test different tunnels conditions in the orchard, and to improve the efficiency of different plastic covers trials in laboratory conditions. In particular, the effect of light intensity (100% PAR, 50% PAR) and composition (50% PAR + red light, 50% PAR + blue light) was tested on Psa growth, motility, biofilm formation, and on the responses of *A. chinensis* alone and following infection with Psa.

MATERIALS AND METHODS

Disease incidence and severity under covers in orchard conditions – The effectiveness of plastic covers on the disease control was tested in two infected commercial *A. chinensis* orchards in Faenza (Italy), with permanently or seasonally closed tunnel. The control was formed by plants under net covers in the same orchard. The orchard with permanently closed tunnel has a plant spacing of 4.7 × 2.4 m (44°12’56.97’’N, 11°51’27.13’’E). The tunnel scaffold, in steel, is supported by the system poles of the orchard. The cover material is LD-PE ANIGOLD LONG LIFE 180 (Aniplast, Bari, Italy), a polyethylene film 0.8 mm thick, with nickel additive (life class "D"). The technical characteristics of the plastic film are: 86.5% total light transmittance, 61.7% direct light, 15.5% UV transmittance and 46.3% greenhouse effect. The orchard with seasonally closed tunnel (44°15’55.79’’N, 11°53’49.54’’E) has a plant spacing of 4.5 × 1.5 m. The tunnel is made with WhiteECOLIGHT (Serroplast, Bari, Italy), a 80 μm thick polyethylene film with Nickel-Quencher additive, characterized by a high diffusion in the range of the visible wavelengths, which ensures a more uniform distribution of light, and a high greenhouse effect. The film hangs from a wire structure connected to the support poles. The tunnels are closed by the end of flowering to early winter (late November).

To check whether covers could influence the microclimate conditions, humidity (EasyLog-UBS-1), temperature, PAR and leaf wetness (Spectrum data-loggers (1000 Series Micro Stations)) sensors were placed in the orchards. Hourly data were transformed to daily cumulates, and values from 10 subsequent days were averaged. In addition to the microclimatic data, the effect of covers on the plant was analyzed with regard to: bud opening, shoot growth, quantum photosynthetic efficiency (PSI equipment, Photon Systems Instruments, Czech Republic), yield and fruit quality. To determine the effect of covers on the progress of the disease, symptoms were observed monthly and rated according the following scale:

0 - asymptomatic plants

1 - leaf spot, chlorosis

2- leaf spot, exudates, cancers, blighted flowers, few shoots wilting;

3- leaf spot, exudates, cancers, blighted flowers, buds withering, pruning interventions, fruiting shoots wilting

- - extensive necrosis;
- - uprooted plant.

In addition, leaf samples (n=24) were monthly analyzed to verify the presence of epiphytic and endophytic bacteria. The leaves were washed in sterile 10 mM $MgSO_4$ solution for 30 minutes, then surface-sterilized by washing in 70% ethanol, 1% NaOCl, and twice in sterile distilled water, and finally grinded in 5 ml of sterile 10 mM $MgSO_4$. The external wash and the leaf extracts were serially diluted 1:10 and plated by placing 10 µl drops in triplicate for each dilution on agarized LB medium, in order to quantify Psa Colony Forming Units per ml (CFU/ml)

Quality and storage life of fruits from infected plants – The fruits were collected from plants under tunnel and controls from the two orchards. The fruits from the orchard with seasonally closed tunnel were analyzed in three different periods: harvesting, two month after cold storage and end cold storage. Flesh firmness was measured by penetrometer (Fruit Texture Analyzer, Güss) with 8 mm tip on two orthogonal faces of the fruit, after peeling. The colour of the flesh was determined with a Minolta colorimeter (CR-400) after removing 2 mm of peel on two portions of the fruit placed orthogonally. Soluble solid content was obtained by a digital refractometer Atago (Optolab, Modena, Italy), by cutting the two end caps of the fruit, squeezing the juice and by averaging the values obtained by each party. Dry matter was determined by drying a 2 mm thick fruit slice at 60 ° C for 48 hours.

Biological material_– The laboratory experiments were carried out with Psa strain CFBP7286 expressing Green fluorescent protein (PsaGFPuv, Spinelli et al 2011), maintained on Luria-Bertani (LB) agarized medium. Micropropagated *A. chinensis* plants (cv. G3) were grown on Murashige and Skoog (MS) medium containing: sucrose (30 g l^{-1}), myo-inositol (100 mg l^{-1}), thiamine-HCl (1 mg l^{-1}), nicotinic acid (1 mg l^{-1}), pyridoxine (1 mg l^{-1}), glycine (1 mg l^{-1}), indolebutyric acid (0.05 mg l^{-1}), benzylaminopurine (1 mg l^{-1}), GA_3 (0.1 mg l^{-1}), adjusted to pH 5.7 with KOH. Shortly before the experiments, the plants were transferred to a minimal medium containing only half-concentration MS inorganic salts, adjusted to pH 5.7.

Lights – For the laboratory tests, growing chambers were built containing LED lights in order to achieve the following growth conditions: 100% (luminous flux of a single LED - 4000-5000 mcd (about 12,5 - 15,5 lm / LED)) and 50% PAR with white LEDs; 50% PAR + red obtained by adding red LEDs ($610 < \lambda < 760$ nm) to white LEDs; 50% PAR + blue obtained with the addition of blue LEDs ($450 < \lambda < 500$ nm) to white LEDs. The chambers were maintained at 22 ± 2 °C, with a 16/8 h light-dark period.

Bacterial growth – Psa was cultured in 20 ml of LB medium. After 6, 24, 30, 48 and 72 hours, a 1 ml aliquot was read at 600 nm by spectrophotometer. At the same time points, the bacterial titre was assessed by producing serial 1:10 diluttions of the culture in sterile of 10 mM $MgSO_4$ and plating 10 µl drops in triplicate for each dilution on agarized LB medium, in order to quantify Psa Colony Forming Units per ml (CFU/ml).

Biofilm formation - The assay was performed as elsewhere described (Taguchi *et al.,* 2006) is based on the ability of bacteria to form biofilms on plastic material, such as on the well of Petri dishes with 3 cm diameter. Five ml of MMMF medium (O'Toole *et al.,*1998) were inoculated with PsaGFPuv. After inoculation, plates were incubated at room temperature for 5 day in the different light conditions. Then the plates were thoroughly rinsed with distilled H_2O three times and dried for 45 min on bench at room temperature. Then 5 ml of a 0.5% (w/v) solution in H_2O of crystal violet (Sigma-Aldrich, St. Louis, MO, USA) was added to each Petri well. The plates were placed for 45 min at room temperature, and subsequently washed thoroughly with distilled H_2O five times to remove aspecific staining. For quantitative analysis of biofilms, crystal violet was re-solubilized with 3 ml of 95% ethanol, and absorbance values at 595 nm were measured (Li *et al.,* 2007).

Bacterium motility – For the evaluation of the motility on semisolid agar surface, Petri dishes of 3 cm diameter containing agarized (0.25%, w/v) MMMF medium (O'Toole *et al.,*1998) were used. A paper disk (3 mm diameter), previously inoculated with 10 µl bacterial culture, was placed in the center of the plate. The plates were incubated at room temperature under the light treatment for 5 days. After this period, the area covered by bacterial growth was measured with the software MacBiophotonics ImageJ 1.48 (MacBiophotonics, Hamilton, ON, Canada).

Transcriptional analysis_– The list of genes tested in this work is provided in table 6, together with their putative function and primers used for transcriptional analysis. Total RNA was extracted from bacteria with the Total Extraction Kit (Norgen Biotek, CA) following manufacturer instruction, and from plants with Spectrum total plant Rna kit (Sigma-Aldrich, St. Louis, USA). Retrotranscription of purified RNA was performed by using the cDNA First-Strand Synthesis kit (Life Technologies, Rockville, USA) according to the manufacturer's recommendations. Real-time PCR was performed with SYBR Green chemistry, with SybrGreen master mix (Life Technologies) on a StepOnePlus (Thermo Scientific) equipment. Fold changes in transcription were referred to Rec A and RpoD genes (for bacteria) and to actin and 16s genes (for plants).

Plant growth and photosynthetic efficiency - Photosystem II quantum yield (QY) was assessed on micropropagated plantlets of *A. chinensis* after 40 days of light treatment, by means of a FluorPen FP 100 (Photon Systems Instruments, Czch Republic) equipment. The plant growth was calculated from the change in weight of the plant between the start and the end of the test.

Enzymatic assays - Soluble proteins were extracted in cold 200 mM potassium phosphate buffer, pH 7.5, including Triton X-100 (0.1%, v/v) and polyvinylpolypyrrolidone (1%, w/v). After centrifugation for 30 min at 12,000 × *g* and 4 °C, the supernatant was desalted on a NAP-10 (GE Healthcare, Little Chalfont, UK) column equilibrated with 50 mM potassium phosphate buffer, pH 7.0, and used for enzymatic assays. GPX assay followed the method by Ushimaru et al (1998), using 0.1 mM H_2O_2 and 50 mM pyrogallol. The reagents were prepared fresh just before use. Absorbance (λ = 430 nm) was taken after 10 min incubation at room temperature, and referred to a blank with no extract added. One GPX unit catalyzes the oxidation of 1 µmol pyrogallol min^{-1} under the described conditions. An absorbance coefficient of 2.47 mM^{-1} cm^{-1} was assumed for calculations. For the NOX assay, the final reaction mixture contained 200 µM nitroblue tetrazolium, 200 µM NADPH, 1 mM $CaCl_2$ and 10 µM $MgCl_2$ in 100 mM potassium phosphate buffer (pH 7.5). The reaction kinetics was monitored at room temperature and λ = 560 nm, compared to a blank consisting in the reaction mix without plant extract added. One NOX unit evolves an amount of superoxide that converts 1 µmol min^{-1} NBT to formazan. Total soluble protein concentrations were measured by means of the Bradford assay (Sigma-Aldrich, St. Louis, USA).

