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Deciphering the cross-talk between *Actinidia* spp. and *Pseudomonas syringae* pv. *actinidiae* (Psa).

Dottorando
Luca Fiorentini

Coordinatore Dottorato
Prof. Giovanni Dinelli

Relatore
Prof. Francesco Spinelli

Correlatori

Dr. Joel Vanneste

Dr. Giampaolo Buriani

Esame finale anno 2016

ABSTRACT

The Quorum-Sensing (QS) is a complex bacterial intra- and inter-communication system which is regulated by signalling molecules produced by cell density dependent mechanisms. Since the QS regulates relevant phenotypes such as virulence, motility and biofilm production, we investigated the traits of the QS-system in *Pseudomonas syringae* pv. *actinidiae* (Psa). Despite Psa was demonstrated unable to produce any canonical QS-signal (e.g. homoserine-lactones, AHLs), recent studies demonstrated the existence of a QS-signal receptors in Psa that could recognize bacterial AHLs (PsaR1, PsaR3) and a still unknown plant molecule (PsaR2).

To study the signalling communication system of *Pseudomonas syringae* pv. *actinidiae* we proceeded with a logical approach, starting the study of the effect of the interspecific communication in pure cultures of *Pseudomonas syringae* pv. *actinidiae* at different cellular densities. Then more complex systems were evaluated in which *Pseudomonas syringae* pv. *actinidiae* was made interact with synthetic molecules (AHLs), microbial *biocoenosis* and the plant-host. We focused our analysis about the signalling communication systems on biofilm formation, motility density *in vitro* and pathogenicity *in vivo*. The gene expression analysis of the genes related to those phenotypes intended to sustain the results obtained from the related bioassays, thus giving further validation of the observations.

First of all we performed a bioinformatics research. About 30 genes involved in different mechanisms (e.g. virulence, density perception, motility and biofilm formation) were selected for the gene expression, performed by qPCR, in *Pseudomonas syringae* pv. *actinidiae* samples obtained from different culture conditions (i.e. density; co-cultures; plant extracts). In qPCR analysis, *rpoD* and *recA* genes are used as reference for gene expression normalization. We also analysed the expression of a putative transcriptional regulator (*Tr*) that was identified through a nucleotide sequence blast analysis of several bacterial signal synthase promoters that responded to PsaR1 and PsaR3 signal receptors. We realized a silenced *Tr* strain via RNA silencing and found that *Tr* is involved in biofilm and motility regulation.

Motility and biofilm formation, crucial phenotypes during host colonization, were dependent from the signalling within the microbial community. The experiments conducted demonstrated *Pseudomonas syringae* pv. *actinidiae* ability of producing biofilms in liquid media only after AHLs signals perception. Furthermore, Psa motility resulted particularly enhanced on supernatants deriving from “neighbouring” bacteria (such as *Pseudomonas fluorescens* and *Pseudomonas syringae* pv. *syringae*) and on *Actinidia* spp. plant extracts or xylem saps. The use of three mutants lacking of *psaR1*, *psaR2* and *psaR3* genes for signal receptors confirmed their involvement in the regulation of phenotypes (motility and biofilm) related to cell-to-cell signalling communication systems.

The quantitative expression of QS-related genes, analysed by qPCR, demonstrated that the expression fold change significantly varied in Psa populations depending on the cell density (from 10^5 up to 10^8 CFU ml^{-1}). The QS-gene expression profiles also significantly changed in *Pseudomonas syringae* pv. *actinidiae* cultures grown in filter-sterilized bacterial spent supernatants of bacteria associated to kiwifruit plants, remarking the importance of interspecific communication on *Pseudomonas syringae* pv. *actinidiae* communication system and potentially on in *Pseudomonas syringae* pv. *actinidiae* virulence. Traits of gene expression in plant extracts indicated that host recognition triggers pathogen arsenal, since virulence genes and plant signal receptor PsaR2 resulted overexpressed.

In conclusion, from our studies we observed that QS- related phenotypes seem to be influenced not only by the cell density, but also by the microbial community sharing the phyllosphere with *Pseudomonas syringae* pv. *actinidiae*. These findings showed that *Pseudomonas syringae* pv. *actinidiae* ecological fitness and virulence can be influenced not only during the interaction with the host, but also by the cross-talk with other bacteria present on the epiphytic, microbial *biocoenosis*. Moreover, since we have evidenced that *Pseudomonas syringae* pv. *actinidiae* can eavesdrop signals coming from its "neighbours", we suggest the hypothesis of a multiple model system, where *Pseudomonas syringae* pv. *actinidiae* interacts both with the plant host and other bacterial species to regulate the phenotypes related to the host colonization and invasion.

Keywords: *Actinidia* spp., Kiwifruit, Virulence, Motility, Biofilm, qPCR, Quorum-Sensing, signalling

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INTRODUCTION

Economic importance of the kiwifruit industry

Italy is the second nation in the world for kiwifruit production after China and the second most important exporting country after New Zealand. In Italy, 6% of the entire surface of fruit orchards is dedicated to kiwifruit crops and the economic value of the entire supply chain is around 10 billion of Euro, equal to the 9% of the total fruit GSP (*gross saleable product*). The 80% of the national kiwifruit industry is located in only four regions: Lazio 32%, Piemonte 21%, Emilia-Romagna 15% and Veneto 13% (Testolin and Ferguson, 2009; CSO 2013).

Effects of the bacterial canker on kiwifruit industry

Commencing from 2007, the kiwifruit cultivation started to face a worldwide crisis due to the spread of the bacterial canker that is caused by the gram-negative bacterium *Pseudomonas syringae* pv. *actinidiae* (Psa), that assumed pandemic characteristics and posed a severe plant health issue (Balestra et al., 2009 a). In Italy, between 2010 and 2012, kiwifruit cultivation area was reduced by about 2000 hectares, with productive losses of 10–50% per hectare (Donati et al., 2014). Moreover, in 2012 the Italian commercial production reached an historical minimum with 376 thousand tons, 26% less than prior the disease spread (CSO, 2014). At that time, private and public institutions and private companies cooperated actively together to understand and solve the problem of Psa threat. For example, just in Emilia-Romagna, 3 millions of euro were overall dispensed in 5 years (2010-2014) by the Region as contributions for nurseries and plant eradications. However, in 2015, the Region suspended the contributions, since Psa was eventually considered under control (CRPV, 2015). In fact, total commercial production in 2015 reached about 496 thousand tons (Apo Conerpo, 2015), thus registering an increase of 4,2% respect to the production in 2009 prior Psa outbreak. Today's good health of kiwifruit industry has been achieved thanks to the research progresses that provided efficient tools for disease control and containment, making possible the introduction of new cultivars, some of which are even less susceptible to the disease.

The bacterial canker of kiwifruit

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*) is aerobic, motile and rod-shaped, with polar flagella, oxidase negative, arginine dihydrolase-negative (Takikawa et al., 1989) and represents the most serious disease that affected *Actinidia* species since their introduction in the Italian territory (Donati et al., 2014). Psa was first reported in Japan negative (Takikawa et al., 1989) and was subsequently reported in South Korea (Koh et al., 1994) and in 1992 in Italy where it was first isolated in Lazio (Scortichini, 1994). Later on, from Latina the pathogen spread all over Italian kiwifruit orchards (Testolin, 2012).

Genomic analyses allowed to associate the Italian pandemic strains in one homogeneous group, named biovar 3. However, early studies on the identification of Psa populations based on molecular analysis, revealed the existence of 4 biovars (Vanneste et al., 2013): *biovar 1* that includes strains of Japanese and Italian 1990s outbreaks; *biovar 2* that is represented by the strains exclusively isolated in South Korea; *biovar 3* represented by the pandemic strains isolated in Europe, New Zealand, Chile and China; and *biovar 4*, that is considered "less-virulent" because it apparently causes only mild symptoms, and included strains isolated only in New Zealand and Australia (Ferrante and Scortichini, 2014). 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). Therefore, biovar 4 was recently identified also in France and its phenotypic, genetic and phylogenetic differences made this biovar to be assigned as a new pathovar, named *Pseudomonas syringae* pv. *actinidifoliorum* (Cunty et al., 2015).

Psa affects all commercial cultivars of kiwifruit, including the green-fleshed kiwifruit (*Actinidia deliciosa* 'Hayward' and 'Summer Kiwi'), and the yellow-fleshed kiwifruit (*Actinidia chinensis* 'Jin Tao', 'Hort16A', and 'Soreli'), representing the most aggressive disease that affected kiwifruit worldwide so far. These species, however, showed different susceptibility: in fact, vines of *A. chinensis* resulted more susceptible than the vines of *A. deliciosa* (Balestra et al., 2009 b). Psa can effectively colonize the kiwifruit plants throughout the year: the epidemic cycle was first described by Serizawa and Ichikawa in 1993 (Serizawa et al., 1993). In particular, late-winter or early-spring, buds and young branches may wither producing cankers due to the infections occurred during the cold period. From the cankers exudates can be released and large amounts of inoculum are consequently spread in the environment. At spring, mild temperatures (12-18°C) and high humidity conditions favour Psa multiplication (Scortichini et al., 2012) and plant colonization by penetration through the flowers and natural openings such as stomata, lenticels and unhealed wounds. Psa can spread systematically along the plant vessels and move from the leaf to the young shoots, causing leaf spots, flower necrosis and fruits collapse with production loss. Since the systemic invasion of the plant may determine the rapid death of plants (Scortichini et al., 2012), a crucial phase of Psa cycle is the ability to migrate from the leaves to shoots and canes via apoplast (Donati et al., 2014). At summer time, high temperatures can either reduce the multiplication and spread of the bacterium in the orchard (Scortichini et al., 2012), but also cause quick withering and death of the heavily infected plants. Eventually fall is a crucial moment in disease spread since the wounds caused by the leaf-drop, fruit-drop and pruning-cuts represent an incredible number of entry points for the bacterium, while mild temperatures promote pathogen multiplication (Gullino and Brunelli, 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). 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 (Spinelli et al., 2012; Donati et al., 2014).

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)and also after administration of some biocontrol agents.

During the PhD, lots of efforts were dedicated in understanding the intimate relationships occurring between the plant and the pathogen. In particular, traits of the bacterial inter-communication, that can lead to the colonization and pathogenesis manifestation, were examined in order to obtain useful information that can be used in the future for the control of the disease.

Reference

Apo Conerpo, 2015. Aumenta la produzione mondiale di kiwi . (http://www.freshplaza.it/article/79031/Aumenta-la-produzione-mondiale-di-kiwi)

Balestra, G. M. et al. 2009 (a). "Current Status of Bacterial Canker Spread on Kiwifruit in Italy." *Australasian Plant Disease Notes* 4(1): 34–36.

Balestra, Giorgio M. et al. 2009 (b). "Occurrence of Pseudomonas Syringae Pv. Actinidiae in Jin Tao Kiwi Plants in Italy." *Phytopathologia Mediterranea* 48(2): 299–301.

Butler, Margi I. et al. 2013. "Pseudomonas Syringae Pv. Actinidiae from Recent Outbreaks of Kiwifruit Bacterial Canker Belong to Different Clones That Originated in China." *PLoS ONE* 8(2).

Cellini, A. et al. 2014. "Elicitors of the Salicylic Acid Pathway Reduce Incidence of Bacterial Canker of Kiwifruit Caused by Pseudomonas Syringae Pv. Actinidae." *Annals of Applied Biology*.

CRPV, 2015. Emilia-Romagna: convegno "La batteriosi del kiwi: i risultati del progetto regionale" (http://www.freshplaza.it/article/78883/Emilia-Romagna-convegno-La-batteriosi-del-kiwi-i-risultati-del-progetto-regionale)

CSO, 2013. Dossier kiwi 2012 (2013) –. Il punto della situazione sulla produzione mondiale, i consumi, i nuovi mercati, le novità. CSO - Centro servizi ortofrutticoli

CSO, 2014. Kiwi: produzione italiana 2014/15 in linea con la stagione precedente. (http://www.freshplaza.it/article/68456/kiwi-produzione-italiana-2014-15-in-linea-con-la-stagione-precedente)

Cunty, A. et al. 2015. "Characterization of Pseudomonas Syringae Pv. Actinidiae (Psa) Isolated from France and Assignment of Psa Biovar 4 to a de Novo Pathovar: Pseudomonas Syringae Pv. Actinidifoliorum Pv. Nov." *Plant Pathology* 64(3): 582–96.

Donati, Irene et al. 2014. "New Insights on the Bacterial Canker of Kiwifruit (Pseudomonas Syringae Pv. Actinidiae)." *Journal of Berry Research* 4(2): 53–67.

Ferrante, P., and M. Scortichini. 2015. "Redefining the Global Populations of Pseudomonas Syringae Pv. Actinidiae Based on Pathogenic, Molecular and Phenotypic Characteristics." *Plant Pathology* 64(1): 51–62.

Gullino L., Brunelli A. (2012) – Prevenzione e difesa del kiwi dalla batteriosi da Psa. Frutticoltura 9: 20-24.

Koh, Y. J., Cha, B. J., Chung, H. J., and Lee, D. H. 1994. Outbreak and spread of bacterial canker in kiwifruit. Korean J. Plant Pathol. 10:68-72.

Serizawa S, Ichikawa T (1993) Epidemiology of bacterial canker of kiwifruit. Annals of the Phytopathological Society of Japan 59, 452–459

Spinelli F., Donati I., Mauri S., Preti M., Fiorentini L., Cellini A., Buriani G., Costa G. (2012) – Osservazioni sullo sviluppo del cancro batterico. Frutticoltura, 9: 32-35.

Scortichini, M. 1994. Occurrence of Pseudomonas syringae pv. actinidiae on kiwifruit in Italy. Plant Pathol. 43:1035-1038.

Scortichini, M. et al. 2012. “Pseudomonas Syringae Pv. Actinidiae: A Re-Emerging, Multi-Faceted, Pandemic Pathogen.” *Molecular Plant Pathology* 13(7): 631–40.

Takikawa, Y., Serizawa, S., Ichikawa, T., Tsuyumu, S., and Goto, M. 1989. Pseudomonas syringae pv. actinidiae pv. nov.: the causal bacterium of canker of kiwifruit in Japan. Ann. Phytopathol. Soc. Jpn. 55:437-444.