Callose determination - The assessment of callose content was carried out as described by Kohler et al (2000). Samples were incubated in 100% ethanol for 4 days. Subsequently, they were grinded to a fine powder and resuspended in ethanol to remove chlorophyll traces. After centrifugation (12,000 × *g*, 20 min), the ethanol was discarded and 400 µl dimethyl sulfoxide were added to each sample, followed by 30 min boiling and centrifugation at max speed for 5 min. A reaction mix was prepared, successively adding: 100 µl of the supernatant, 200 µl of 1 M NaOH, 590 µl of 1 M glycine-KOH buffer (pH 9.5), 210 µl of 1 M HCl, and 400 µl of an aniline blue solution (0.1%, v/v) in water. After an 20 minutes incubation at 50°C, the samples were read on a spectrofluorimeter (excitation: 393 nm; emission: 479 nm). For each sample, a blank was prepared in the same way, adding 400 µl of water instead of the aniline solution, to normalize for aspecific fluorescence.

Statistical analysis - STATISTICA Software 5 (StatSoft Inc, Tulsa, USA) was used to analyze the

collected data. ANOVA and LSD test were applied to the experiments with 4 light treatments and real time data sets. The Student's T test was applied to orchard experiments.

RESULTS

Bacterial canker control by plastic covers in orchard

Bacterial canker progression was monitored in two infected commercial *A. chinensis* orchards in Faenza (Italy), covered with a permanently closed tunnel or with a seasonally closed tunnel (fig.1). In both orchards, there were no significant temperature variations between the plants placed under the cover and the control plants (fig.4). Only in the orchard with seasonal closed tunnel, a significantly reduction of the incident radiation in plants placed under tunnel was found (fig.2b). In the same tunnel, a lower humidity was noticed in the presence of more precipitation (September, October, and November) (fig.3b). As for the leaf wetness, the orchard with seasonally closed tunnel did not show significant differences (5b). On the contrary, the orchard with permanently closed tunnel showed a lower leaf wetness under the tunnels in the period of lower rainfall (May to September) (fig.5a). Bud opening of the plants under the permanent tunnel attained similar ratios (70%) as the control plants, in spite of an initial delay (tab.1a). Instead, the plants under the seasonal tunnel had a reduction of the buds opening of 40% (tab.1b). The analysis of fruits quality of the orchard with seasonal closed tunnel showed that the fruits under the tunnel had delayed ripening and a reduction in dry matter (-10%) and sugar content (-2° Brix) (tab.4). A reduction of endophytic Psa population was observed in plants under tunnels from both orchards (fig.9-10). The permanent cover (tab. 5a), but not seasonal covers (tab.5b), also reduced the bacterial canker symptom incidence (fig. 8).

Bacterial growth, motility and biofilm formation in different light regimes

The data show only a significant difference 6 hours from inoculation between 50% and red thesis; and 24 hours from inoculation the blue thesis differs from the other theses. In general, light treatments did not cause significant differences in the growth of the bacterium (fig.11), in fact after 48 hours all four theses have reached the same growth. Bacterial cultures placed under coloured lights showed an increase in biofilm formation. In particular, the 50% PAR thesis shows a

significant reduction of biofilm compared to the other theses (fig.12). Both red and blue lights theses enhanced bacterial motility compared to white lights regardless of their intensity (fig.13).

Influence of light on plant responses to infection

Micropropagated infected plantlets of *A. chinensis* showed a reduced light quantum yield; the 50% PAR thesis recorded the lowest reduction of this parameter (fig. 14). An increased callose deposition was noticed after infection in the blue thesis (fig. 15); however, this reaction did not prevent a higher endophytic bacterial growth (fig. 16). NOX activity was enhanced by 50% illumination, but partially reduced after plant infection (fig. 17). An increased NOX activity was also observed in the red light thesis compared to the 100% PAR and blue theses in the infected plants. GPX activity was not affected by light or infection (not shown).

Light-dependent activation of plant and bacterial responses

Infected plants under a 100% PAR illumination show an induction of PR1 and ETR1 genes. In contrast, only genes related to ethylene perception (ETR1, ERF1, EIN2) were up-regulated with 50% PAR. Both red and blue light promoted PR8 and ETR1 genes more than 100% PAR; in addition, PR1 transcription was found in red-treated plants, while ERF1 was stimulated by blue light (fig.18).

The genes tested in bacterial cultures are mostly expressed at high bacterial titres (24 hours). Red (fig.19b) and blue (fig.19c) light greatly enhance the expression of the pathogenesis factors LysR, HrpM, HopZ5 and PAMTADA. In addition, the genes related to biofilm formation (AlgD and AefR) are also promoted. The photoreceptors for red (bPHP) and blue (LOV) light are activated in the respective light treatments (fig.19).

DISCUSSION

Several studies have shown that infection by the pathogenic microorganisms and insects can be influenced by the leaf wetness, temperature and light of the host plant (Roberts et al., 2006). In this view, plastic covers may modulate microclimatic conditions and affect the development of the bacterial canker of kiwifruit. In this study, lower leaf wetness was observed under the permanently closed tunnel, resulting in a reduction of the disease progression. Under the seasonally closed tunnel, a lower efficiency was observed on the reduction of the leaf wetness

in comparison with the permanently closed tunnel, together with a significant reduction in the incident radiation. These conditions may explain the disease incidence under seasonal covers, similar to that in control plants. Besides, plant growing under seasonally closed tunnel showed a lower bud break ratio, possibly explained with the competition for resources between growth and defence.

In addition to the modulation of microclimatic conditions, we tested whether a stimulation of plant defences, or the reduction of bacterial pathogenicity could be obtained by the modification of PAR intensity or wavelength composition. In fact, recent experiments conducted with a low R: FR ratio (<1) showed a greater susceptibility of *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC 3000, due to a change in the modulation effects of PhyB and Pfr on the signalling networks activated by the major defence hormones JA and SA (Ballare *et al.,* 2012). The results of this study confirmed that a reduction in luminous intensity is detrimental for infected plants.

Light intensity and quality may also influence bacterial pathogenicity by regulating bacterial motility (Wu *et al.,* 2013). Some bacteria, such as *Pseudomonas syringae* pv. *tomato* DC3000, may actively move on leaf surfaces (Rìo-Alvarez *et al.,* 2013; Quinones *et al.,* 2005) by chemotaxis towards nutrients. Therefore, the switch from an epiphytic to an endophytic, pathogenic lifestyle would require the entry into the plant apoplast, which is a motility dependent process, and may be influenced by light conditions (Rìo-Alvarez *et al.,* 2013). The blue, but not the red component of white light is responsible for the inhibition of swarming motility in *Pseudomonas syringae* pv. *tomato* DC3000 (Rìo-Alvarez *et al.,* 2013). A similar observation was made in this work on *Pseudomonas syringae* pv. *actinidiae*, where the red light stimulated the bacterial motility compared to the white light. The biofilm formation and adherence to plant surfaces require the inhibition of bacteria motility and, therefore, the switch from a motile state to a sessile state (Verstraeten *et al.,* 2008). The ability of a bacterium to form a biofilm is thought to be important for its survival in a variety of environments (Hinsat *et al.,* 2006) and light might regulate the transition from one state to another (Gomelsky *et al.,* 2011). In this work, biofilm formation in Psa was promoted by both blue and red lights.

Environmental cues, both biotic and abiotic, are perceived by a large number of plant receptors, and the resulting information is integrated by a complex signalling apparatus (Genoud *et al.,* 2002; Schenk *et al.,* 2000). For instance, the initial recognition phase in plant-pathogen interaction may be mediated by a NOX- or GPX-dependent oxidative burst, followed by the

activation of defence reactions including the synthesis of various proteins (pathogenesis-related proteins, PRs) and phytoalexins, changes in the wall structure of cells, and a localized and active cell death referred to as hypersensitive reaction (HR; Durner *et al.,* 1997), but the induction of plant defences against pathogens can be affected by light conditions (Roberts *et al.,* 2006). An induction of NOX activity was found in plants subjected to several stressing factors, both biotic and abiotic ones (Boon *et al.,* 2003), as one of the early oxidative signals leading to the onset of more specific responses. The adaptation to shade requires metabolic and morphologic adjustments, and the increased NOX activity may transduce this signal. In contrast, the pathogen may suppress NOX to escape its own recognition by the plant. The induction of Pal and pr1, the accumulation of salicylic acid and the development of the hypersensitivity are plants defence responses, activated at the site of bacterial infection in dependence of light (Ukness *et al.,* 1992). Gene expression analysis in Psa-inoculated plants showed that, in the 100% PAR-treated samples, ETR1 and pr1 were slightly induced. In contrast, none of the pr genes tested was activated under 50% PAR. An even stronger induction of ethylene perception-related genes emerged in red light-, and above all in blue light-treated plants, together with PR8. Taken together, these data suggest that lower PAR conditions all stimulate ethylene sensing, thus inactivating plant defences against bacterial pathogens. Rahman *et al.,* 2003 and Islam et al. 2011 noticed an involvement of the red light in the induction of SA-dependent responses, and in the synthesis of PR1 in leaf tissues. In this work, the main difference between red and blue light is represented by the activation of the transcription factor ERF1, demonstrating the existence of light-specific responses.

Another molecule involved in the plants defence processes is callose. This heterogeneous β-1,3-glucan is involved in a variety of plant developmental process, such as cell division and ripening of pollen mother cell (Kauss, 1992). It is believed that the callose accumulates in the cells in response to pathogen attack (Kauss, 1989) to strengthen plant cell walls and clog xylematic vessels, thus restricting its movement. Blue light led to an increase in callose production in infected plants, but this increase could not prevent bacterial multiplication. This may be due to the fact that the bacterium can move through both parenchimatic apoplast and xylem, but only the latter pathway is probably efficiently blocked by callose. Interaction of *Arabidopsis thaliana* with an avirulent strain of *Pseudomonas syringae pv. maculicola* in the dark resulted an increased apoplastic bacterial growth and therefore reduced local resistance as compared to an infection process in the presence of light (Islam *et al.,* 2011). This is also in agreement with the increased bacterial population within plants. Overall, it may be suggested that in plant-bacterium

pathosystem a reduced PAR drives the plant to implement defences against necrotrophic rahter than biotrophic pathogens (such as Psa), thus favouring the latter's virulence.