Testolin, R, and A R Ferguson. 2009. 37 New Zealand Journal of Crop and Horticultural Science *Kiwifruit (Actinidia spp.) Production and Marketing in Italy*. http://www.tandfonline.com/doi/abs/10.1080/01140670909510246\npapers2://publication/doi/10.1080/01140670909510246.

Testolin R. (2012) – Il bilico fra batteriosi e innovazione varietale. Frutticoltura, 9:2-10.

Vanneste, Joel L et al. 2012. “Identification, Virulence and Distribution of Two Biovars of Pseudomonas Syringae Pv. Actinidiae in New Zealand.” *Plant Disease* 97(6): 121114115754006.

AIM OF THE THESIS

Since we extensively worked (2010-2012) on the signalling pathways involved during the pathogenesis, demonstrating relevant host physiological implications during the pathogenesis, such as the elicitation of the salicylic acid pathway that was able to reduce plant symptoms (Cellini et al., 2014), in 2013 a PhD project was set up with a particular objective on plant/microbe interaction. In particular, the project was centred on the pathogen molecular patterns during the host infection and the cross-talk with its microbial "neighbours", present in the phyllosphere during colonization of the plant. We had few crucial questions to which we intended to find an answer: *may be the density responsible for long latency of the disease in apparently asymptomatic plants? What is the role of microbial biocoenosis in interfering - positively or negatively – with Psa during first stages of plant surfaces colonization? How interkingdom signalling communication can be described in Actindia sp. and Psa?*

In order to study how the pathogen related to its host, we decided to consider a particular mechanism, present both in gram negative and gram positive bacteria, that had never been studied for Psa before: the Quorum-Sensing (QS). The QS is a communication system that is based on the production, secretion and perception of small signal molecules, allowing the bacteria to coordinate group behaviours as a function of cell density (Fuqua & Greenberg, 2002). Following the signal perception, gene transcription is modulated and phenotypes such as virulence, biofilm formation, antibiotic production, motility, siderophore synthesis etc. are regulated (Wagner, Bushnell, Passador, Brooks, & Iglewski, 2003). Since the QS may regulate phenotypes involved in epiphytic fitness and host colonization, we investigated the traits of the "signalling" in Psa, considering the virulence, motility and biofilm production in particular. Despite Psa was demonstrated unable to produce any canonical QS-signal (e.g. homoserine-lactones, AHLs), recent studies demonstrated the existence of QS-signal receptors in Psa, named PsaR1, PsaR2, PsaR3, that could recognize bacterial AHLs and a still unknown plant molecule (Patel et al., 2014), thus enforcing the hypothesis that microbial community and the host plant may play a significant role in determining the expression of QS traits.

The steps of the PhD project followed a logical approach, starting the study of the effect of the interactions first in pure cultures of Psa at different densities, then in more complex systems in which Psa was made interact with synthetic molecules, microbial biocoenosis and the host. The gene expression analysis by qPCR of biofilm, motility density and virulence genes was intended to sustain the results obtained from the related phenotypic bioassays, thus giving further validation of the observations.

The research conducted represents one of the first most comprehensive studies on the intercommunication systems of *Actinidia* sp. bacterial canker, since it included phenotypic and molecular analysis applied to different levels of biologic interactions. Since the scientific research for Psa around this topic is still very limited, innovative findings will be presented in the Thesis.

Reference

Cellini, A., Fiorentini, L., Buriani, G., Yu, J., Donati, I., Cornish, D. A., … Spinelli, F. (2014). Elicitors of the salicylic acid pathway reduce incidence of bacterial canker of kiwifruit caused by Pseudomonas syringae pv. actinidae. *Annals of Applied Biology*. http://doi.org/10.1111/aab.12150

Fuqua, C., & Greenberg, E. P. (2002). Listening in on bacteria: acyl-homoserine lactone signalling. *Nature Reviews in Molecular Cell Biology*, *3*(September), 685–695. http://doi.org/10.1038/nrm907

Patel, H. K., Ferrante, P., Covaceuszach, S., Lamba, D., Scortichini, M., & Venturi, V. (2014). The kiwifruit emerging pathogen Pseudomonas syringae pv. actinidiae does not produce AHLs but possesses three luxR solos. *PLoS ONE*, *9*(1). http://doi.org/10.1371/journal.pone.0087862

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., & Iglewski, B. H. (2003). Microarray Analysis of *Pseudomonas aeruginosa* Quorum-Sensing Regulons : Effects of Growth Phase and Environment. *Journal of Bacteriology*, *185*(7), 2080–2095. http://doi.org/10.1128/JB.185.7.2080

Cell density signaling system in *Pseudomonas syringae* pv. *actinidiae* and its implications in host colonization and virulence

Luca Fiorentini[1], Giampaolo Buriani[1], Joel Vanneste[2], Guglielmo Costa[1] and Francesco Spinelli[1]

[1] Department of Agricultural Sciences, Alma Mater Studiorum -University of Bologna, viale Fanin 46, 40127 Bologna – Italy,

[2] Bioprotection, The New Zealand Institute for Plant & Food Research Ltd.,
Ruakura Research Centre, Hamilton, New Zealand

Abstract

Many bacteria present a complex intraspecific communication system, often referred as quorum-sensing, which allows them to coordinate cell functions on the perception of the population density *via* diffusible chemical signals such as N-acyl homoserine lactones. The aim of this work was to investigate the presence of a cell density dependent behaviour in *Pseudomonas syringae* pv. *actinidiae. Pseudomonas syringae* pv. *actinidiae* is the causal agent of the bacterial canker of kiwifruit(*Actinidiae* spp) a pandemic disease which is threatening kiwifruit production worldwide. *Pseudomonas syringae* pv. *actinidiae* displays three quorum sensing -signal receptors (*psaR1, psaR2 and psaR3,* being R1 and R3 involved in cell density perception), but it does not possess any gene for the signal synthase. Bacterial motility, biofilm formation and virulence are phenotypes known to be regulated in many bacteria by quorum sensing. In this study, we evaluated the effect of cell density and exogenous application of N-acyl homoserine lactones on these phenotypes. Moreover, the expression of key genes related with these phenotypes was also characterized by qPCR. Cell density influenced virulence, but it had only negligible effects on motility and biofilm formation. Finally, defective mutants for the cell dependent receptors (*psaR1*$^{-}$ and *psaR3*$^{-}$) and strain impaired via target gene silencing for biofilm formation (*algD*$^{-}$) were used to investigate their effect on motility and biofilm formation. The application of N-acyl homoserine lactones increased biofilm formation and cell motility, but not the pathogen virulence. The analysis of gene expression corroborated the observed effect with only a modest influence of cell density on gene regulation. Mutation of *psaR1* and *psaR3* revealed that motility and biofilm rely on a fine regulation mediated by both receptors. *AlgD* is implicated in alginate synthesis and in *Pseudomonas syringae* pv. *actinidiae*. The silencing of this gene increased cell motility and impaired biofilm formation.

Keywords: quorum sensing, N-acyl homoserine lactones (AHL), motility, biofilm formation, virulence

Introduction

Signalling in bacteria

Bacteria present a complex intraspecific communication system, often referred as quorum-sensing (QS), which allows them to coordinate cell functions on the perception of the population density *via* diffusible chemical signals such as N-acyl homoserine lactones (AHLs) (Venturi, 2006). Once single bacterial cells form aggregates form an initial, dispersed planktonic live, the concentration of diffusible signals increases molecules and the cells start to coordinate gene expression and modulate phenotypes in order to modify their environment (A. Rezaei et al., 2011). QS signal transduction relies on a number of self-produced signals or autoinducers. Among these signals, the most characterized are AHLs. AHLs are synthetized by the LuxI-protein family and bind selectively to the LuxR-like receptors (Miller and Bassler, 2001). When the AHL binds the receptor, the newly formed complex functions both as transcriptional factor and as autoinducer, recognizing the signal synthase promoter, thus increasing the signal production as function of the cell density, and also target gene promoters of which it activates the transcription (Fuqua and Greenberg, 2002). Other common communication signals are (i) diketopiperazines (DKPs) that were found in Gram-negative bacteria where it functions as input signals for the two-component histidine protein kinase *gacS/gacA* response regulators (Holden *et al*., 1999); (ii) autoinducing peptides (AIPs) that are exploited by Gram-positive; (iii) the furanosyl borate diester autoinducer AI-2 is used by both Gram-positive and Gram-negative species (Di Cagno *et al*., 2011).

Cell motility, biofilm formation, production of secondary metabolites, virulence, plasmid transfer, bioluminescence, production of extracellular enzymes are regulated by QS both in Gram-negative and Gram-positive bacteria (Fuqua and Greenberg, 2002). Therefore, QS pays a crucial role in bacterial adaptability, fitness and survival, especially in harsh or oligotrophic environments such as the phyllosphere (Venturi and Fuqua, 2013). QS has been found to influence pathogenesis in many phytopathogen such as *Agrobacterium tumefaciens*, *Pantoea stewartii*, *Erwinia carotovora*, *Ralstonia solanacearum and Xanthomonas campestris* (Von Bodman *et al*., 2003). Furthermore, QS regulatory systems have been well characterized in several *Pseudomonad* species (Patel *et al*., 2014). For this reason, interfering with QS can an important tool for the control of plant pathogens (Hentzer and Givskov, 2003). Among emerging plant pathogens, in recent years the pandemic outbreak of *Pseudomonas syringae* pv. *actinidiae* (Psa) was one of the major threat to temperate fruit production. Psa is a vascular pathogen of kiwifruit (*Actinidia* spp.) where it can cause severe symptoms and the plant death (Koh *et al*., 2010). Psa affects all commercial cultivars of kiwifruit causing important production losses (Balestra *et al*. 2009, Donati *et al*. 2014).

This study investigated the presence in Psa of density-dependent behaviours. Recent studies showed that Psa harbours three LuxR signalling receptors involved in *in planta* survival (PsaR1, PsaR2 and PsaR3) but lacks of their cognate signal synthase gene (Patel *et al*., 2014). Specifically, PsaR1 and PsaR3 recognize AHLs molecules and PsaR2 belongs to the sub-family of PAB-solos which putatively respond to plant signals

(Subramoni *et al*., 2011). Thus, Psa, similarly to other plant pathogens, might respond to both cell density and also to plant signals (Subramoni and Venturi, 2009).

We evaluated the effect of cell density and exogenous application of N-acyl homoserine lactones on these phenotypes. Moreover, the expression of key genes related with these phenotypes was also characterized by qPCR. Cell density influenced virulence, but it had only negligible effects on motility and biofilm formation. Finally, defective mutants for the cell dependent receptors (*psaR1*$^-$ and *psaR3*$^-$) and for biofilm formation (*algD*$^-$) were used to investigate their effect on motility and biofilm formation.

Cell motility and biofilm production are two social features enhancing pathogen ability to colonize the host plant. Indeed, motility is an essential feature for the invasion of new habitats (Sauer *et al*. 2002). Motility mechanisms consist of swarming, twitching, swimming and gliding (Danhorn and Fuqua, 2007) and are mediated by flagella, pila and fimbriae (Van Gerven *et al*., 2011). Bacterial motility is typically promoted at high cell densities showing peculiar characteristic when driven by QS or QS-like responses (Quiñones *et al*., 2005). In fact, swarming is typically QS-dependent, while swimming and twitching motility seem to be triggered by the physical contact between the cells and therefore are linked to a *non-social* behaviour which is only moderately affected by cell density signals (Beatson et al., 2002). Swarming motility is mediated by flagella and it is crucial in establishing contacts on plant surfaces (Fujishige *et al*. 2006). Swarming is distinct from flagella-dependent swimming motility that represents individual cell movement (Inoue et al., 2008). Moreover, twitching motility are mediated by type IV pili and appears to be used by *Pseudomonas* spp. to spread across the surface (Mattik, 2002; Klausen *et al*. 2003) and form micro-aggregates within the biofilm (Pratt and Kolter, 1999). Biofilms formation is an adaptive behaviour allowing the cells to adhere to surfaces, increasing their fitness during environmental colonization (Prakash *et al*., 2003).

Materials and methods

Bacterial species, culture conditions and bacteria quantification

The bacterial species, strains and plasmids used in this study are listed in table 1. The *Pseudomonas syringae* pv. *actinidiae* (Psa) strains belong to the high virulent biovar 3 which is responsible for the present pandemic. *Escherichia coli* and all other strains tested, including Psa wild type, mutants and silenced strain, were grown on Luria-Bertani (LB) medium (Sambrook *et al*., 1989) at appropriate temperature (37°C *E. coli*; 27°C all the other strains) under moderate shaking (120 rpm). Each actively growing culture was initiated by inoculating the appropriate sterile medium with a two days old single colony grown in LB plates. Low density cultures were obtained by inoculating 100 ml LB till bacterial growth reached a turbidity lower than OD_{600} < 0.010. High density cultures were obtained in 20 ml LB from OD_{600} > 0.100-0.200, when Psa reached the late log phase. Optical density was measures in cuvettes at 600 nm wavelength using spectrophotometer (Biochrom Libra, Cambridge UK). Bacterial population was also enumerated by direct plating 3 replicates of each 10-fold serial dilution. Colonies were counted after two days incubation.

Two Psa mutants, Psa-mR1 and Psa-mR3 (courtesy provided by dr. V. Venturi, ICGEB Trieste, IT) were used in order to elucidate the role of PsaR1 and PsaR3 receptors that responded to homoserine (Patel *et al*., 2014) in the regulation of QS-dependent phenotypes such as biofilm and motility. *In vivo* virulence was not assessed since it was already proved to be impaired by the mutations (Patel *et al*., 2014).

AHLs used in the experiments

Previous studies showed that N-acyl-homoserine lactones (AHLs) signal receptors PsaR1 and PsaR3 responded to hydroxylated AHLs and in particular to OH-C6, OH-C8, OH-C10 and OH-C12 AHLs which present an hydroxy group at position C3, whose working concentration was 1μM (Patel *et al*., 2014; Steindler et al., 2008). Therefore we applied 1μM AHLs in motility, biofilm, virulence and gene expression experiments. Relative gene expression was evaluated at low (10^5 CFU ml^{-1}) and high (10^8 CFU ml^{-1}) cell densities in order to establish if the signal perception occurred independently from the population density. Then, since OH-C6- and OH-C8-AHL were the only two signals which had significant effect on biofilm and motility induction, Psa was grown to high density in the presence of OH-C6 and OH-C8-AHL and used to assess the level of infection inoculating micropropagated plants of *Actinidia deliciosa* cv. Hayward. In addition, the effect of different dosages, lower (0.1, 0.25 and 0.5μM) or higher (10μM) than the optimal concentration of 1μM, was tested with the aim of studying the relation between the two phenotypes.

Motility assay

Motility was assessed according to (Kinscherf and Willis 1999). A sterile disk of filter paper disk (6 mm Ø) was centrally placed on a LB plates (Sigma-Aldrich, Saint Louis, St. Louis, MO, USA) containing 0.4% agar. The paper disk was inoculated with a 10μl drop of low (10^5 CFU ml^{-1}) or high density bacterial suspension (10^8 CFU ml^{-1}) adjusted to contain 1 x 10^7 cells ml^{-1}. Plates were then incubated at 27°C for 5

days. Bacterial spread and the surface of the plate covered by the colony was evaluated using the MacBiophotonics ImageJ 1.48 software (MacBiophotonics, Hamilton, ON, Canada).

To assess the influence of AHLs on Psa motility, 1 ml of different concentration of AHLs was spread on the soft LB plate till complete absorption. Once the plate dried up, the sterile paper disk was added and inoculated. Each ALH was diluted in PBS buffer till the desired concentration (0.10, 0.25, 0.50, 1.00 and 10.00 μM). For each of the different concentration of each AHL, 6 plates were prepared and the experiments were repeated three independent times.

Microscopic visualization of motility in soft agar was performed using a binocular Nikon SMZ25 fluorescence microscope (Nikon Instruments Corporation, Tokyo, JAPAN) The BHS (GHS) filterset and 40× and 60× objectives were used for imaging (excitation wavelength of GFP-B: 460-500 nm, emission wavelength: 510–560 nm). Images acquisition and processing was obtained by using the Nikon NIS ELEMENTS V. 4.30.02 software (Nikon Instruments Corporation, Tokyo, JAPAN).

Biofilm assay

The assay was performed as described by Taguchi *et al*., 2006. A volume of 3 ml of LB medium was added into a 35 mm polystyrene Petri dish and inoculated with 50 μl of a 10^8 CFU ml^{-1} fresh liquid culture of Psa.

After inoculation, plates were sealed with parafilm and incubated at 27°C with slow shaking at 70 rpm for 5 days. Successively, the bacterial population in each tube was assessed by serial dilutions and plating as previously described. Plates were thoroughly rinsed with distilled sterile water and dried for 45 min under laminar hood at room temperature. Thereafter, 3 ml of a crystal violet water solution (0.5% w/v) (Sigma-Aldrich) were added to each plate. The plates were incubated for 60 minutes at room temperature under shaking (70 rpm) and subsequently washed thoroughly with distilled water to remove nonspecific staining. For quantitative analysis of biofilms, crystal violet was re-solubilized by adding 3 ml of ethanol 95%. The solution was transferred to cuvette and absorbance at 595 nm was measured by using spectrophotometer. Psa biofilm formation was compared with the one of bacterial species known to produce biofilm (*Pseudomonas syringae* pv. *syringae* strain 4364 and *Pseudomonas fluorescens* strain A506). Aspecific staining was also quantified by with the same procedure on sterile, non-inoculated LB petri dishes. Unspecific absorbance was subtracted from all the measures. For each species and conditions 10 plates were inoculated with 50 μl of a 10^8 CFU ml^{-1} fresh liquid culture of Psa and let grow to high density for efficient cell adhesion and quantification (Ghods et al. 2015). To quantify the effect of exogenous AHLs on biofilm production, different AHL, each at different concentrations (0.10, 0.25, 0.50, 1.00 or 10.00 μM) were added to the growing medium before inoculation with Psa.

Virulence assay

The effect of cell density and application of exogenous AHLs were assessed on Psa virulence by performing *in vivo* inoculation of kiwifruit plants. Three months old micropropagated plants of *Actinidia deliciosa* cv. Hayward. The plants were grown on MS medium (Murashige and Skoog, 1962) 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}), GA3 (0.1 mg l^{-1}), adjusted to pH 5.7 with KOH. The plants were kept in a growing chamber for the whole duration of the experiments (22°C, 70% RH and a light/dark cycle of 16:8 hours). *Inocula* were prepared starting form LD and HD Psa cultures. Before the inoculation, LD e HD cultures were pelleted by centrifugation at 9000 rpm, 18°C for 10 minutes and re-suspended in sterile 10mM $MgSO_4$ in order to adjust them to the same cell concentration in the suspension (OD_{600} 0.200 corresponding approx. to 10^8 CFU ml^{-1}). The cell concentration in the suspension was successively confirmed by sequential dilutions and plating. The effect of two AHLs (OH-C6-AHL and OH-C8-AHL) was tested since PsaR1 and PsaR3 had the greatest induction after their administration (Patel *et al*., 2014). Psa was grown to a high density in LB with 1 μM of AHL added. Plants were individually inoculated by soaking them in the Psa suspension for 5 seconds. Fifteen plants were used for each treatment and divided into three repeats of three plants. At 12, 24, 72, 240 and 360 hours plant weight, symptoms and endophytic bacterial population was assessed. Each plant was ground 5 ml of sterile $MgSO_4$ (10 mM) addition, the suspension was filtered and centrifuged for 5 minutes at 14000 rpm. Bacterial pellets were frozen in liquid N_2 and stored at -80°C for subsequent molecular quantification epiphytic populations. At the same time points, plant material was frozen in liquid N_2 and stored at -80°C for subsequent molecular quantification endophytic populations.

Bioinformatics

In order to detect a possible target for Psa LuxR-solos receptors (PsaR1, PsaR2 and PsaR3), 8 gene promoters (*traI*, *cepI*, *cviI*, *rhlI*, *phzI*, *ahlI*, *ppuI* and *luxI*) were blasted PsaCFBP7286 genome. All these promoters contain *lux*-boxes that are positively regulated by the cognate LuxR-family proteins in the presence of AHL (Steindler *et al*., 2008). Promoters DNA binding boxes at position -35 and -10 were calculated for all the primers using BPROM free online software (Solovyev and Salamov, 2011) that is a software specific for bacterial promoter recognition with about 80% accuracy and specificity. The 8 promoters gene sequences are reported in table 5. The nucleotidic sequences of the promoters were blasted in PsaCFBP7286 genome using FASTA sequence similarity searching tool (EMBL-EBI, Cambridge, UK). Entry sequences always align to the forward strands present in the databank by default of the program. Therefore, when as result the reverse complement of the entry sequence aligns, it means that the sequence of interest is on the reverse strand. Standard parameters were maintained. Only the position of the alignments with a sequence identity greater than 70% were searched in PsaCFBP7286 genome using Geneious software (V. R8) (Kearse et al., 2012). In order to characterize *Tr*, a preliminary *Tr* structure prediction (fig. S1) was obtained *in silico* using I-TASSER, V.4.4 (Yang ET AL., 2015). PROSITE (Sigrist et al., 2012), Pfam (Finn et al., 2016), InterPro (Mitchell et al., 2015) and GenomeNet (Bioinformatics Center, Kyoto University, Japan) databases that include large collections of protein families were used to predict the function of Tr.

Transcriptional analysis

The effect of cell density and application of exogenous AHLs on gene expression was investigated by qPCR. A number of genes putatively responding to cell density and social behavior were selected according to the current knowledge on Psa related bacterial species. The list of selected genes is reported in table 2. The aminoacidic sequences of the reporter genes were blasted in PsaCFBP7286 genome using FASTA sequence similarity searching tool (EMBL-EBI, Cambridge, UK). Standard parameters were maintained. Only identities greater than 60% were accepted (table 2). The corresponding nucleotidic sequences in Psa genome were elaborated with Geneious software (version R8) (Kearse *et al*., 2012).

Gene expression studies were performed on a comparable amount of bacterial cells obtained from four cell densities cultures: 10^5, 10^6, 10^7, 10^8 CFU ml^{-1}. Fresh Psa cultures were centrifuged (14000rpm, 4°C, 10min) and the pellet obtained were suspended with differential amount of $MgSO_4$ (10 mM) in order to obtain the same OD_{600} 0.200 corresponding approx. to 10^8 CFU ml^{-1}. The cell concentration in the suspension was successively confirmed by sequential dilutions and plating.

Total bacterial RNA was extracted form Psa culture using Total RNA Purification kit (Norgen Biotek Corp., Thorold, CA), whereas were extracted Spectrum Plant Total RNA kit (Sigma-Aldrich) was used for Psa suspension obtained from infected plants. RNA purity and quantity was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). An aliquot of 1 μg of purified RNA was reverse-transcripted using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem Life Technologies, Carlsbad USA) according to the manufacturer's recommendations. cDNA samples were used as template for qPCR which was performed with Quick SybrGreen chemistry (Applied Biosystem) in a 96 well spectrofluorometric thermal cycler StepOnePlus (Thermo Fisher Scientific Inc.). Each template was adjusted to a final concentration of 100 ηg of cDNA per reaction and run in triplicate. qPCR cycles were performed as follows: 1 cycle of 50°C 2 min, 1 cycle of 95°C 10 min, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute Melting-curve analysis was performed immediately after completion of the real-time PCR (95°C for 15 seconds, 60°C for 15 seconds). Gene expression was expressed as relative expression to reference housekeeping genes *recA*, *rpoD* that used in *Pseudomonad* (Shi-En Lu et al., 2005; Narusaka et al., 2011; Greenwald et al., 2012) The relative quantification of gene expression was evaluated using the comparative C_t method (Pfaffl, 2001). Prior of raw C_t analysis all primers efficiency was assessed using LingRegPCR software (Ruijter *et al*. 2011). The qPCR primers developed for each gene are listed in table 3. Beacon Designer V 8,0 (PREMIER Biosoft, Palo Alto CA, USA) and Primer3Plus (Untergasser *et al*., 2007; Thornton and Basu, 2011) were used to design the primers. All primers pair were checked for specificity by end point PCR (performed as described for qPCR with Psa Genomic DNA as template).

RNA-silencing of *algD* gene

Since *algD* plays key roles in colonization process and pathogenesis and it is strictly QS-related in many *Pseudomonas syringae* species (Yu *et al*., 1999; Keith *et al*., 2003; Penaloza-Vazquez *et al*., 2010), we obtained *via* RNA silencing an *algD* silenced strain (strain name: Psa-AlgDsile) and evaluated biofilm formation and motility by the silenced strain.

To silence *algD* gene the plasmid pHN678, kindly provided by Dr. N. Nakashima was used as scaffold (Nakashima and Tamura, 2009). Following the author's specifications, the plasmid was digested with the appropriate enzymes (NEB, New England Biolabs, Massachusetts, USA) (table 4). *algD* was amplified with complementary restriction site at 5' and 3' ends (primers table 2), followed by amplicons purification using the QIAquick Gel Extraction Kit (Qiagen, Redwood City, USA) according to the manufacturer's instructions. The purified amplicon was digested as described above. Linearized vector and the purified amplicon were checked for purity and digestion on a 1% agarose gel stained with GelRED (Biotium Inc., Hayward, USA). The linearized vector and the amplicon (1:6 ratio) were ligated in a volume of 15 µl with Quick Ligation Kit (NEB). An aliquot of 2 µl was used to transform electrocompetent *E.coli* DH5α cells. Transformed cells were plated on selective LB amended with chloramphenicol (25µg/ml). The presence of the plasmid inside the growing colonies was confirmed by sequencing. The plasmid pHN678-AlgD was extracted and purified from the selected colonies by using a QIAprep minipre kit (Qiagen). Plasmid quantity and purity was verified by NanoDrop spectrophotometer and agarose gel analysis. The purified plasmid was used to transform by electroporation competent Psa-CFBP7286 cells as described above. The efficacy of the silencing was tested on transformed Psa-AlgDsile cultures grown on liquid LB amended with 0.5% IPTG for plasmid expression. From these cultures, RNA was (i) extracted, (ii) purified using total RNA extraction KIT (Sigma-Aldrich), (iii) reverse-transcripted to cDNA by using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.) (iv) and amplified with gene specific primers.

Psa algD silenced strain was preliminary tested in biofilm and motility assays.

Statistical analysis

Each experiment was independently repeated at least twice. Significance of correlations was assessed with Fisher's exact test, assuming a confidence level of 0.1 or 0.05. Where indicted, difference between data are calculated with the Student's *t* test. ANOVA and SNK test were applied to gene expression analysis data. Statistically significant differences were assumed for $P \leq 0.05$.

Results

Identification of a putative LuxR dependent transcriptional factor

Through a bioinformatics research we looked on PsaCFBP7286 whole genome for possible targets for Psa LuxR-solos receptors (PsaR1, PsaR2 and PsaR3) that responded to 8 homoserine-lactones gene promoters (*luxI*, *cviI*, *ahlI*, *rhlI*, *cepI*, *phzI*, *traI* and *ppuI*) (Patel et al., 2014). Most of the alignments were discarded because or too far from a gene or were positioned inside gene sequences. Out of a total of about 300 alignments one promising homology from the blast of *P. syringae phaseolica* 1448A (Joardar *et al*.,2005) *ahlI* promoter sequence was found with 72.4% of identity 135 base pairs (bp) upstream a 450bp long putative transcriptional regulator coding sequence, here named *Tr*. The result of the alignment is represented in table 6. In the supplementary information *Tr* promoter boxes position confirmed by BPROM analysis (table S1) and *in silico* structure prediction using I-TASSER (fig. S1) are presented.