The light is involved in the regulation of a wide variety of mechanisms of gene expression associated with stress responses (Arnanz-Elias *et al.*, 2011). From the analysis of genes related to the pathogenesis, the sensitivity of Psa to quality of light emerges. In fact, LOV and PHPI genes, encoding for receptors of blue and red radiation, respectively, are expressed according to the corresponding light conditions. This finding is in agreement with previous works (Rio-Alvarez *et al.,* 2013), showing the importance of blue light in controlling the style of life of the bacteria. This ability of the bacteria to sense the red and blue light is confirmed by studies (Bonomi *et al.,* 2012), in which it is reported that the discovery of protein with LOV and PHY photo-reactive domains involved in the perception of blue and red light in plant pathogenic bacteria.

In conclusion, the use of covers may be useful to protect plants from Psa by varying the microclimatic conditions such as moisture and temperature and reduce leaf wetness, however, covers should be chosen in order not to reduce the light intensity or shift its wavelength to blue and red.

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TABLES AND FIGURES

Fig.1 – Two infected commercial *A. chinensis* orchards in Faenza (Italy): (a) – orchard with permanently closed tunnel; (b) - orchard with seasonally closed tunnel.

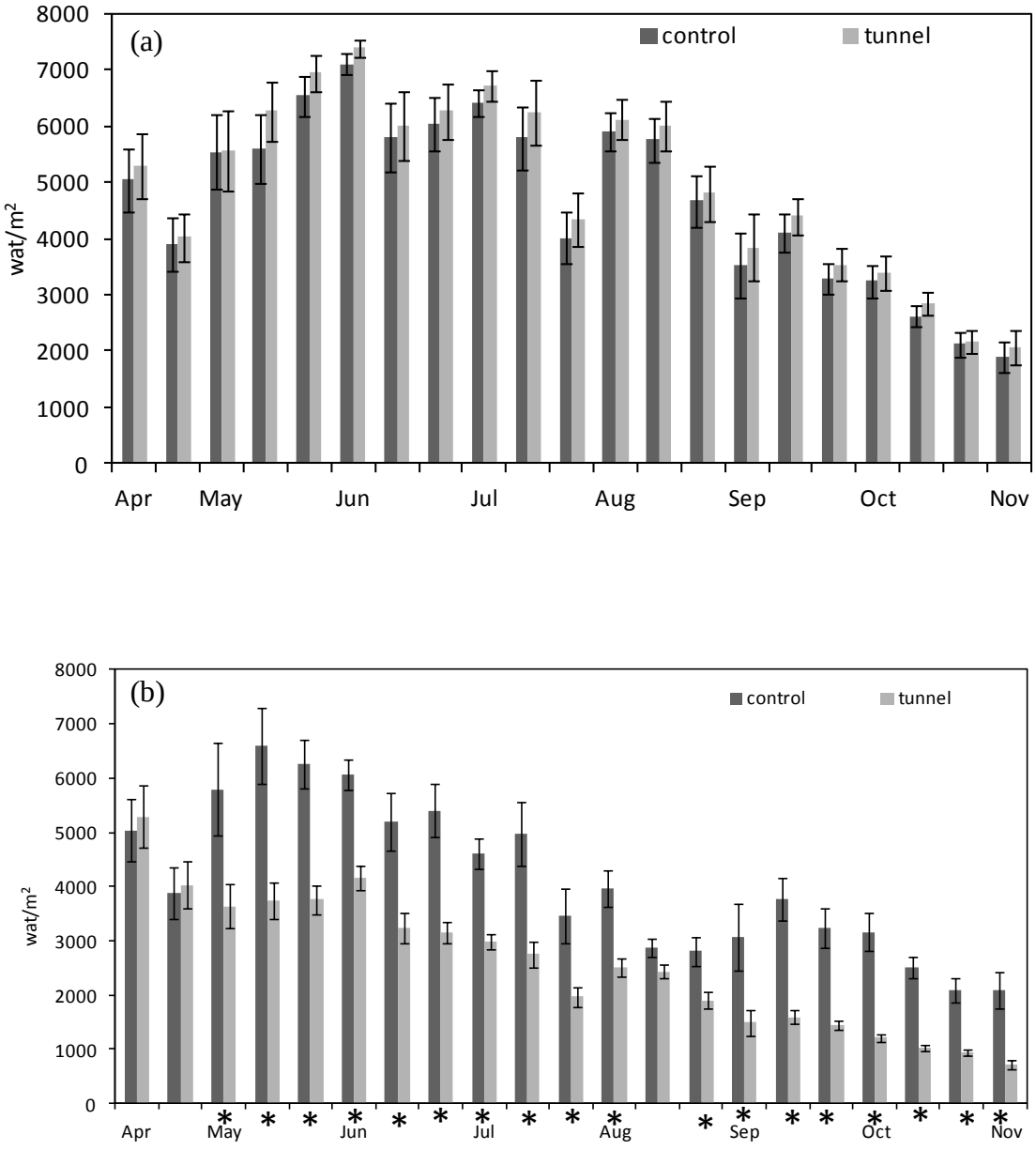

Fig.2 – Solar radiation in two infected commercial *A. chinensis* orchards in Faenza (Italy): (a) – orchard with permanently closed tunnel; (b) - orchard with seasonally closed tunnel. Data are reported as the mean of 10 days of daily cumulates (mean ± SE). Those marked with an asterisk (*) were significantly different (T-test; $P<0.05$) between covered and uncovered rows.

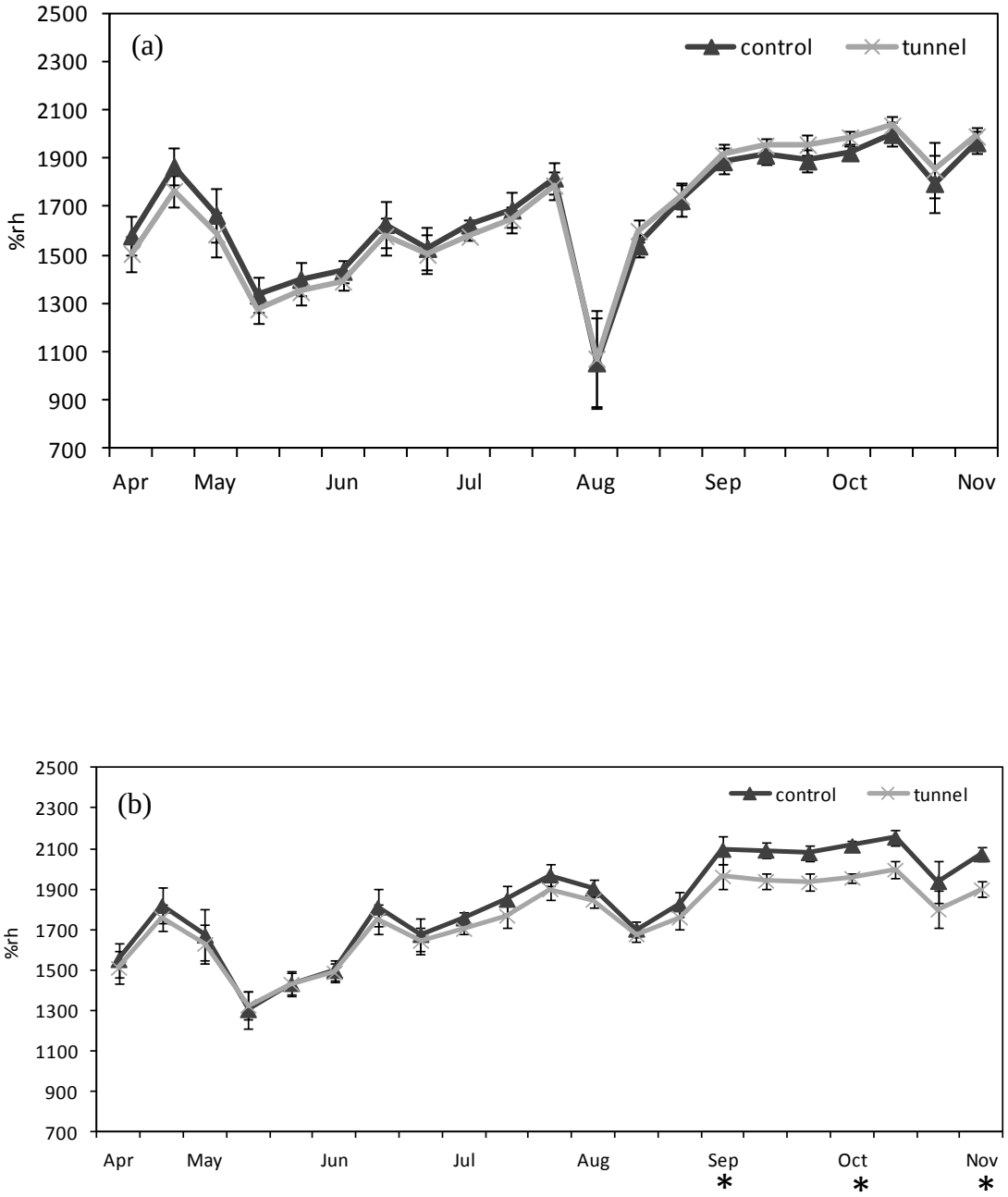

Fig. 3 – Humidity in two infected commercial *A. chinensis* orchards in Faenza (Italy): (a) – orchard with permanently closed tunnel; (b) - orchard with seasonally closed tunnel. Data are reported as the mean of 10 days of daily cumulates (mean ± SE). Those marked with an asterisk (*) were significantly different (T-test; $P<0.05$) between covered and uncovered rows