One of the closest genes to *Tr* is a *lysR*-type transcriptional regulator that belong to a family of proteins (LTTRs) that regulate a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility (Maddocks and Oyston, 2008). According to PROSITE, Pfam and InterPro databases, the *Tr* harbours one DNA binding domain related to cro/C1-type helix-turn-helix domain (HTH) (Steinmetzer et al., 2002). The HTH DNA-binding motif is generally 50-60 residue long, located in the N-terminal part, and involved in DNA-binding into the major groove, where the recognition helix makes most DNA-contacts. Moreover, GenomeNet highlighted that another functional domain predicted at Tr N-terminus resulted to belong to MqsA antitoxin family which plays an essential role in motility, biofilm regulation and cell signalling in *E. coli* (Ren et al., 2004; Barrios et al., 2006).

Cell density perception

Motility

Motility was evaluated at different cell densities. Colonies from low density *inocula* were obtained from a 10^5 CFU ml^{-1} and displayed only non-social swimming motility (100% of the colonies). Whereas swarming, swimming and twitching motilities were differentially showed by colonies derived from high density *inocula* (10^8 CFU ml^{-1}) (fig. 1). In swarming motility, the colonies showed characteristic branching edges (fig. 1a), whereas in swimming motility the colony substantially expanded without forming branching edges, but showing subtle bacterial streams inside the expanding zone (fig. 1b). Finally, twitching motility was characterized by the formation on numerous offspring microcolonies (fig. 1c). Colonies often showed more than one type of motility at the same time (fig. 1d). *P. syringae syringae* 4364 and *P. fluorescence* A506, two bacterial strains known to show a strong cell density dependent motility, were used as comparison (Table 7). In Pss4364 and PfA506 swarming motility was the only observed, while in Psa, the swarming motility was observed only in about the 15% of the colonies.

Biofilm production

In comparison to *P. syringae syringae* 4364 and *P. fluorescence* A506, which form a relevant amount of biofilm, Psa CFBP7286 showed little biofilm production (fig. 2).

Virulence

Regardless from the fact that plants were infected by using *inocula* with the same cell concentration, the cell density in the starting culture influenced PsaCFBP7286 virulence (fig. 3). Starting from 10 days after inoculation, in plants inoculated with a bacterial suspension originated by an HD culture (10^8 CFU ml^{-1}) both epiphytic and endophytic populations were significantly higher than in plants inoculated with cell from a LD culture (10^5 CFU ml^{-1}) (fig. 3). Epiphytic population in HD inoculated plants remained constant during the whole experiment, while in LD inoculated ones it sharply decreased starting from 10 days after inoculation (fig. 3a). Endophytic population was below the detection level for the first 24 hours after inoculation, thus showing that infection was at the very early stage and no invasion of the host tissues occurred. Between 3 and 15 days post inoculation, Psa population inside the host plants constantly increased. At 10 and 15 days, plants inoculated with cells derived from HD cultures harboured a significantly higher Psa population in comparison to the ones inoculated with cell derived from LD cultures (fig. 3b). At each time point, symptoms were evaluated before processing the plants, but not visible spots or exudates were observed.

Gene expression

Analysis of density-related genes expression by qPCR revealed that *aefR* (epiphytic fitness regulator), *fur* (virulence, pila and QS regulator) and *Tr* were moderately induced by the increase in cell density (fig. 4a). Acylases genes (*hacA, hacB, hacC*) and *psaR1*, *psaR2* and *psaR3* receptors were not expressed at any of the tested cell densities. Also *gacS/gacA* two-component system, was not expressed.

The analysis of motility-related genes showed a moderate expression at all the tested cell density (fig. 4b). rpoN and flip expression were slightly increased by the two highest cell densities (fig. 4b).

Concerning biofilm-related genes, *algD*, *clpP* and *wssB* expression were induced by the two highest cell densities (fig. 4c). *mdoH* and *wspR* showed the highest expression in colture with a concentration of 10^7 CFU ml^{-1}.

Virulence related genes were all moderately expressed during the growth on artificial medium (fig. 4d). Nonetheless, the increase in the cell concentration in the culture increased the expression of all genes with the exception of *paMTAda* and *oprM* (fig. 4d). The enolase gene was highly induced in culture with a cell concentration of 10^7 CFU ml^{-1}.

Perception of exogenous AHLs

The effect of exogenous application of OH-C6, OH-C8, OH-C10 and OH-C12 AHLs on Psa behaviors was studied. Moreover, for the AHLs inducing the most relevant effects, a dose-effect study was performed.

Effect of different AHLs on motility, biofilm formation and virulence

The effect of AHLs on motility depended on the signal administered. In particular, swarming motility was enhanced by the application of OH-C6 and OH-C8 AHLs, rather than OH-C10 and OH-C12 (table 8). The number of twitching colonies in AHLs were comparable to the control with no signal added, with the exception of the treatment with OH-C6. When we evaluated biofilm production in the presence of AHLs, the greatest induction derived only from the treatment with OH-C6 (fig. 5).

Since OH-C6- and OH-C8-AHLs greatly induced swarming motility and OH-C6 affected biofilm formation, and also because in previous studies these two AHLs at 1μM determined the greatest induction of PsaR1 and PsaR3 signal receptors (Patel et al., 2014), they were chosen also to test the effect of exogenous AHLs on virulence. OH-C6- and OH-C8-AHLs did not influence the epiphytic growth of Psa (fig. 6). Endophytic populations of Psa treated with OH-C6 and OH-C8 in the first 240 hours were less than untreated Psa. However, OH-C6 treated Psa reached the same population after 360 hours, while OH-C8 treated Psa still resulted significantly less than the untreated control.

We also investigated the dose effect of OH-C6- and OH-C8-AHL on biofilm production and motility in order to understand how biofilm and motility phenotypes were related. The two molecules showed comparable dose-effect performances on motility and biofilm production (fig. 7 and 8). Increasing concentration of AHLs induced swarming motility, being the concentration of 1μM the most effective (fig. 7). The opposite effect was observed on biofilm formation. In this case, the highest induction was observed at the lowest concentrations (0.1 - 0.25μM), while the higher concentration did not influence biofilm in comparison to the untreated control (fig. 8).

Gene expression in the presence of AHLs at high and low cell density

The effect of exogenous application of OH-C6-, OH-C8-, OH-C10- and OH-C12-AHLs on Psa gene expression was also tested. All AHLs were applied at a concentration of 1μM, which it is a typical subsaturating concentration of AHL. The relative gene expression was evaluated in two conditions: at low (LD, 10^5 CFU ml^{-1}) and high (HD, 10^8 CFU ml^{-1}) cell densities in order to establish if the signal perception depended on the population density.

Concerning density related genes, OH-C8 and OH-C12 caused a significant expression of *fur*, *gacS*/*gacA* and acylases (*hacA, hacB, hacC*), both at low and high cell densities (fig. 9a-b). OH-C6-AHL was the only molecule inducing the receptors *psaR1* and *psaR3*, but only at high cell density (fig. 9b). *Tr* expression was not significant.

As far as motility-related genes is concerned, the flagellar protein *flip* resulted particularly expressed only in the presence of OH-C8-AHL. However, *pilA* and *pilO* genes were induced by OH-C8 and OH-C12 at LD, and by OH-C6, OH-C8 and OH-C12 in HD conditions (fig. 10a-b). No effect on *pilC* was observed.
Concerning biofilm-related genes, only *algD* and *mdoH* seemed to be activated at LD by OH-C8 and OH-C12 respectively (fig. 11a). At high density, AHLs application did not influence the expression of biofilm-related genes (fig. 11b). *wssB* relative expression in all treatments with AHLs resulted inhibited.
Virulence genes are expressed in AHLs both at high and low density (fig. 12a-b). The most active AHLs are the OH-C8 and OH-C12. A summary of the most significant inductions is represented in table 9.

Effect of psaR1 and psaR3 mutation on AHLs perception

In *Pseudomonas syringae* pv. *actinidiae* PsaR1 and PsaR3 are known to respond to AHLs (Patel et al., 2014), thus the ability of these receptors to perceive AHLs was tested in *in vitro* assays. Since in our experiments motility and biofilm formation were the two phenotypes responding to AHLs application, the research concentrated on the effect of *psaR1* and *psaR3* mutation on these two phenotypes.
All the AHLs at 1μM stimulated motility in the wild type strain. In Psa-mR1 OH-C8, OH-C6 and OH-C12 AHLs were the signals that induced the swarming phenotype (table 10). No significant effects of the addition of AHLs were registered by the mutant Psa-mR3. In fact, the deletion of *psaR3* gene resulted in a total lack of expression of swarming phenotype, except for OH-C10 that was comparable to the wild type.
The biofilm was tested at the optimal inducing concentration of 0.25μM with OH-C6 and OH-C8, the two AHLs that induced swarming motility and biofilm formation in PsaCFPB7286 wild type (fig. 13). Biofilm was stimulated by both AHLs in the mutant deleted of *psaR3* signal receptor gene, whereas it was inhibited in Psa-mR1 mutant.

Psa-AlgDsile silenced strain phenotyping bioassays

algD plays key roles in colonization process and pathogenesis (Yu *et al*., 1999; Keith *et al*., 2003; Penaloza-Vazquez *et al*., 2010). Moreover, *algD* relative expression of *Pseudomonas syringae* pv. *actinidiae* resulted to be density related (fig. 4c). Here we report our preliminary results on motility and biofilm formation in order to outline the importance of this gene in Psa.
As far as motility is concerned, the colonies of Psa-AlgDsile showed a greater expansion due to swarming motility (fig. 14 a-b). Moreover, the *algD*$^{-}$ silenced strain always produced compact colonies with dendritic edges (swarming), but not floccular formations typical of the twitching (fig. 18).
Concerning biofilm production, the *algD*$^{-}$ silenced strain produced almost 5 time less biofilm than the wild type strain (fig. 15).

Discussion

Psa exploited both swarming and swimming motilities

The results demonstrated that Psa is capable of motility forms such as swimming and swarming, presumably as result of the contact between the cells or the production of yet indefinite signals respectively. This suggested that Psa would perceive its own population, perhaps as consequence of substances released in the media that could play a role in regulating the motility. Analysis of the motility-related genes at different cell densities revealed that their weak expression can find an explanation in the bioassay, where movement of Psa resulted very mild compared to canonical motility strains (Pss4364 and PfA506) that swarmed greatly.

Psa in liquid cultures produced low amounts of biofilm

When the ability of forming biofilms in LB liquid culture on plastic surface was tested, we observed that Psa produced low amounts of biofilm. We also evaluated typical biofilm stimulant media, such as HSg-CF (Penaloza-Vazquez *et al*., 2010) or MMMF (O'Toole and Kolter, 1998) but the results were not significantly different (data not shown). This should not be surprising: in fact, in a study conducted on several *Pseudomonas* spp., the environmental isolates produced more biofilms than plant pathogens strains that typically produced fewer biofilms instead (Ude *et al.*, 2006). However, the greatest expression of Psa genes related to cellular aggregation and biofilm biosynthesis was detected only at high cell densities. Therefore, we can assume that Psa exploits mechanisms of perception of the community to induce "social" behavior such as the formation of biofilm. However, we have also to take into consideration that Psa possesses at least two signal receptors for bacterial signals (PsaR1, PsaR3) and one for a plant signal (PsaR2). Therefore, these *in vitro* tests, where we considered Psa on its own, may be implemented with experiments where Psa interacts with other bacteria or the plant host in order to verify if biofilm formation in this pathogen is also regulated by external stimuli.

Density does not affect QS-related genes expression

Through the analysis of gene expression at different cell densities we attempted to study traits of the intraspecific communication, with a focus on the quorum-sensing mechanisms. In literature, the density thresholds at which the signal is perceived by the population vary depending on the bacterial species (Fuqua *et al*., 1996; Kabir *et al*., 2010). For example, *P. syringae* pv. *syringae* (Pss) triggers a coordinated expression of specific target genes when the quorum is reached at 10^7 CFU ml^{-1} (Quiñones *et al*., 2005). Thus, we analysed four cell densities of liquid cultures of Psa, covering a range that goes from 10^5 up to 10^8 CFU ml^{-1}. Our results showed that very little resulted density-dependent. In particular, In fact, only *fur* and *aefR* expressed at high cell density (10^7-10^8). This can be due by the fact that these two regulators, other than quorum-sensing, are involved in key events of colonization stages: *aefR* is a regulator of EPS production, oxidative stress tolerance, disease development and swarming motility in *Pss* (Quiñones *et al*., 2005), whereas *fur* is involved in the regulation of iron uptake, storage genes, virulence and protection against

oxidative stress in *Pseudomonas syringae* pv. *tomato* (Butcher *et al*., 2011). Moreover, the lack of expression of *gacS*/*gacA* in Psa would indicate that this regulatory system, that in other *Pseudomonads* regulates the synthesis of *N*-acyl-homoserine lactone (Chancey *et al*., 1999; Kitten and Willis, 1996), but in several species responds to environmental stimuli (Heeb and Haas, 2001) such as small organic molecules (Koch *et al*. 2005), could be dedicated to the signal transmission at more complex levels of communication signals (i.e. Psa-host, Psa-bacteria).

Interestingly, when in the growing media AHLs are added, the gene expression revealed that the signals are recognized and trigger, both at LD and HD, the expression of signal acilases (*hacA*, *hacB, hacC*), *fur* and the two component system *gacS*/*gacA*, indicating a "priming effect" of AHLs independently to population density.

Virulence is affected by cell density

Microbial virulence has been often associated with group behavior and with signaling systems, for example when at high cell densities the signal accumulates and triggers the activation of the quorum-sensing. In our experiments where we compared *in vivo* the virulence of two inoculums harvested at low (LD) and high (HD) cell densities, the epiphytic colonization of an LD inoculum suffered the biggest drop after 10 days. Also, the LD endophytic populations resulted slightly, but statistically significative, lower than the HD ones. Perhaps this was due to the intraspecific signaling that did not occur in the LD populations. In fact, we can suppose that an HD culture experienced the perception of the community and therefore a more effective armament. These results are in agreement with the expression of virulence genes of planktonic cultures of Psa at different cell densities: the fact that at LD the genes resulted poorly induced, and that this induction increased with the increase of the density would confirm that the community perception somehow triggered the virulence. How this perception takes place is still under investigation.