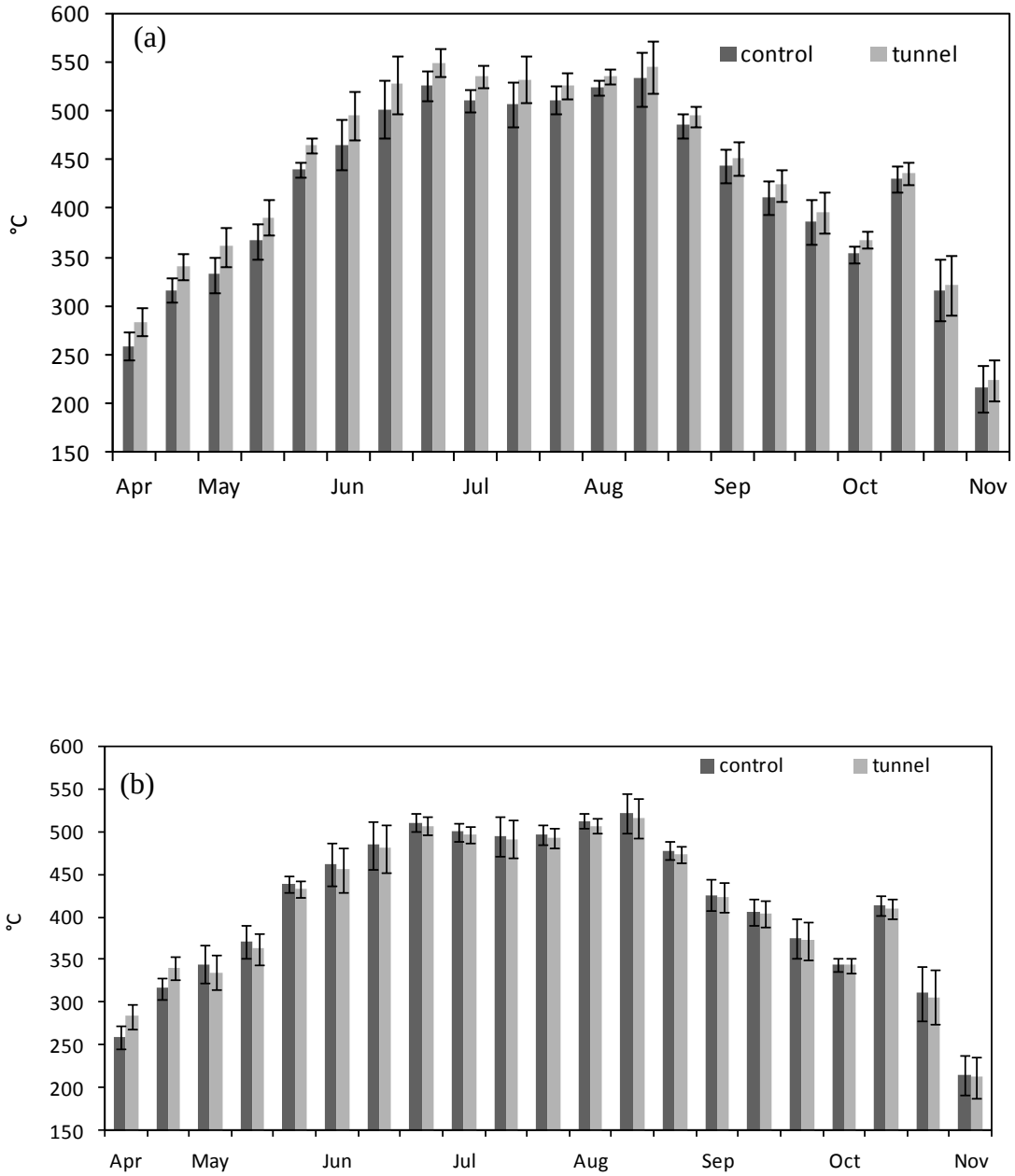

Fig. 4 – Temperature in two infected commercial *A. chinensis* orchards in Faenza (Italy): (a) – orchard with permanently closed tunnel; (b) - orchard with seasonally closed tunnel. Data are reported as the mean of 10 days of daily cumulates (mean ± SE). Those marked with an asterisk (*) were significantly different (T-test; $P<0.05$) between covered and uncovered rows.

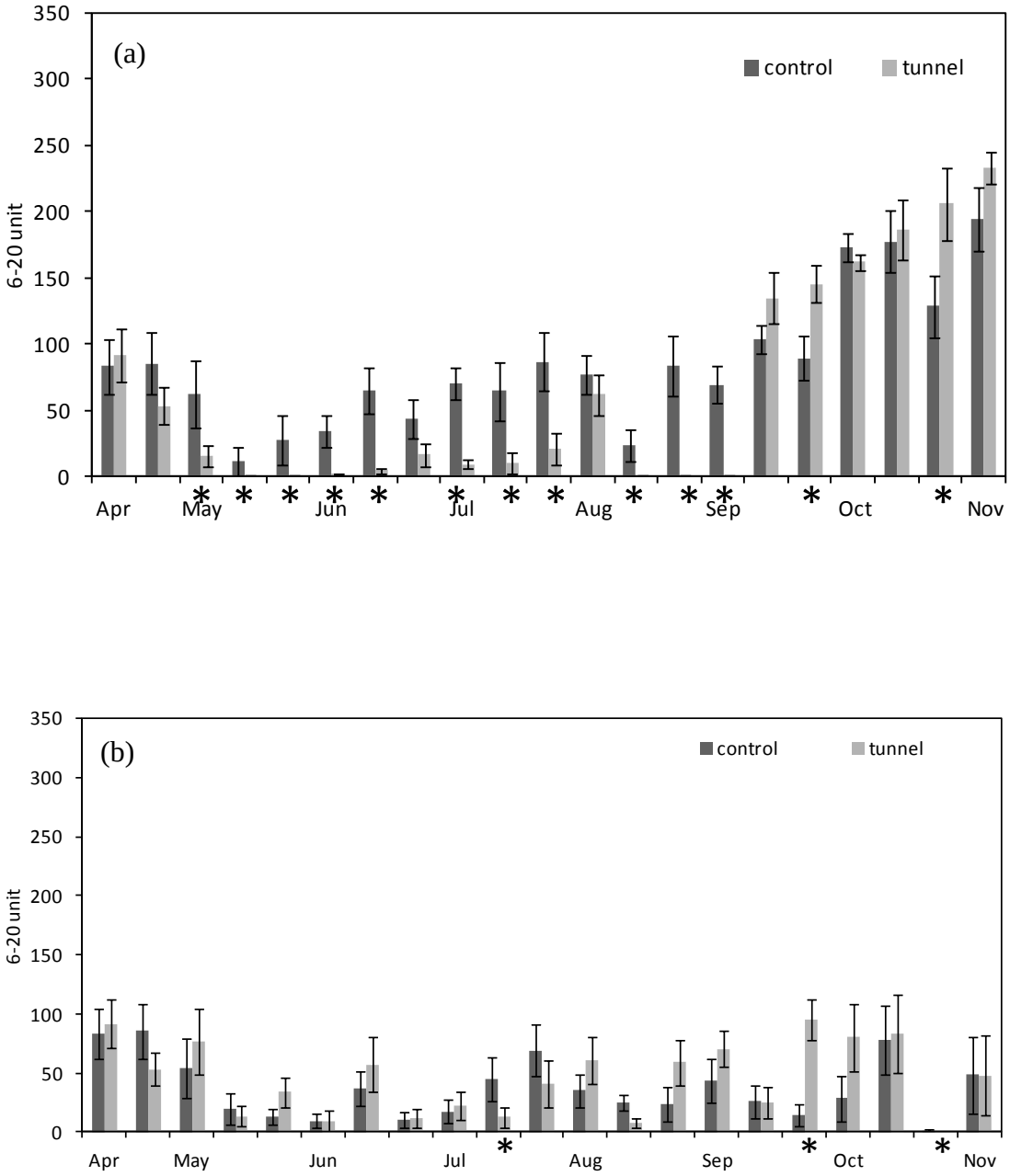

Fig. 5 - Leaf wetness in two infected commercial *A. chinensis* orchards in Faenza (Italy): (a) – orchard with permanently closed tunnel; (b) - orchard with seasonally closed tunnel. Data are reported as the mean of 10 days of daily cumulates (mean ± SE) in arbitrary (instrumental) units. Data marked with an asterisk (*) were significantly different (T-test; $P<0.05$) between covered and uncovered rows.

Thesis (a)	may		early jun		end of jun		jul	
	% bud active	lenght of stems	% bud active	lenght of stems	% bud active	lenght of stems	% bud active	lenght of stems *
control	61.1%	15.1 ± 1.0	63.9%	42.4 ± 2.7	63.9%	78.7 ± 5.6	69.4%	116.7 ± 10.0
tunnel	5.6%	16.5 ± 1.5	5.6%	60.0 ± 2.4	5.6%	110.0 ± 2.4	72.2%	32.0 ± 7.3

Thesis (b)	jul		aug	
	% bud active	length of stems *	% bud active	length of stems
control	100.0%	41.0 ± 3.4	100.0%	141.9 ± 14.6
tunnel	63.0%	26.1 ± 3.4	67.0%	170.6 ± 16.2

Tab. 1 – Percentage of bud active and long of stems below plastic cover and in uncovered rows in two infected commercial *A. chinensis* orchards in Faenza (Italy): (a) – orchard with permanently closed tunnel; (b) - orchard with seasonally closed tunnel (n=24; mean ± SE). Data marked with an asterisk (*) were significantly different (T-test; P<0.05) between covered and uncovered rows.

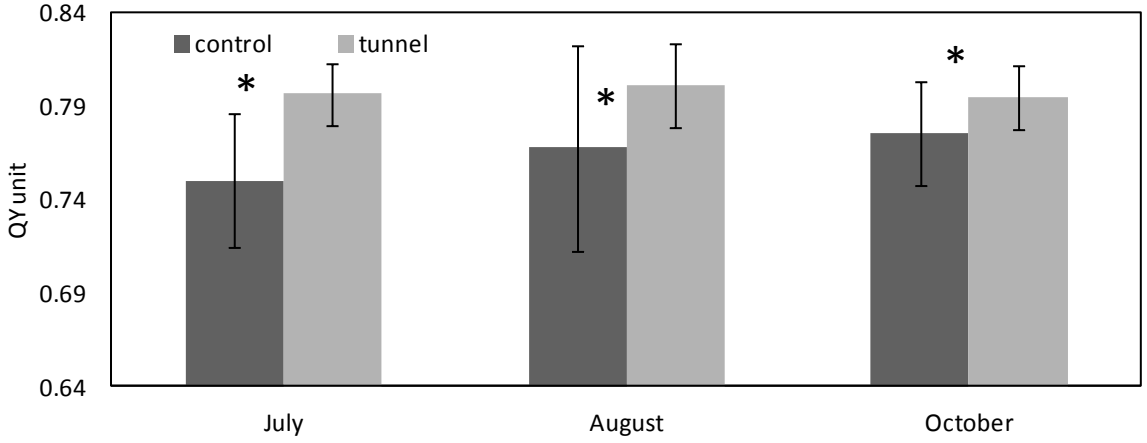

Fig. 6 – Quantum yield in an infected commercial *A. chinensis* orchard in Faenza (Italy) with seasonally closed tunnel (mean ± SE, n=24). Data marked with an asterisk (*) were significantly different (T-test; P<0.05) between covered and uncovered rows.

Thesis		Weight (g)		average fruits per plant	average kg per plant
Pre-harvest	Control	100.79 ± 16.78	*		
	Tunnel	124.17 ± 23.55			
Harvest	Control	96.43 ± 19.10	*	163.8 ± 16.2	15.8 ± 1.8
	Tunnel	123.18 ± 25.75		312.0 ± 38.0	38.4 ± 5.4
Storage	Control	95.13 ± 20.95	*		
	Tunnel	125.61 ± 25.44			

Tab. 2 – Fruit weight and number in an infected commercial *A. chinensis* orchard in Faenza (Italy) with seasonally closed tunnel, in pre-harvest, harvest, and storage (mean ± SE). Data marked with an asterisk (*) were significantly different (T-test; P<0.05) between covered and uncovered rows.

Thesis	Weight (g)	Hue angle (°)	firmness (kg)	dry metter (% of fresh)	sugar content (°Brix)
Control	94.0 ± 8.3	104.1 ± 0.4	7.1 ± 0.0	20.0% ± 75.0%	14.6 ± 0.6
Tunnel	89.2 ± 3.7	103.1 ± 0.6	6.7 ± 0.2	20.4% ± 6.4%	15.3 ± 0.7

Tab. 3 – Fruit qualitative analysis at the harvest in an infected commercial *A. chinensis* orchard in Faenza (Italy) with permanently closed tunnel (mean ± SE). The data show no significant differences according to T-test (P<0.05).

Thesis		Hue angle (°)		firmness (kg)		dry matter (% of fresh)		sugar content (°Brix)	
Pre-harvest	Control	105.29 ± 1.70	*	5.62 ± 0.53		16.36% ± 1.09%		8.32 ± 1.27	*
	Tunnel	107.64 ± 2.42		5.52 ± 0.44		16.15% ± 1.17%		6.31 ± 0.70	
Harvest	Control	102.65 ± 2.28	*	5.58 ± 0.54		14.58% ± 1.18%	*	10.34 ± 1.67	*
	Tunnel	103.74 ± 2.57		5.52 ± 0.57		13.71% ± 1.03%		7.69 ± 1.16	
Storage	Control	115.34 ± 0.61		1.94 ± 0.71	*	18.88% ± 0.90%	*	16.60 ± 1.03	*
	Tunnel	115.29 ± 1.07		3.91 ± 0.95		17.04% ± 0.85%		14.23 ± 1.09	

Tab. 4 – Fruit qualitative analysis at the pre-harvest, harvest and storage –in an infected commercial *A. chinensis* orchard in Faenza (Italy) with seasonally closed tunnel (mean ± SE, n=30). Data marked with an asterisk (*) were significantly different (T-test; P<0.05) between covered and uncovered rows.

(a)

	% plant can infected
Control	3%
Tunnel	0%

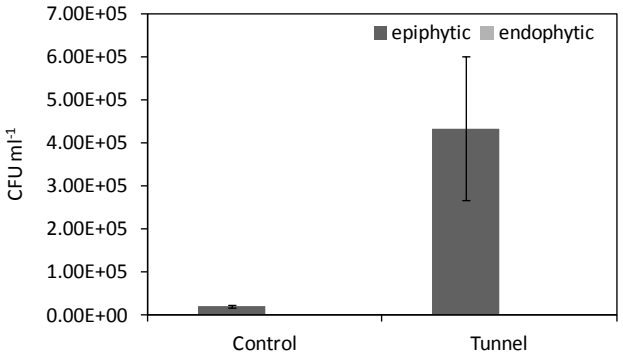

Fig. 6 – Infected buds in an infected commercial *A. chinensis* orchard in Faenza (Italy) with permanently closed tunnel (a) percentage of plant can infected; (b) epiphytic and endophytic bacterial population in infected buds (mean ± SE).

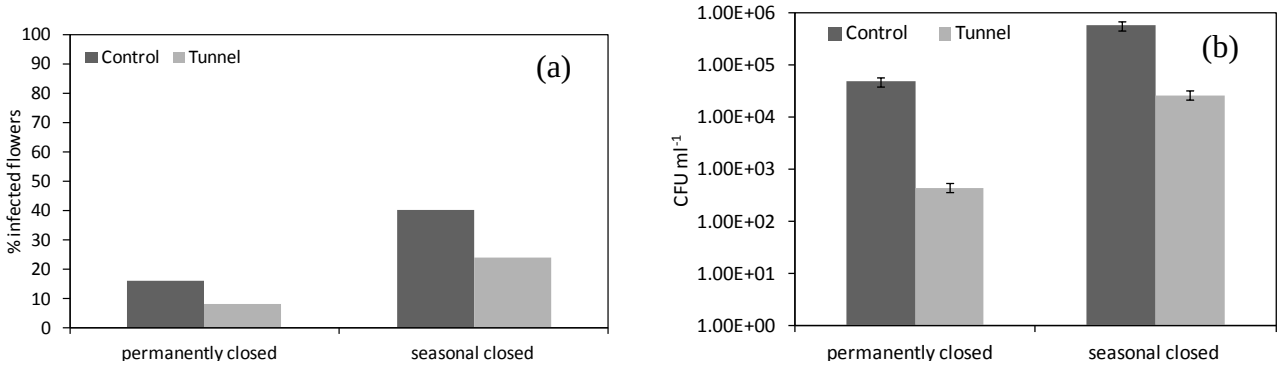

Fig. 7 – Infected flowers in two infected commercial *A. chinensis* orchards in Faenza (Italy) in orchard with permanently or seasonally closed tunnel (mean ± SE) (a) percentage of infected flowers; (b) epiphytic and endophytic bacterial population in infected flowers.

(a)	thesis	total plants	March	early April	end April	May	increase disease (t_f-t_0)
% diseased plants	tunnel	603	0.00%	0.33%	0.33%	0.33%	0.33%
	control	622	6.59%	8.04%	11.58%	11.58%	4.99%

(b)	thesis	total plants	May	June	July	August	October	increase disease (t_0-t_f)
% diseased plants	tunnel	527	49.6 %	55.9 %	59.6 %	61.5%	64.9%	15.3%
	control	413	51.8 %	54.5 %	55.4 %	55.6%	65.1%	13.3%

Tab. 5 – Percentage of plants with symptoms in two infected commercial *A. chinensis* orchards in Faenza (Italy): (a) – orchard with permanently closed tunnel; (b) - orchard with seasonally closed tunnel.

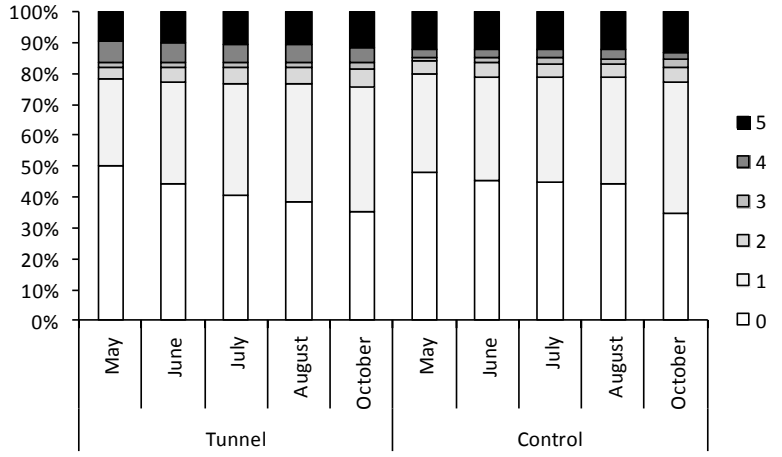

Fig. 8 – Distribution of symptom severity in covered and uncovered plants in infected commercial *A. chinensis* orchards in Faenza (Italy) with seasonal closed tunnel. Symptoms scale: 0 – asymptomatic plants; 1 - leaf spot, chlorosis; 2- leaf spot, exudates, cancers, blighted flowers, few shoots wilting; 3- leaf spot, exudates, cancers, blighted flowers, buds withering, pruning interventions, fruiting shoots wilting; 4- extensive necrosis; 5- uprooted plant.