Motility and biofilm were differentially affected by AHLs

Swarming motility resulted enhanced when external signals (AHLs) were provided to the media of the plates, especially at high concentrations. On the other hand, biofilm production was particularly stimulated by OH-C6- and OH-C8-AHL when low concentrations were administered to the media. Acilases genes, that resulted inducted by the presence of AHLs, may be involved in this mechanisms by degrading long-chain AHLs, thus making available shorter C6 and C8 chains. Comparing these results, it would appear that if the biofilm is induced by low concentrations of signals, motility is not. Vice-versa, when the AHLs are at a concentration such as to induce motility, biofilm production is comparable to the control with no signals added. Therefore, it seems that biofilm and motility are affected by the signal concentration and they would mutually exclude each other (Caiazza *et al*., 2007). Moreover, the results of the bioassay were confirmed by the addition of AHLs to liquid cultures of Psa whose gene expression was examined at low and high cell densities. In fact, if the AHLs had no or little effect on biofilm genes expression, they enhanced motility gene

expression at both densities tested. In conclusion, the administration of signaling molecules appeared to regulate especially the motility and this is confirmed both from *in vitro* results and gene profiles.
However, the inverse regulation of motility and biofilm could have an interesting ecological significance. Psa would be able to perceive the AHLs from the environment with the aim to sense who is close by. Since the signal is perceived when it is produced by large amount of cells, its perception would trigger the motility in order increase chance of survival and reduce the effects of the competition for space and nutrients. In the case that the signal is little or nothing, Psa would register this information as there are no competitors around, and consequently stimulates biofilm formation in order to enlarge the colonies and enable the pathogenesis.

Virulence is not affected by AHLs

Bacterial virulence may be triggered by intracellular signalling, resulting to be associated to quorum-sensing mechanisms such as the production and perception of communication signals (Holden *et al*., 1999). Interestingly, when we evaluated the effects of OH-C6- and OH-C8-AHL on triggering virulence of Psa towards *A. deliciosa* microcuttings, it was found that the AHLs had no effect on virulence (OH-C8 even reduced the endophytic population). Furthermore, if we consider the virulence-gene expression in the presence of AHLs had no or little effect both at high and low cell density, we can conclude that the signalling mediated by AHLs is not able to influence the virulence of Psa, at least not in a direct way. However, the concentration of AHLs administered prior inoculation may have influenced the test result. Further investigation is needed to ascertain the effect on virulence of differential concentrations of AHLs.

Role of PsaΔR1 and PsaΔR3 receptor

When PsaΔR1 and PsaΔR3 mutants were tested for biofilm production with or without AHLs added, it resulted a greater production than the wild type also without the signal. Moreover, the two mutants grown in addition of AHLs were found not statistically different, indicating that these receptors are relatively involved in the manifestation of this phenotype. Instead, the motility was completely different in the two mutants: the deletion of PsaR3 receptor resulted in a non-motile mutant, even when signal was added. On the other hand, deletion of PsaR1 and addition of AHLs have resulted in a stimulation both of swarming and swimming motility (PsaΔR1 mutant manifested motility also in PBS, but the AHLs accentuated the phenotype). We can conclude that PsaR1 would act as repressor and PsaR3 would instead favour motility by binding to OH-C6-, OH-C8- and OH-C12-AHL. Therefore, motility of *Psa* could rely on a fine regulation mediated by both receptors: the moment when one of the two is repressed, the adjustment is biased towards a motile or non-motile phenotype depending on PsaR1 or PsaR3 is missing respectively.

algD is involved in biofilm and motility

Our first evaluations using an AlgD mutant strain revealed that *algD* is implicated directly or indirectly in the regulation of motility and biofilm phenotypes. We can suppose that in the wild type, *algD* would have a

positive effect on the biofilm formation and, at the same time, a negative effect on the expression of motility. These results also confirm that biofilm and motility in Psa are two phenotypes that operate in a mutual exclusion. Further investigations of virulence *in vivo* will ensure its role in virulence, and QPCR analysis will elucidate which genes are potentially affected.

Conclusions

This study that aimed at investigating aspects of Psa intraspecific communication system, highlighted aspects entirely innovative. At the present time we do not know whether or not Psa produces its own communication signal that of course differ from AHLs but would work with a similar principle by activating community behaviours. However, Psa seemed to be able to perceive itself, even if in a slightly manner and to respond with well-defined phenotypes, in particular moving, producing biofilms, but also activating virulence in a density-dependent manner. These aspects would suggest that the tested phenotypes, which are typical of the QS-circuits and therefore of the intraspecific communication, could be induced in another way, different from the homoserine-lactones. It is not yet possible to exclude that Psa communication is based con cell-cell contact, thus the answers to population density are not mediated by signal molecules.

As experimentally observed, after administration of homoserine-lactones, made us assume that Psa is able to perceive the canonical QS-signals, the AHLs, and that some of these signals appeared to influence both the motility and the biofilm, with a peculiar mutual exclusion effect. Motility and biofilm, that ensure the success of the dissemination and environmental colonization, would result to be tuned according to the perceived environmental conditions and could be potentially influenced by interspecific interactions. The effect of QS-signals on virulence resulted surprisingly poor both from gene expression and *in vivo* observations.

In conclusion, it has been defined that Psa possesses the machinery for typical bacterial community behaviours and also that this pathogen is able to respond to external synthetic signals. Hence, we can suppose that external signals produced by other bacteria or the plant host can be perceived and therefore trigger in Psa important traits of host colonization and invasion. These hypothesis will be the object of further investigations.

Reference

Appleby, Jeryl L., John S. Parkinson, and Robert B. Bourret. 1996. “Signal Transduction via the Multi-Step Phosphorelay: Not Necessarily a Road Less Traveled.” *Cell* 86(6):845–48.

Argenio, David a D. et al. 2002. “Autolysis and Autoaggregation in.” *Microbiology* 184(23):6481–89.

Balestra, G. M. et al. 2009. “Increased Spread of Bacterial Canker of Kiwifruit in Italy.” *Informatore Agrario* 65(24):58–60.

Barrios, G. et al. 2006. “Autoinducer 2 Controls Biofilm Formation in Escherichia Coli through a Novel Motility Quorum-Sensing Regulator (MqsR, B3022).” *Journal of Bacteriology* 188(1):305–16.

Beatson, Scott a, Cynthia B. Whitchurch, B. T. Annalese, John S. Mattick, and Annalese B. T. Semmler. 2002. “Quorum Sensing Is Not Required for Twitching Motility in Pseudomonas Aeruginosa Quorum Sensing Is Not Required for Twitching Motility in Pseudomonas Aeruginosa.” *Journal of Bacteriology* 184(13):3598–3604.

von Bodman, Susanne, Dietz Bauer, and David Coplin. 2003. “Quorum Sensing in Plant-Pathogenic Bacteria.” *Annual Review of Phytopathology* 37(1):473–91. Retrieved (http://www.annualreviews.org/doi/abs/10.1146/annurev.phyto.37.1.473).

Butcher, Bronwyn G. et al. 2011. “Characterization of the Fur Regulon in Pseudomonas Syringae Pv. Tomato DC3000.” *Journal of Bacteriology* 193(18):4598–4611.

Di Cagno, Raffaella, Maria De Angelis, Maria Calasso, and Marco Gobbetti. 2011. “Proteomics of the Bacterial Cross-Talk by Quorum Sensing.” *Journal of Proteomics* 74(1):19–34. Retrieved (http://dx.doi.org/10.1016/j.jprot.2010.09.003).

Caiazza, Nicky C., Judith H. Merritt, Kimberly M. Brothers, and George A. O’Toole. 2007. “Inverse Regulation of Biofilm Formation and Swarming Motility by Pseudomonas Aeruginosa PA14.” *Journal of Bacteriology* 189(9):3603–12.

Cha, Ji Young, Jun Seung Lee, Jeong Il Oh, Jang Won Choi, and Hyung Suk Baik. 2008. “Functional Analysis of the Role of Fur in the Virulence of Pseudomonas Syringae Pv. Tabaci 11528: Fur Controls Expression of Genes Involved in Quorum-Sensing.” *Biochemical and Biophysical Research Communications* 366(2):281–87.

Chancey, S. T. and D. W. Wood. 1999. “Two-Component Transcriptional Regulation of N -Acyl-Homoserine Lactone Production in Pseudomonas Aureofaciens.” 65(6):2294–99.

Danhorn, Thomas and Clay Fuqua. 2007. “Biofilm Formation by Plant-Associated Bacteria.” *Annual Review of Microbiology* 61(1):401–22. Retrieved (http://www.annualreviews.org/doi/abs/10.1146/annurev.micro.61.080706.093316).

Donati, Irene et al. 2014. “New Insights on the Bacterial Canker of Kiwifruit (Pseudomonas Syringae Pv. Actinidiae).” *Journal of Berry Research* 4(2):53–67.

Finn, Robert D. et al. 2015. “The Pfam Protein Families Database: Towards a More Sustainable Future.” *Nucleic acids research* gkv1344. Retrieved (http://nar.oxfordjournals.org/content/early/2015/12/15/nar.gkv1344.full).

Fujishige, Nancy A., Neel N. Kapadia, Peter L. De Hoff, and Ann M. Hirsch. 2006. “Investigations of Rhizobium Biofilm Formation.” *FEMS Microbiology Ecology* 56(2):195–206.

Fuqua, Clay and E. Peter Greenberg. 2002. “Listening in on Bacteria: Acyl-Homoserine Lactone Signalling.” *Nature reviews in Molecular cell biology* 3(September):685–95.

Fuqua, Clay, Stephen C. Winans, and E. Peter Greenberg. 1996. “CENSUS AND CONSENSUS IN BACTERIAL ECOSYSTEMS: The LuxR-LuxI Family of Quorum-Sensing Transcriptional Regulators.” *Annu. Rev. Microbiol* 50:727–51.

Van Gerven, Nani, Gabriel Waksman, and Han Remaut. 2011. *Pili and Flagella: Biology, Structure, and Biotechnological Applications*.

Ghods, Shirin, Ian M. Sims, M. Fata Moradali, and Bernd H. A. Rehma. 2015. “Bactericidal Compounds Controlling Growth of the Plant Pathogen Pseudomonas Syringae Pv. Actinidiae, Which Forms Biofilms Composed of a Novel Exopolysaccharide.” *Applied and Environmental Microbiology* 81(12):4026–36.

Greenwald, Jessica W., Charles J. Greenwald, Benjamin J. Philmus, Tadhg P. Begley, and Dennis C. Gross. 2012. “RNA-Seq Analysis Reveals That an ECF ?? Factor, AcsS, Regulates Achromobactin Biosynthesis in Pseudomonas Syringae Pv. Syringae B728a.” *PLoS ONE* 7(4).

Guan, Rong et al. 2012. “Methylthioadenosine Deaminase in an Alternative Quorum Sensing Pathway in Pseudomonas Aeruginosa.” *Biochemistry* 51(45):9094–9103.

Heeb, Stephan and Dieter Haas. 2001. “Regulatory Roles of the GacS / GacA Two-Component System in Plant-Associated and Other Gram-Negative Bacteria.” *Molecular Plant-Microbe Interactions* 14(12):1351–63.

Hentzer, Morten and Michael Givskov. 2003. “Pharmacological Inhibition of Quorum Sensing for the Treatment of Chronic Bacterial Infections.” *J. Clin. Invest.* 112(9):1300–1307.

Holden, Matthew T. G. et al. 1999. “Quorum-Sensing Cross Talk: Isolation and Chemical Characterization of Cyclic Dipeptides from Pseudomonas Aeruginosa and Other Gram-Negative Bacteria.” *Molecular Microbiology* 33(6):1254–66.

Inoue, Tetsuyoshi, Ryuji Shingaki, and Kazuhiro Fukui. 2008. “Inhibition of Swarming Motility of Pseudomonas Aeruginosa by Branched-Chain Fatty Acids.” *FEMS Microbiology Letters* 281(1):81–86.

Joardar, Vinita et al. 2005. “Whole-Genome Sequence Analysis of.” *Society* 187(18):6488–98.

Kabir, Ahmad Humayan;, Anindya; Ghosh Roy, Mohammad; Firoz Alam, and Rafiul Islam. 2010. “Detection of Quorum Sensing Signaks in Gram-Negative Bacteria by Using Reporter Strain CV206.” *Notulae Scientia Biologicae* 2(4):72–75.

Kalia, Vipin Chandra. 2013. “Quorum Sensing Inhibitors: An Overview.” *Biotechnology Advances* 31(2):224–45. Retrieved (http://dx.doi.org/10.1016/j.biotechadv.2012.10.004).

Kawakita, Yuichiro et al. 2012. “Characterization of Each aefR and mexT Mutant in Pseudomonas Syringae Pv. Tabaci 6605.” *Molecular Genetics and Genomics* 287(6):473–84.

Kearse, Matthew et al. 2012. “Geneious Basic: An Integrated and Extendable Desktop Software Platform for the Organization and Analysis of Sequence Data.” *Bioinformatics* 28(12):1647–49.

Keith, Ronald C., Lisa M. W. Keith, Gustavo Hernández-Guzmán, Srinivasa R. Uppalapati, and Carol L. Bender. 2003. “Alginate Gene Expression by Pseudomonas Syringae Pv. Tomato DC3000 in Host and Non-Host Plants.” *Microbiology* 149(5):1127–38.

Kinscherf, Thomas G. and David K. Willis. 1999. “Swarming by Pseudomonas Syringae B728a Requires gacS (lemA) and gacA but Not the Acyl-Homoserine Lactone Biosynthetic Gene ahlI.” *Journal of Bacteriology* 181(13):4133–36.

Klausen, Mikkel et al. 2003. “Biofilm Formation by Pseudomonas Aeruginosa Wild Type, Flagella and Type IV Pili Mutants.” *Molecular Microbiology* 48(6):1511–24.

Koch, B. et al. 2005. “The LuxR Receptor: The Sites of Interaction with Quorum-Sensing Signals and Inhibitors.” *Microbiology* 151(11):3589–3602.

Koh, Y. J., G. H. Kim, J. S. Jung, Y. S. Lee, and J. S. Hur. 2010. “Outbreak of Bacterial Canker on Hort16A (Actinidia Chinensis Planchon) Caused by Pseudomonas Syringae Pv . Actinidiae in Korea.” *New Zealand Journal of Crop and Horticultural Science* 38(4):275–82.

Lu, Shi-En, Nian Wang, Jianlin Wang, Z. Jeffrey Chen, and Dennis C. Gross. 2005. “Oligonucleotide Microarray Analysis of the salA Regulon Controlling Phytotoxin Production by Pseudomonas Syringae Pv. Syringae.” *Molecular plant-microbe interactions : MPMI* 18(4):324–33.

Maddocks, Sarah E. and P. C. F. Oyston. 2008. “Structure and Function of the LysR-Type Transcriptional Regulator (LTTR) Family Proteins.” *Microbiology* 154(12):3609–23.

Marcelletti, Simone, Patrizia Ferrante, Milena Petriccione, Giuseppe Firrao, and Marco Scortichini. 2011. “Pseudomonas Syringae Pv. Actinidiae Draft Genomes Comparison Reveal Strain-Specific Features Involved in Adaptation and Virulence to Actinidia Species.” *PLoS ONE* 6(11).

Martin, Paul R., Alison A. Watson, Thomas F. McCaul, and John S. Mattick. 1995. “Characterization of a Five-Gene Cluster Required for the Biogenesis of Type 4 Fimbriae in Pseudomonas Aeruginosa.” *Molecular Microbiology* 16(3):497–508.

Mattick, John S. 2002. “Type IV Pili and Twitching Motility.” *Annual review of microbiology* 56:289–314. Retrieved (http://www.ncbi.nlm.nih.gov/pubmed/12142488\nhttp://www.annualreviews.org/doi/abs/10.1146/annurev.micro.56.012302.160938).

McCann, Honour C. et al. 2013. “Genomic Analysis of the Kiwifruit Pathogen Pseudomonas Syringae Pv. Actinidiae Provides Insight into the Origins of an Emergent Plant Disease.” *PLoS Pathogens* 9(7).

Miller, Melissa B. and Bonnie L. Bassler. 2001. “Quorum Sensing in Bacteria.” *Annual Review of Microbiology*.

Mitchell, Alex et al. 2015. “The InterPro Protein Families Database: The Classification Resource after 15 Years.” *Nucleic Acids Research* 43(D1):D213–21.

Murashige, T. and F. Skoog. 1962. “A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures.” *Physiologia plantarum* 15:473–97. Retrieved (http://onlinelibrary.wiley.com/doi/10.1111/j.1399-3054.1962.tb08052.x/full).

Nakashima, Nobutaka and Tomohiro Tamura. 2009. “Conditional Gene Silencing of Multiple Genes with Antisense RNAs and Generation of a Mutator Strain of Escherichia Coli.” *Nucleic Acids Research* 37(15).

Narusaka, Mari, Tomonori Shiraishi, Masaki Iwabuchi, and Yoshihiro Narusaka. 2011. “rpoD Gene Expression as an Indicator of Bacterial Pathogens in Host Plants.” *Journal of General Plant Pathology* 77(2):75–80.

O’Toole, G. A. and R. Kolter. 1998. “Initation of Biofilm Formation in Pseudomans Fluorescens WC365 Proceeds via Multiple, Convergent Signalling Pathways: A Genetic Analysis.” *Molecular Microbiology* 28:449–61.

Patel, Hitendra Kumar et al. 2014. “The Kiwifruit Emerging Pathogen Pseudomonas Syringae Pv. Actinidiae Does Not Produce AHLs but Possesses Three luxR Solos.” *PLoS ONE* 9(1).

Penaloza-Vazquez, Alejandro, Aswathy Sreedharan, and Carol L. Bender. 2010. “Transcriptional Studies of the hrpM/opgH Gene in Pseudomonas Syringae during Biofilm Formation and in Response to Different Environmental Challenges.” *Environmental Microbiology* 12(6):1452–67.

Pernestig, Anna Karin, Öjar Melefors, and Dimitris Georgellis. 2001. "Identification of UvrY as the Cognate Response Regulator for the BarA Sensor Kinase in Escherichia Coli." *Journal of Biological Chemistry* 276(1):225–31.

Pfaffl, M. W. 2001. "A New Mathematical Model for Relative Quantification in Real-Time RT-PCR." *Nucleic acids research* 29(9):e45. Retrieved (http://www.ncbi.nlm.nih.gov/pubmed/11328886).

Phan, Gilles et al. 2010. "Structural and Dynamical Insights into the Opening Mechanism of P. Aeruginosa OprM Channel." *Structure* 18(4):507–17.

Prakash, B., B. M. Veeregowda, and G. Krishnappa. 2003. "<Biofilms- A Survival Strategy of Bacteria.pdf>." 85(9).

Pratt, L. a and R. Kolter. 1999. "Genetic Analyses of Bacterial Biofilm Formation." *Current opinion in microbiology* 2(6):598–603.

Quiñones, Beatriz, Glenn Dulla, and Steven E. Lindow. 2005. "Quorum Sensing Regulates Exopolysaccharide Production, Motility, and Virulence in Pseudomonas Syringae." *Molecular plant-microbe interactions : MPMI* 18(7):682–93.

Ren, D., L. A. Bedzyk, S. M. Thomas, R. W. Ye, and T. K. Wood. 2004. "Gene Expression in Escherichia Coli Biofilms." *Applied Microbiology and Biotechnology* 64(4):515–24.

Rezaei, Afsaneh et al. 2011. "Original Molecular Screening of Anti-Quorum Sensing Capability of Salvadora Persica on Enterococcus Faecalis." *Journal of Hard Tissue Biology* 20(2):115–214.

Ruijter, J. M., S. van der Velden, and A. Ilgun. 2011. "LinRegPCR (11.0) - Analysis of Quantitative RT-PCR Data." *Heart Failure Research Center* 1–29. Retrieved (papers://476b58c2-a19a-46f3-a8ef-897be4da28ce/Paper/p17039).

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.*

Sauer, Karin et al. 2002. "Pseudomonas Aeruginosa." *Society* 184(4):1140–54.

Segura, A., E. Duque, A. Hurtado, and J. L. Ramos. 2001. "Mutations in Genes Involved in the Flagellar Export Apparatus of the Solvent-Tolerant Pseudomonas Putida DOT-T1E Strain Impair Motility and Lead to Hypersensitivity to Toluene Shocks." *Journal of Bacteriology* 183(14):4127–33.

Sheng, Li, Dan Gu, Qiyao Wang, Qin Liu, and Yuanxing Zhang. 2012. "Quorum Sensing and Alternative Sigma Factor RpoN Regulate Type VI Secretion System I (T6SSVA1) in Wsh Pathogen Vibrio Alginolyticus." *Archives of Microbiology* 194(5):379–90.

Shepherd, Ryan W. and Steven E. Lindow. 2009. "Two Dissimilar N-Aeyl-Homoserine Lactone Acylases of Pseudomonas Syringae Influence Colony and Biofilm Morphology." *Applied and Environmental Microbiology* 75(1):45–53.

Sigrist, Christian J. A. et al. 2013. "New and Continuing Developments at PROSITE." *Nucleic Acids Research* 41(D1).

Solovyev, V. and a Salamov. 2011. "Automatic Annotation of Microbial Genomes and Metagenomic Sequences." *Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies (Ed. RW Li), Nova Science Publishers* 61–78.

De Souza, Alessandra A. et al. 2004. "Gene Expression Profile of the Plant Pathogen Xylella Fastidiosa during Biofilm Formation in Vitro." *FEMS Microbiology Letters* 237(2):341–53.

Spiers, Andrew J. et al. 2013. "Cellulose Expression in Pseudomonas Fluorescens SBW25 and Other Environmental Pseudomonads." *Cellulose - Medical, Pharmaceutical and Electronic Applications* 1–26.

Steinmetzer, Katrin, Joachim Behlke, Sabine Brantl, and Mike Lorenz. 2002. “CopR Binds and Bends Its Target DNA: A Footprinting and Fluorescence Resonance Energy Transfer Study.” *Nucleic acids research* 30(9):2052–60.

Subramoni, Sujatha et al. 2011. “Bacterial Subfamily of LuxR Regulators That Respond to Plant Compounds.” *Applied and Environmental Microbiology* 77(13):4579–88.

Subramoni, Sujatha and Vittorio Venturi. 2009. “LuxR-Family ‘Solos’: Bachelor Sensors/regulators of Signalling Molecules.” *Microbiology* 155(5):1377–85.

Taguchi, Fumiko et al. 2006. “Identification of Glycosylation Genes and Glycosylated Amino Acids of Flagellin in Pseudomonas Syringae Pv. Tabaci.” *Cellular Microbiology* 8(6):923–38.

Thornton, Brenda and Chhandak Basu. 2011. “Real-Time PCR (qPCR) Primer Design Using Free Online Software.” *Biochemistry and Molecular Biology Education* 39(2):145–54.

Tian, Yang et al. 2008. “Role of RpoS in Stress Survival, Synthesis of Extracellular Autoinducer 2, and Virulence in Vibrio Alginolyticus.” *Archives of Microbiology* 190(5):585–94.

Ude, Susanne, Dawn L. Arnold, Christina D. Moon, Tracey Timms-Wilson, and Andrew J. Spiers. 2006. “Biofilm Formation and Cellulose Expression among Diverse Environmental Pseudomonas Isolates.” *Environmental Microbiology* 8(11):1997–2011.

Untergasser, Andreas et al. 2007. “Primer3Plus, an Enhanced Web Interface to Primer3.” *Nucleic Acids Research* 35(SUPPL.2):71–74.

Vanneste, J. L., D. A. Cornish, F. Spinelli, and J. Yu. 2004. “Colonisation of Apple and Pear Leaves By Different Strains of Biological Control Agents of Fire Blight.” *Society* 53:49–53.

Venturi, Vittorio. 2006. “Regulation of Quorum Sensing in Pseudomonas.” *FEMS Microbiology Reviews* 30(2):274–91.

Venturi, Vittorio and Clay Fuqua. 2013. “Chemical Signaling between Plants and Plant-Pathogenic Bacteria.” *Annual review of phytopathology* 51:17–37. Retrieved (http://www.ncbi.nlm.nih.gov/pubmed/23915131).

Yu, Jing, Alejandro Penaloza-Vázquez, Ananda M. Chakrabarty, and Carol L. Bender. 1999. “Involvement of the Exopolysaccharide Alginate in the Virulence and Epiphytic Fitness of Pseudomonas Syringae Pv. Syringae.” *Molecular Microbiology* 33(4):712–20.

Supplementary information

Figure S1. Transcriptional regulator *Tr in-silico* 3D protein structure from I-TASSER software analysis. (a) Protein structure with DNA ligand binding site represented in the box; (b) the HTH DNA-binding motif is represented to bind the strand of DNA.

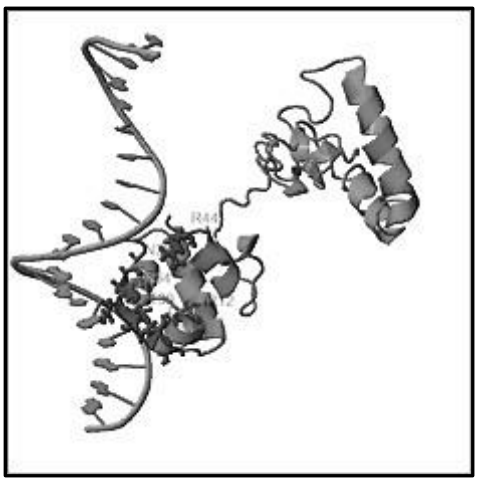
a)  b) 

Table S1.

Sequence of PsaCFPB7286 that aligned to *ahlI* promoter boxes:
5'_GTACCTGATTGCACCGAACAGACGGCTTTCCA_3'

BPROM prevision of *Tr* promoter boxes:
5'_GTACCTGATTGCACCGAACAGACGGCTTTCCATATT_3'

Homologies between *Tr* promoter and *ahlI* promoter sequences:

5'_**GTACCT**G**A**T**TGCA**CCG**AACAGA**C**GG**C**T**T**T**CCA**T**ATTT**GT**TCGA_3' *Tr*

5'_**GTACCT**A**A**G**TGCA**AAT**AACAGA**A**GG**T**T**A**T**ATT**T**CAAG**GT**GTTG_3' *ahlI*

Tables and figures

Table 1. Bacterial species, strains and plasmids used in this study. The most relevant characteristics, the growing medium and temperature are also reported. Km^r, Nf^r and Chl^r indicate resistance to kanamycin, nitrofurantoin and chloramphenicol respectively.

Strain or plasmid	Relevant characteristics	Reference/Source
Pseudomonas syringae pv. *actinidiae* (*Psa*)		
Psa wild type strain CFBP7286	Cultivated in LB at 27°C	Spinelli et al 2011
Psa-mR1 (psaR1::pKNOCK); derivative of wild type	Cultivated in LB at 27°C Km^r (100 μg/ml)	Patel et al., 2014
Psa-mR3 (psaR3:: in-frame deletion mutant generated by pEX19Gm plasmid); derivative of wild type	Cultivated in LB at 27°C Nf^r (150 μg/ml)	Patel et al., 2014
Psa-AlgDsile (Psa harbouring pSileAlgD)	Cultivated in LB at 27°C; gene silencing is induced with IPTG 0.5%; Chl^r (25 μg/ml)	This work.
Pseudomonas syringae pv. *syringae* (*Pss*) Pss wild type strain 4364	Cultivated in LB at 27°C. Strain used as control for biofilm and motility experiments	This lab
Pseduomonas fluorescens (*Pf*) Pf wild type strain A506	Cultivated in LB at 27°C. Strain used as control for biofilm and motility experiments	Vanneste et al., 2004
Escherichia coli (*E. coli*) strain DH5α	Cultivated in LB at 37°C; electrocompetent cells are used for multiplication of pHN678-AlgD	Sigma-Aldrich
pSile AlgD	Derived from pHN678, expressing the N terminal part and putative Shine-Delgarno sequence of AlgD gene; Chl^r (25 μg/ml)	Nakashima and Tamura 2009

Table 2. Genes selected from gene expression studies. The genes were divided into 4 classes according to their function. The gene function/s, the source of the original sequence used to screen Psa genome and the homology are also reported. Reference for each gene is indicated.