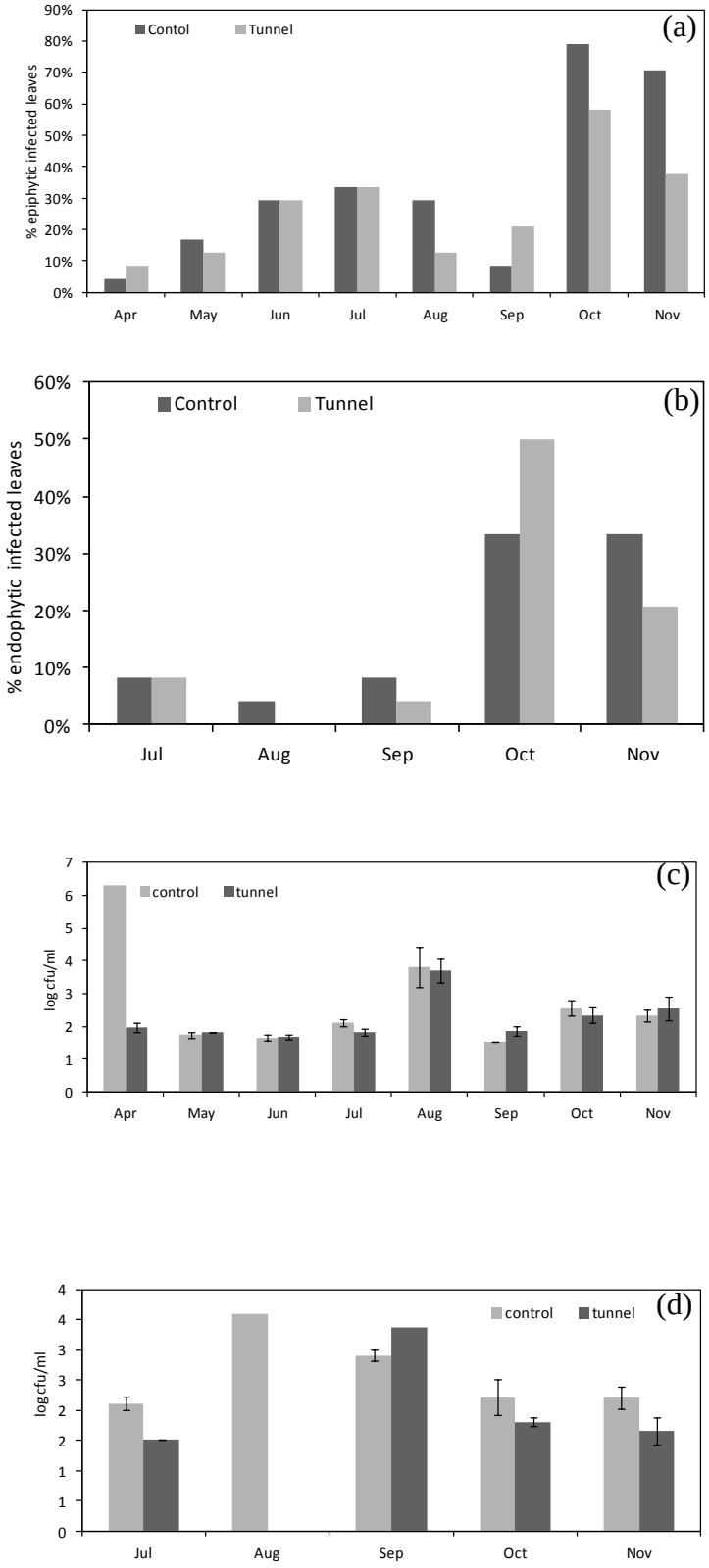

Fig. 9 – Bacterial populationin the leaves of an infected commercial *A.chinensis* orchard in Faenza (Italy) with permanently closed tunnel (mean ± SE, n=24) percentage of epiphytic (a) and endophytic (b) infected leaves; epiphytic (c) and endophytic (d) bacterial population in infected leaves.

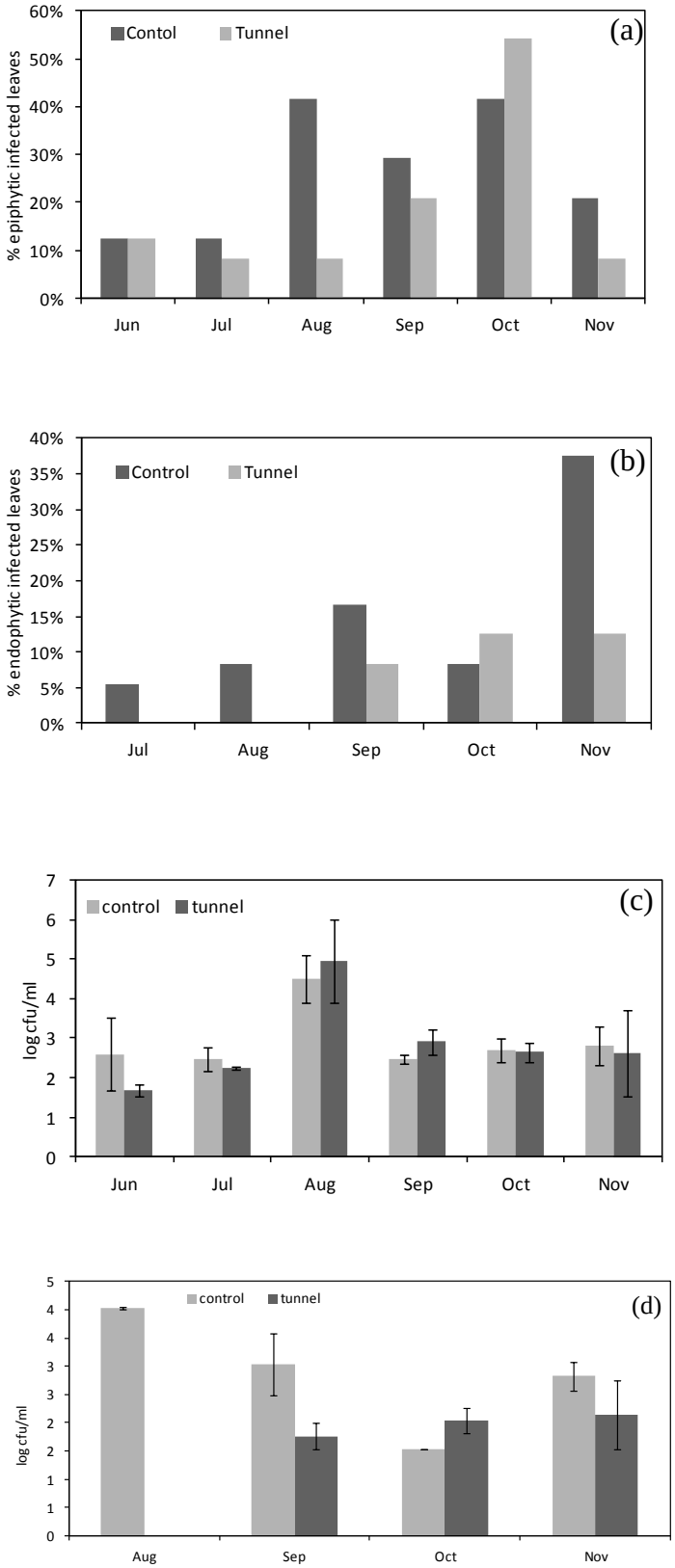

Fig. 10 – Bacterial population on the leaves of an infected commercial *A. chinensis* orchard in Faenza (Italy) with seasonal closed tunnel (mean ± SE, n=24) percentage of epiphytic (a) and endophytic (b) infected leaves; epiphytic (c) and endophytic (d) bacterial population in infected leaves.

Gene	Function	Forward primer	Reverse primer	Origin
AlgD	Biofilm formation	GACCTGGAACTGGACTACATC	TGCTGCGAACCACGATAG	This work
AefR	Biofilm formation/virulence	AACTGCTGGAATTGCTCTG	TGTATCGTGGCACCTACC	This work
MexE	Virulence factor	TGTACGCACGGCTGAAACTG	TCCTTGTCCATCACCAGCAC	This work
LysR	Virulence factor	TGCGGAAGTTGAAGCGGATTACG	ACCGAAATGTTGCTGCCTCCC	This work
Pamtada	Virulence factor	ACACATGACCCAGATCAG	CAGCTTGAGGTTGGATTC	This work
Enolase	Virulence factor	CATCGCCAACCTCAATGG	CCTGGATGTCGATGTTGTTAT	This work
HrpM	Virulence factor	TCCAGATAGGCTCGATCA	GACATAACTGCCGATGCT	This work
HopZ5	Virulence factor	TCAGGCTACAATACTTACGCATCA	CAGGAATAGAACGGAACTCAGGAT	This work
Lov	Blue light receptor	GGCAGAAGTTGCCTTGCTGAACAT	ACCGCAATAGAGACATAACGGCCA	Wu *et al.,* 2013
bPHO	Far red light receptor	TGGAACGGCCTTTCTCGATGTGTA	GAGCCAGTGCTCGAAACATGCAAA	Wu *et al.,* 2013
bPHP1	Red light receptor	TTTCGACGTTGCGCAGTGTTTCAC	AATCAGCGACACACTCATGGACGA	Wu *et al.,* 2013
RecA	Recombinase A	CGCACTTGATCCTGAATACG	CATGTCGGTGATTTCCAGTG	This work

Tab. 6 – The list of genes tested in transcriptional analysis.

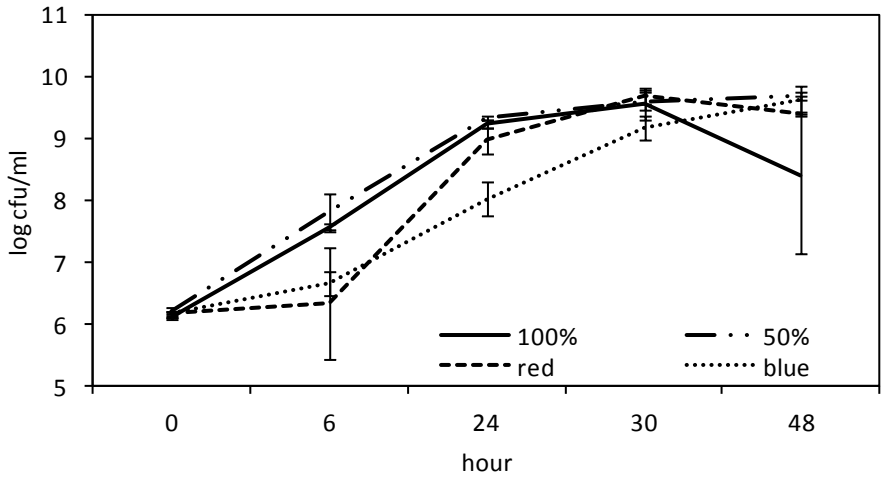

Fig. 11– Bacterial growth of Psa strain 7286 expressing the Green fluorescent protein GFP-uv in LB after 0, 6, 24, 30, 48, 72 hour under light treatment (100% PAR, 50% PAR, red and blue) (n=5). The data show only significant differences at t6 between red and 50% treatments; at t 24 in blue thesis, according to Fisher's LSD test (P<0.05).