	Gene	Identity	Organism	Strain	Function/s	Reference
Biofilm	*algD*	99,3	*Pseudomonas syringae* pv. *tomato*	DC3000	*algD* is involved in biofilm formation both *in vitro* and *in planta*. First gene to be transcribed during the biosynthesis of alginate, a virulence factor in *Ps. syringae*.	Penaloza-Vazquez et al., 2010
	clpP	87,6	*Pseudomonas fluorescens*	SBW25	Codes for a protein required for biofilm formation of *P.fluorescens*.	O'Toole and Kolter, 1998
	mdoH	99,5	*Pseudomonas syringae* pv. *tomato*	DC3000	Membrane derived oligosaccharides, a family of glucans present in periplasmic space of Gram negative bacteria. Required for biofilm formation.	Penaloza-Vazquez et al., 2010
	wspR	85,2	*Pseudomonas fluorescens*	SBW25	Is involved in colony morphology and cellular aggregation; regulator of genes that encode a putative fimbrial adhesin required for biofilm formation	D'Argenio et al., 2002 Ude et al., 2006
	wssB	68,3	*Pseudomonas fluorescens*	SBW25	Cellulose synthase catalytic subunit. Cellulose is used by *P. fluorescens* SBW25 in the colonization of plant surfaces.	Spiers et al., 2013

	Gene	Identity	Organism	Strain	Function/s	Reference
Motility	*fliP*	81,9	*Pseudomonas putida*	W619	Flagellar biosynthetic protein FliP	Segura et al., 2001
	pilA	92,4	*Pseudomonas syringae* pv. *tabaci*		involved in the attachment of the bacteria and/or biofilm formation	A. de Souza et al., 2004
	pilC	90,4	*Pseudomonas syringae* pv. *tomato*	DC3000	type IV pilus biogenesis protein PilC	A. de Souza et al., 2004
	pilO	74,9	*Pseudomonas aeruginosa*	PAO1	type 4 fimbrial biogenesis protein PilO	Martin et al., 1995
	rpoN	60,0	*Vibrio alginolyticus*		Involved in polar flagellar formation.	Sheng et al., 2012

	Gene	Identity	Organism	Strain	Function/s	Reference
Density-related genes	*aefR*	91,6	*Pseudomonas syringae* pv. *syringae*	B728a	Potential regulator of mexEF/oprN in *Ps. tabaci*; Synthesis of 3OC6-HSL. Gene product: "AHL and epiphytic fitness regulator"	Quinones et al., 2005; Kawakita et al., 2012
	fur	100,0	*Pseudomonas syringae* pv. *tabaci*		Ferric uptake regulator. Role in the control of genes involved in QS.	Cha et al., 2007
	gacA	99,1	*Pseudomonas syringae* pv. *syringae*	B728a	The GacS/GacA system interacts positively with the PhzI/PhzR quorum sensing system by regulating the synthesis of N-acyl-homoserine lactone	Chancey et al.,1999
	gacS	100,0	*Pseudomonas syringae* pv. *syringae*	B728a	Membrane-bound sensor kinase protein that recognizes specific environmental stimuli and activates GacA	Appleby et al ., 1996 Heeb and Haas, 2001 Pernestig et al., 2001
	hacA	88,4	*Pseudomonas syringae* pv. *syringae*	B728a	AHL acylase; Peptidase S45, penicillin amidase	Shepherd and Lindow, 2009
	hacB	94,3	*Pseudomonas syringae* pv. *syringae*	B728a	AHL acylase; Penicillin amidase. Can inactivate 3OC6HSL	Shepherd and Lindow, 2009
	hacC	97,0	*Pseudomonas syringae* pv. *syringae*	B728a	Aspartate transaminase: it is an AHL acylase that degrades AHL signal	Kalia, 2013
	mexE	93,7	*Pseudomonas syringae* pv. *phaseolicola*		Related with resistance to antimicrobial agents component of multidrug efflux system; multidrug efflux RND transporter, membrane fusion protein MexE.	Kawakita et al., 2012
	oprM	73,1	*Pseudomonas aeruginosa*	PAO1	Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM precursor	Phan et al., 2010
	psaR1	-	*Pseudomonas syringae* pv. *actindiae*		Highly similar to LuxRs which bind AHLs and are part of the canonical LuxI/R AHL QS systemslo that bind to AHLs	Patel et al., 2014
	psaR2	-	*Pseudomonas syringae* pv. *actindiae*		Binds and responds to yet unknown plant signal molecules.	Patel et al., 2014
	psaR3	-	*Pseudomonas syringae* pv. *actindiae*		Highly similar to LuxRs which bind AHLs and are part of the canonical LuxI/R AHL QS systemslo that bind to AHLs	Patel et al., 2014
	rpoS	69,4	*Vibrio alginolyticus*		Part of the regulatory networks of virulence and LuxS quorum sensing system; RpoS is cell density related; RpoS positively regulates AHL levels.	Tian et al., 2008
	Tr	-	*Pseudomonas syringae* pv. *actindiae*		Transcriptional regulator derived from promoter blast analysis. Potentially involved in signaling transduction and biofilm regulation.	This work

	Gene	Identity	Organism	Strain	Function/s	Reference
Virulence	*avrpt01*	-	*Pseudomonas syringae* pv. *actindiae*		Unknown	This work
	enolase	-	*Pseudomonas syringae* pv. *actindiae*		Pathogenesis related genes	McCann et al., 2013
	hopD1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	McCann et al., 2013
	hopQ1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopR1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopS2	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopZ5	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	McCann et al., 2013
	lysR	93,5	*Pseudomonas syringae* pv. *tabaci*		LysR-type transcriptional regulators (LTTRs) regulate a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility.	Kawakita et al., 2012
	pamTada	74,7	*Pseudomonas aeruginosa*		Potential target for QS in most Gram-negative bacteria.	Guan et al., 2012
	virB4	-	*Pseudomonas syringae* pv. *actindiae*		Secretion system	Marcelletti et al., 2011

Table 3. Primers used for PCR and qPCR. The genes are grouped in 4 classes according to their function. The source of primer sequences is also reported.

	Gene	Forward primer	Reverse primer	Source
Biofilm				
	algD	GACCTGGAACTGGACTACATC	TGCTGCGAACCACGATAG	This work
	clpP	CTTATATTCAGCAGAACTCT	GCGAATAGATGTCATAGG	This work
	mdoH	ACGGTAACCTTGAACTTGC	CACCATCGTTCTGCTGTT	This work
	wspR	ACGACTATCTGGTCAAACTG	ATAGGCTTCATCACGCTG	This work
	wssB	CGCTGGTGATGATGATGGT	CTGACGCTCAACGCTGTG	This work
Motility				
	fliP	TCAAGACGGCGTTTCAGA	CGGCGAGAGCATCATCAT	This work
	pilA	GCCATTCCTTCCTATCAA	GTAAGACCATTGCTCCAG	This work
	pilC	CGCTGGACATCGCATTCT	GCACCTTCGGCAATGATG	This work
	pilO	CCTACAGAAAGCAGATGGA	GTGATGTCTTCAAGCAGTC	This work
	rpoN	GCACCGACTCCTGATTGA	GAATCCACAGAAGCCGAATAC	This work
Density-related				
	aefR	AACTGCTGGAATTGCTCTG	TGTATCGTGGCACCTACC	This work
	fur	TGAAAATAGCGAACTACGAAAAGC	TGTAAACATCCTCGGCACTC	This work
	gacA	GATGACCATGACCTTGTTC	TCTTCAGCGATTCCTCAC	This work
	gacS	AGAACCTGGAAACCATCG	ATCTCGTGGCTCATGTTG	This work
	hacA	AGTTCACTGAAGCCTTTGC	CCAGTTGTAGCGCCTGAA	This work
	hacB	ACGGCATCAACCAGTATC	ATGCTGACCGTGTCCTCT	This work
	hacC	GACCTTCTTCGCCTCCAG	TTCCTTCGATTCGGTGATGA	This work
	mexE	TGTACGCACGGCTGAAACTG	TCCTTGTCCATCACCAGCAC	This work
	oprM	CGCTGGACATCGCATTCT	GCACCTTCGGCAATGATG	This work
	psaR1	ATACCTGGTCAGTAGTCTCA	GCAGCACTTCAAGTTCAC	This work
	psaR2	ACTGTTTGACCAGAAGATG	CTGAACGGTTGAGTTGAT	This work
	psaR3	GGTTCGCTCATTATCTGAT	GCAATGCTTGAGGATAGG	This work
	rpoS	CGTCGCTCAAACAACACAAAT	GAGACAGCAGAGGGGAAAAC	This work
	Tr	ACATTTACCATGTCACCCGCC	TGATGGCTTCTGCGTCGTTC	This work
Virulence				
	avrpt01	GGAGCGAATCTTGCCATT	GGAGCGATATGCGTGAAG	This work
	enolase	CATCGCCAACCTCAATGG	CCTGGATGTCGATGTTGTTAT	This work
	hopD1	CAGTAGACAGCAGTAGCC	CGGGTTATCGGAAACAAG	This work
	hopQ1	GGCATTCCACTTCGTATAG	CAACGCACTCTTCTGAAC	This work
	hopR1	GACATAACTGCCGATGCT	TCCAGATAGGCTCGATCA	This work
	hopS2	CCTTAAACGGCTGGCAGAG	CGAAGTGATGCTTGAGGTGAA	This work
	hopZ5	TCAGGCTACAATACTTACGCATCA	CAGGAATAGAACGGAACTCAGGAT	This work
	lysR	TGCGGAAGTTGAAGCGGATTACG	ACCGAAATGTTGCTGCCTCCC	This work
	pamTada	ACACATGACCCAGATCAG	CAGCTTGAGGTTGGATTC	This work
	virB4	TTTGAAGACACCACTGTTTC	CTGCGTCACCTACTACTC	This work
Reference				
	rpoD	CCGAGATCAAGGACATCAAC	GAGATCACCAGACGCAAGTT	Narusaka et *al.*, 2011
	recA	CGCACTTGATCCTGAATACG	CATGTCGGTGATTTCCAGTG	This work

Table 4. Enzymes for *algD* cohesive-ends digestion (*Xho*I and *Bam*HI) are reported in the cleavage site.

Primer_For (5'-3')	CCG	CTCGAG *Xho*I	ACATCTGCTGGCCAAAATTAA
Primer_Rev (5'-3')	CG	GGATCC *Bam*HI	GATATCCACACCCACTACGTCG

Table 5. Summary of the species, strain names and total promoter lengths used in the bioinformatics research. Promoter's core binding sites of RNA polymerase were calculated with BPROM (underlined). Nucleotidic (nt) length of the sequences blasted in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 is also reported.

Species	**Strain**	**Promoter**	**Promoter lenght**	**Promoter sequence with DNA binding boxes underlined (-35 left; -10 right) 5'-3'**	**Lenght blasted**
Agrobacterium tumefaciens	NTL4	*traI*	299 nt	TTGAGGTAATTTCGGTTGTAGCTCGGTTAACCT	33 nt
Burkholderia cepacia	ATCC25416	*cepI*	228 nt	TTTCCGCAGTGCTGGCGCTCTTTATAAG	28 nt
Chromobacterium violaceum	ATCC31532	*cviI*	246 nt	TTAAATAAATCTATAAATATTAAATTT	27 nt
Pseudomonas aeruginosa	PUPa3	*rhlI*	150 nt	TTGCCTGCCGTTCATCCTCCTTTAGTCT	28 nt
Pseudomonas aureofaciens	30-84	*phzI*	168 nt	TCTTGCAGGTGCCAAG-CCGGTACAAGTCCTCTATAAA	37 nt
Pseudomonas syringae phaseolicola	1448A	*ahlI*	185 nt	GTACCTAAGTGCAAATAACAGAAGGTTATATT	32 nt
Pseudomonas putida	WCS 358	*ppuI*	134 nt	TGGCCGACATTAACCAGACTTGTTAATTT	29 nt
Vibrio fischeri	pSB401	*luxI*	196 nt	GTGACAAAAATCCAATTTATTAGAAT	26 nt

Table 6. Alignment of *Pseudomonas syringae* pv. *phaseolica* 1448A *ahlI* promoter sequence with *Pseudomonas syringae* pv. *actinidiae* CFBP7286 produced with EMBL FASTA tool with standard parameters. Identity of *ahlI* promoter sequence alignment: 72.4%.

ahlI prom reverse strand	AATATAACCTTCTGTTATTTGCACTTAGGTAC
	: : :: :::::: :::: : ::::::
PsaCFPB7286 forward strand	**TGGAAAGCCGTCTGTTCGGTGCAATCAGGTAC**

Table 7. Percentages of colonies showing swarming swimming or twitching motility on soft LB plates (0.4% agar) derived from high density *inocula* (10^8 CFU ml^{-1}).

	Social	*Non-social*	
Strain	**% of swarming colonies**	**% of swimming colonies**	**% of twitching colonies**
PsaCFBP7286	15.2%	48.3%	36.4%
Pss4364	75.0%	25.0%	0.0%
PfA506	95.0%	5.0%	0.0%

Table 8. Summary of the results concerning motility with 1 μM hydroxylated acyl-homoserine lactones added (OH-C6, OH-C8, OH-C10, OH-C12). Values represent the number of colonies that displayed swarming, twitching and swimming, in percentage. The control (NO AHL) consisted of PsaCFBP7286 motility on plates with no addition of synthetic signals.