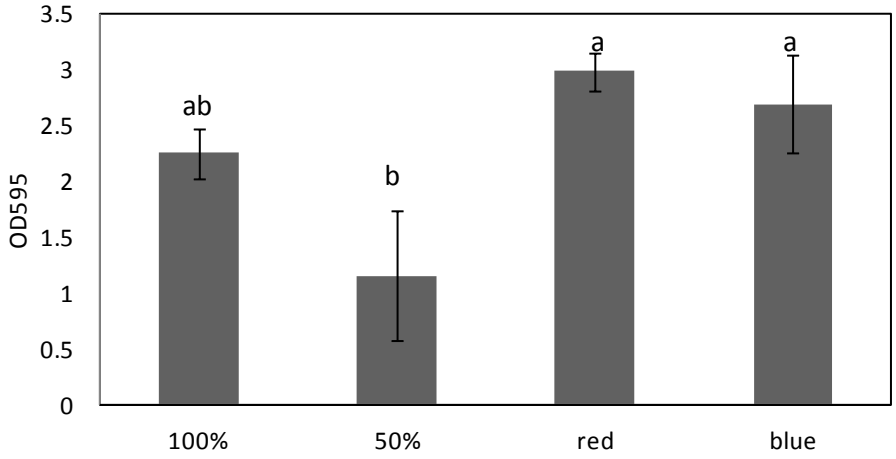

Fig. 12 – Biofilm formation of Psa strain 7286 expressing the Green fluorescent protein GFPuv (MMMF) under light treatment (100% PAR, 50% PAR, red and blue) (mean ± SE, n=6). Value with different letter are significantly different according to Fisher's LSD test (P<0.05).

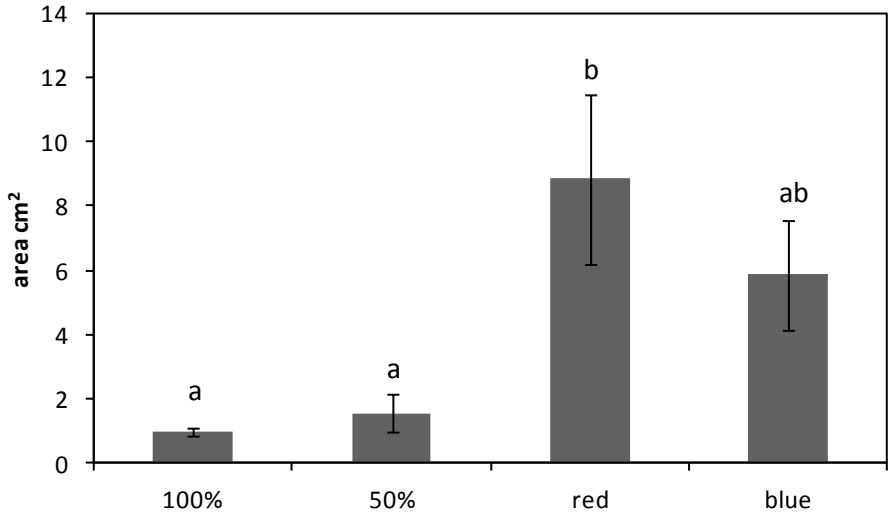

Fig. 13 – Bacterial motility of Psa strain 7286 expressing the Green fluorescent protein GFPuv in LB under light treatment (100% PAR, 50% PAR, red and blue) (mean ± SE, n=6). Value with different letter are significantly different according to Fisher's LSD test (P<0.05).

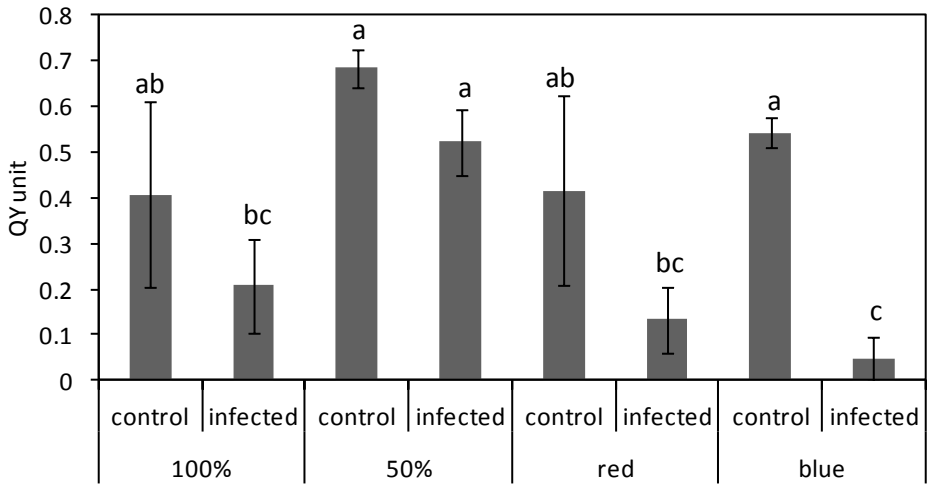

Fig. 14 – Light quantum yield in Psa-infected and non infected micropropagated plants of *A. chinensis* after light treatment (100% PAR, 50% PAR, red and blue) (mean ± SE, n=6). The control sample is consisting of healthy plants. Value with different letter are significantly different according to Fisher's LSD test (P<0.05).

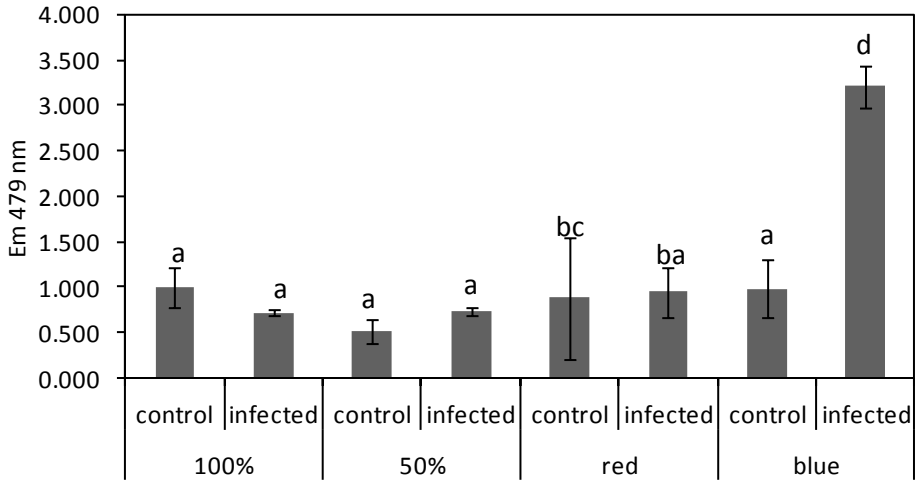

Fig. 15 – Callose formation in Psa-infected and non infected micropropagated plants of *A. chinensis* after light treatment (100% PAR, 50% PAR, red and blue) (mean ± SE, n=6). The control sample is consisting of healthy plants. The samples were read on a spectrofluorimeter (excitation: 393 nm; emission: 479 nm). Value with different letter are significantly different according to Fisher's LSD test (P<0.05).

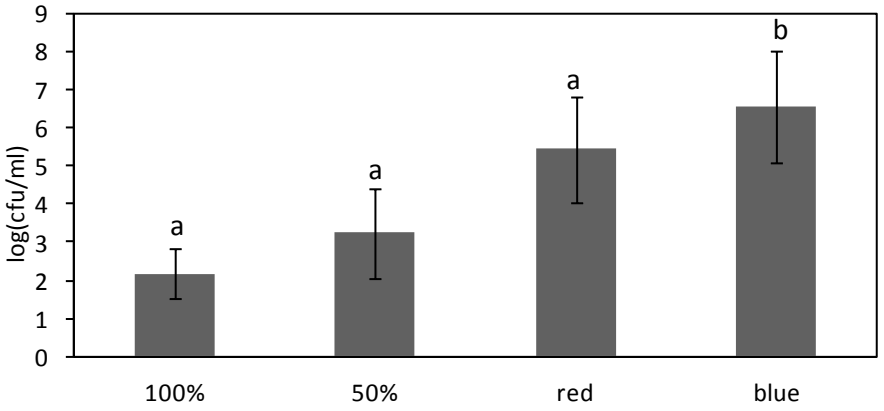

Fig. 16 – Endophytic bacterial populations in infected micropropagated plants of *A. chinensis* after light treatment (100% PAR, 50% PAR, red and blue) (mean ± SE, n=6). Value with different letter are significantly different according to Fisher's LSD test (P<0.05).

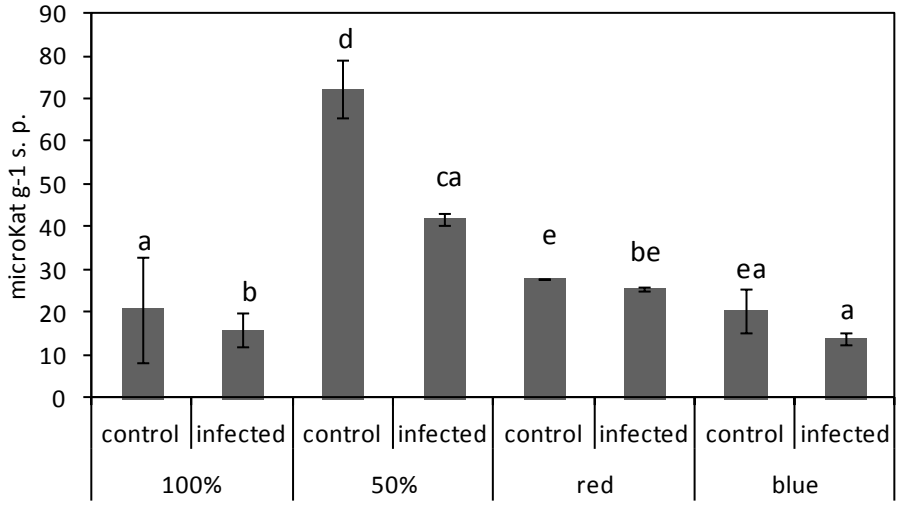

Fig. 17 - – NOX activity in Psa-infected and non infected micropropagated plants of *A. chinensis* after light treatment (100% PAR, 50% PAR, red and blue) (mean ± SE, n=6). One NOX unit evolves an amount of superoxide that converts 1 µmol min-1 NBT to formazan. Value with different letter are significantly different according to Fisher's LSD test ($P<0.05$).

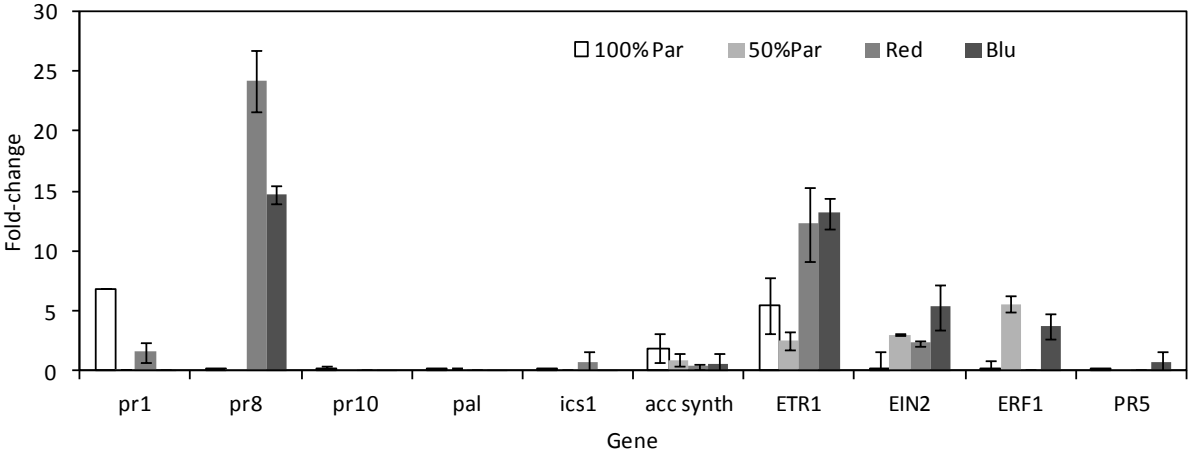

Fig. 18 – Expression of genes related to defences in Psa-infected and non infected micropropagated plants of *A. chinensis* after light treatment (100% PAR, 50% PAR, red and blue) (mean ± SE, n=6) as relative fold-change compared to actin gene.

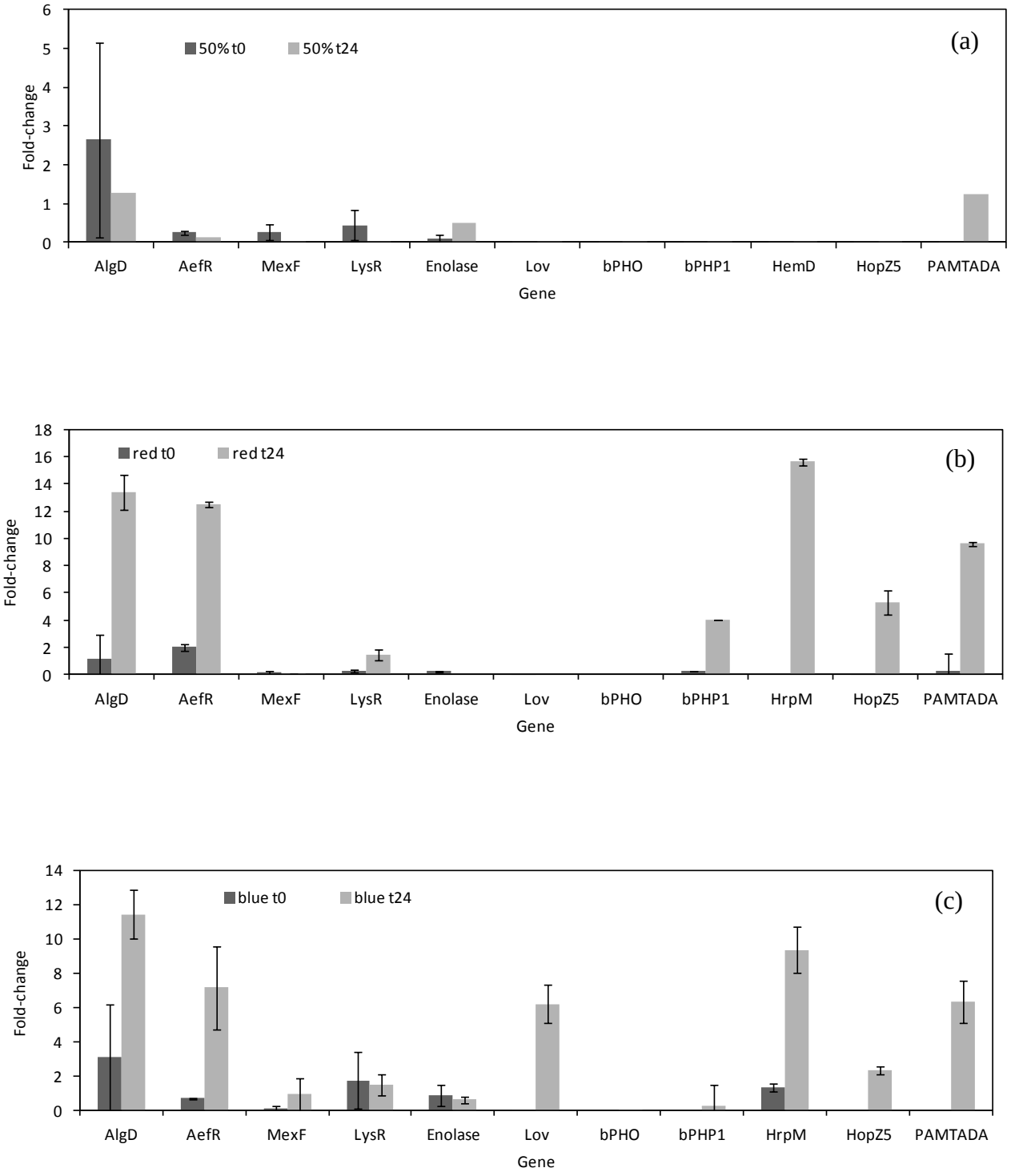

Fig. 19 – Expression of genes related to biofilm formation, motility, light perception and pathogenicity in Psa, grown under different light conditions. Data are expressed as fold-change expressed as relative fold-change compared to rec A gene. Theses: (a) 50% PAR light; (b) red light; (c) blue light (mean ± SE, n=6).

GENERAL CONCLUSIONS

This research work focused on the impact of agricultural practices on the incidence and severity of the bacterial canker of kiwifruit, defining which ones are significant in limiting the bacterium diffusion in the orchard. This allows developing the integration of different practices in order to minimize the risk of disease.

As regards the nitrogen fertilization, it has a direct effect on the pathogen's endophytic growth, in particular, NPK fertilization has demonstrated a lower incidence and severity of the disease, but an excess of N favors the disease development in the plant. Furthermore, the depletion of some micronutrients, such as iron, increases the disease. The water stress consequent to a reduced irrigation resulted in higher symptoms. In addition, stagnant water sources seem to favor the bacteria spread more than running water supplies. The study on the role of pruning in the spread of the bacteria showed that the open cuts remain a possible entry point for more than 30 days. As regards the timing of intervention, pruning during the middle of winter (January) appeared to be the safest with the lowest incidence of the disease. The GDC showed a better accessibility of the canopy to water sprays, due to a greater openness of the canopy at the top next to the leader. Pruning forms that allow better aeration and irradiation of the foliage could be useful in the bacterial control, by increasing the effectiveness of treatments and reducing humidity and the time of leaf wetting, thus disadvantaging the epiphytic bacterial growth. Several species were tested for their susceptibility to Psa in order to develop a tolerant rootstock: *A. arguta* resulted the most tolerant to the disease and further experiments are needed to test its possible exploitation as a commercial rootstock. The application of synthetic cytokines (Forchlorferon) does not negatively influence on the disease development, showing indeed a potential action of slowing it down. Conversely, the application of synthetic auxins (Triclopyr) was strongly negative, possibly due to the formation of lesions, which also create a pathogen entry point, weaken the plant defence and/or stimulate bacterial multiplication. The influence of the disease on fruit yield, quality and storability showed that the fruits from symptomatic plants had a smaller size and a shorter storability.

The study of *M. pruinosa* showed the capacity of the insect to spread the disease in controlled conditions, in fact, the insects could vector the bacterium from plant to plant. Furthermore, the number of infected insects feeding on a plant, rather than their bacterial load, is

determinant for the disease transmission. This is particularly important in the light of *M. pruinosa*'s gregarious behavior. However, the capability and the importance of *M. pruinosa* as a vector of Psa in field conditions has not yet been evaluated and further experiments are needed to fully understand the contribution of *M. pruinosa* to Psa disease spread.

Tunnels can help controlling the spread of Psa, mainly due to the decrease of leaf wetness. Interestingly, some aspects of Psa's pathogenicity are promoted by PAR reduction, blue and/or red light. Therefore, in the choice of cover materials, transparent films should be preferred, to avoid favoring the virulence of the bacterium in the pathosystem.

In conclusion, it is recommended to avoid excess nitrogen fertilization and water stresses to the plants, favoring the use running water supplies. Pruning of vegetation is useful to allow better ventilation of the canopy, but should be performed when environmental conditions are less permissive for Psa. It is very important to control insect population in the orchard, to reduce the number of possible bacterial vector. The use of tunnels in the orchard can be a useful technique to the bacterium containment, paying attention to the material used.