	Social	*Non-social*	
Thesis	**% of swarming colonies**	**% of twitching colonies**	**% of swimming colonies**
NO AHL	10.6%	25.5%	63.8%
OH-C6 1μM	23.1%	33.3%	43.6%
OH-C8 1μM	21.1%	28.9%	50.0%
OH-C10 1μM	17.9%	25.0%	57.1%
OH-C12 1μM	19.4%	29.0%	51.6%

Table 9. Summary of the virulence genes that were significantly induced by AHLs in LD and HD conditions.

	in LD			in HD		
	OH-C6	OH-C8	OH-C12	OH-C6	OH-C8	OH-C12
avrpt01	x	x				x
hopD1	x	x	x		x	x
hopS2	x		x	x		
hopZ5		x				
paMTAda		x			x	x
enolase					x	
lysR			x			
oprM			x			x

Table 10. Effect of the exogenous application of 1 μM hydroxylated acyl-homoserine lactones (OH-C6, OH-C8, OH-C10, OH-C12) on the swarming motility of *Pseudomonas syringae* pv. *actinidiae* wild type strain CFBP7286, Psa-mR1 and Psa-mR3 mutants. Values represent the number of colonies that displayed swarming, in percentage. The control (NO AHL) consisted of motility on plates with no addition of synthetic signals.

	% of swarming colonies		
	PsaCFBP7286	Psa-mR1	Psa-mR3
NO AHL	10.6%	0.0%	0.0%
OH-C6	23.1%	40.0%	0.0%
OH-C8	21.1%	100.0%	0.0%
OH-C10	17.9%	0.0%	20.0%
OH-C12	19.4%	40.0%	0.0%

Figure 1. Swarming motility of PsaCFBP7286 on soft LB plates (0.4% agar). Photographs were taken binocular Nikon SMZ25 fluorescence microscope. (a) swarming motility (zoom magnification 1x); (b) swimming motility (zoom magnification 6.3x); (c) twitching motility (zoom magnification 6.3x); (d) twitching and swimming motility (zoom magnification 6.3x)

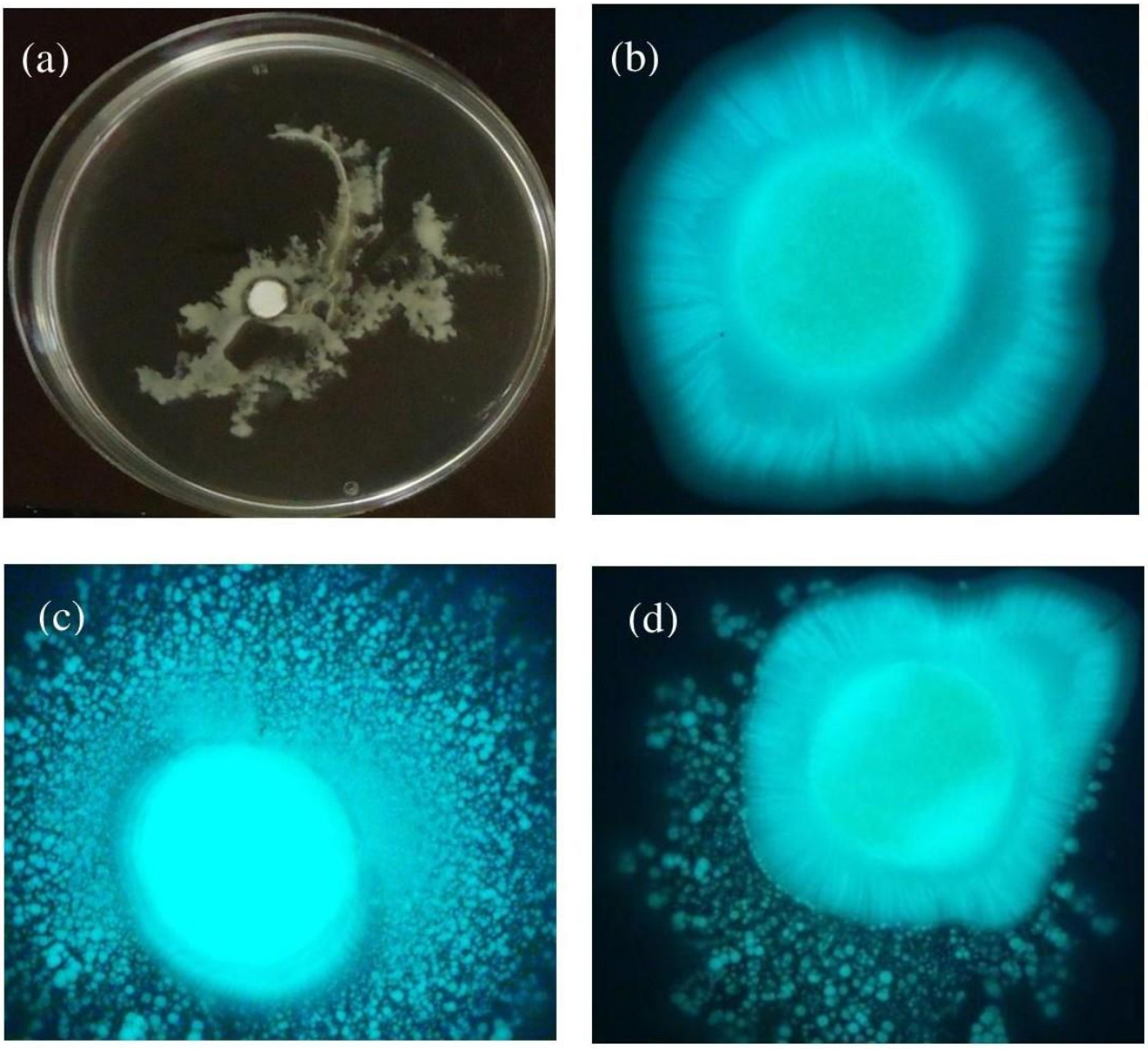


Figure 2: Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 compared with the biofilm forming strains *P. syringae syringae* 4364 and *P. fluorescens* A506. Biofilm was quantified after staining with crystal violet (0.5% w/v). Standard error is shown. Bar marked with an asterisk (*) were significantly different according to Student's *t* test ($P < 0.05$).

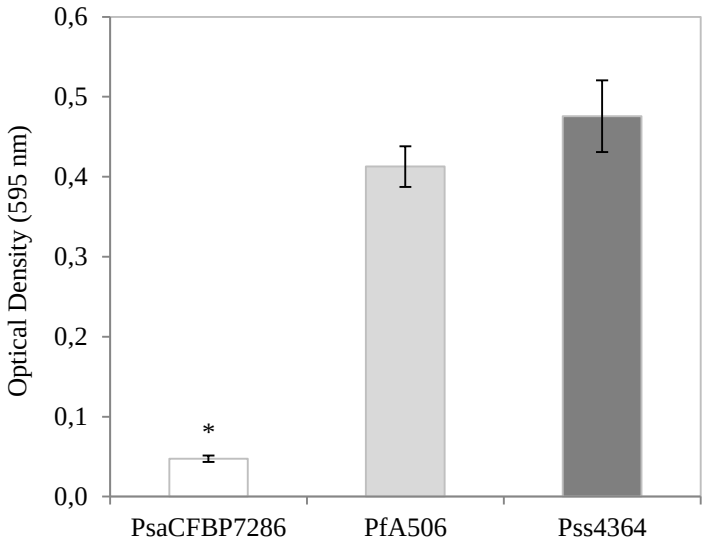


Figure 3. Epiphyte (a) and endophyte (b) populations of *Actinidia deliciosa* cv. Hayward plantlets inoculated with an equal amount of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 cells (10^8 CFU ml^{-1}) derived either from low density (10^5 CFU ml^{-1}) or high density (10^8 CFU ml^{-1}) cultures. Bacterial population was estimated by qPCR. Data are the average of three independent replicates of three plants each. The standard error is shown. Time points marked with an asterisk (*) indicate that LD and HD are significantly different according to the Student's *t* test, with $P < 0.05$. Values with different letters are significantly different according to Fisher's LSD test ($P < 0.05$) and indicate the effect of the two treatment (LD or HD) for each time point.

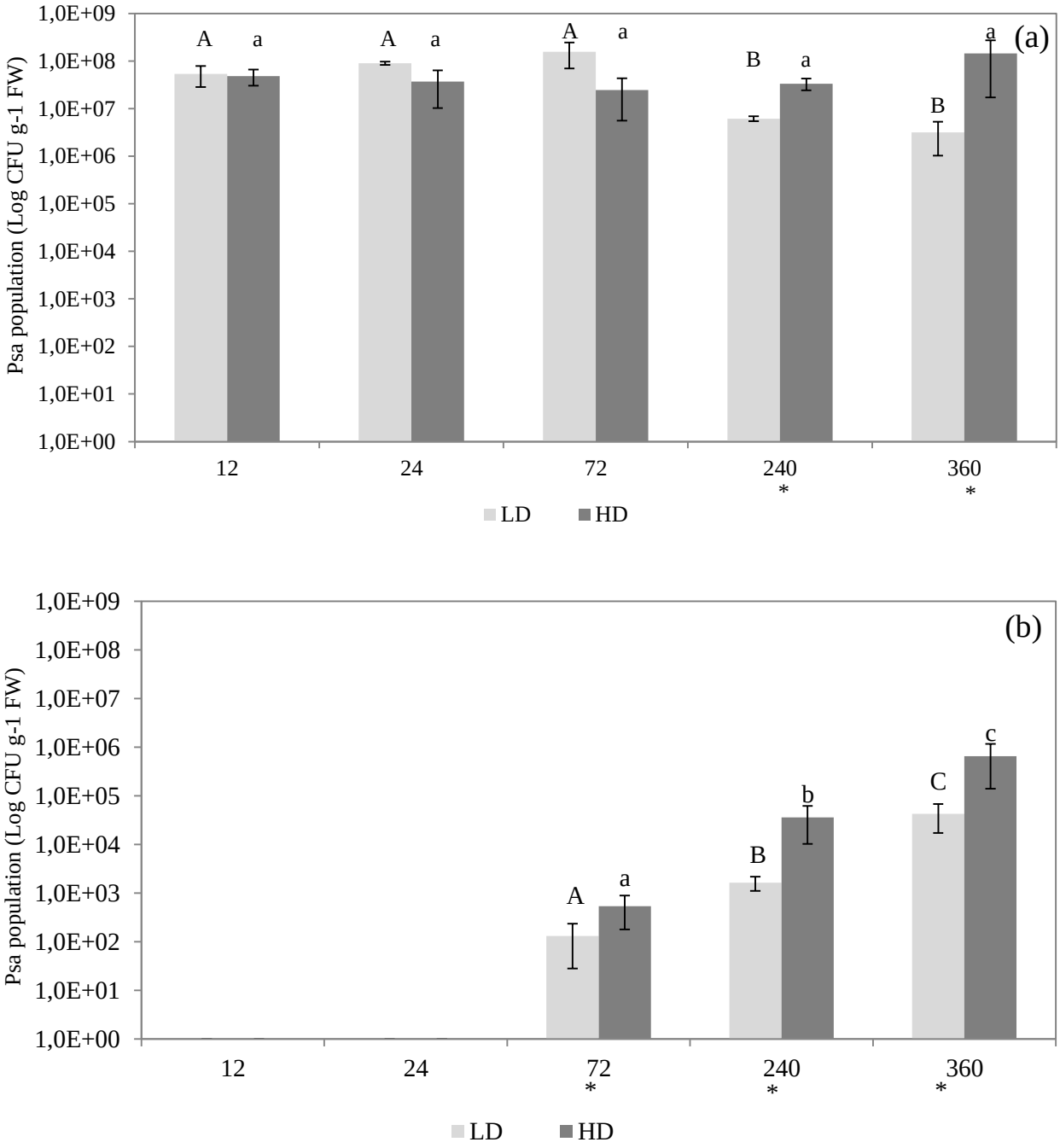
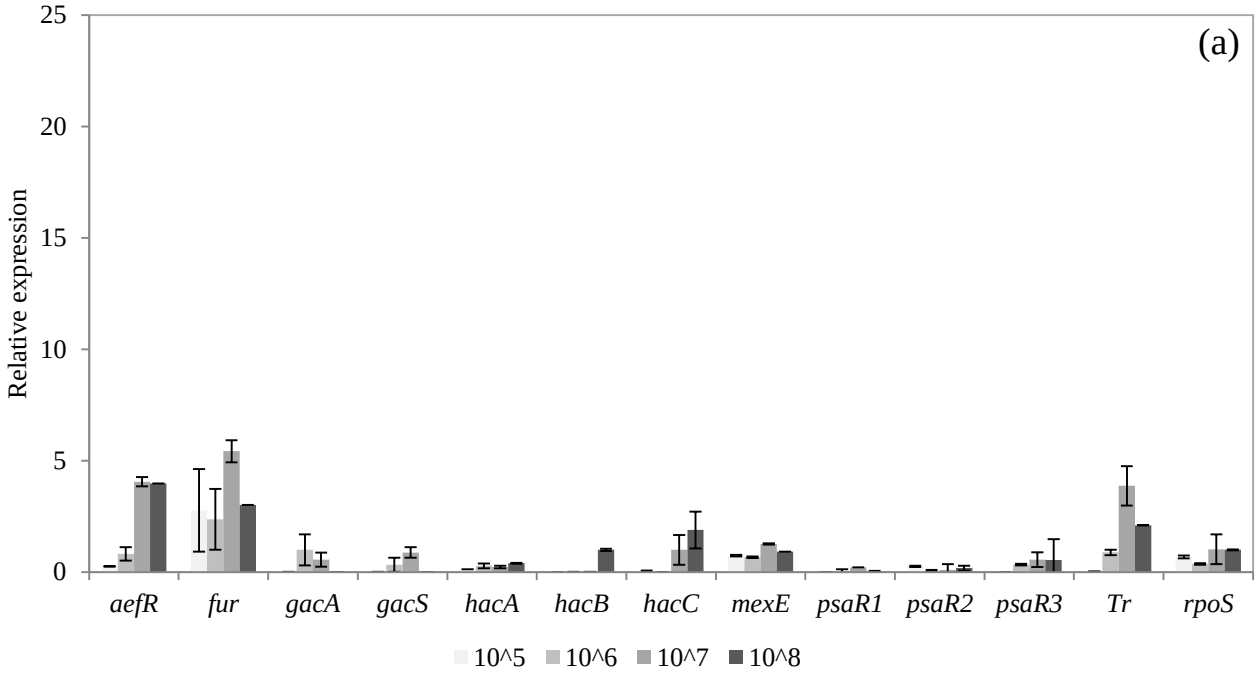
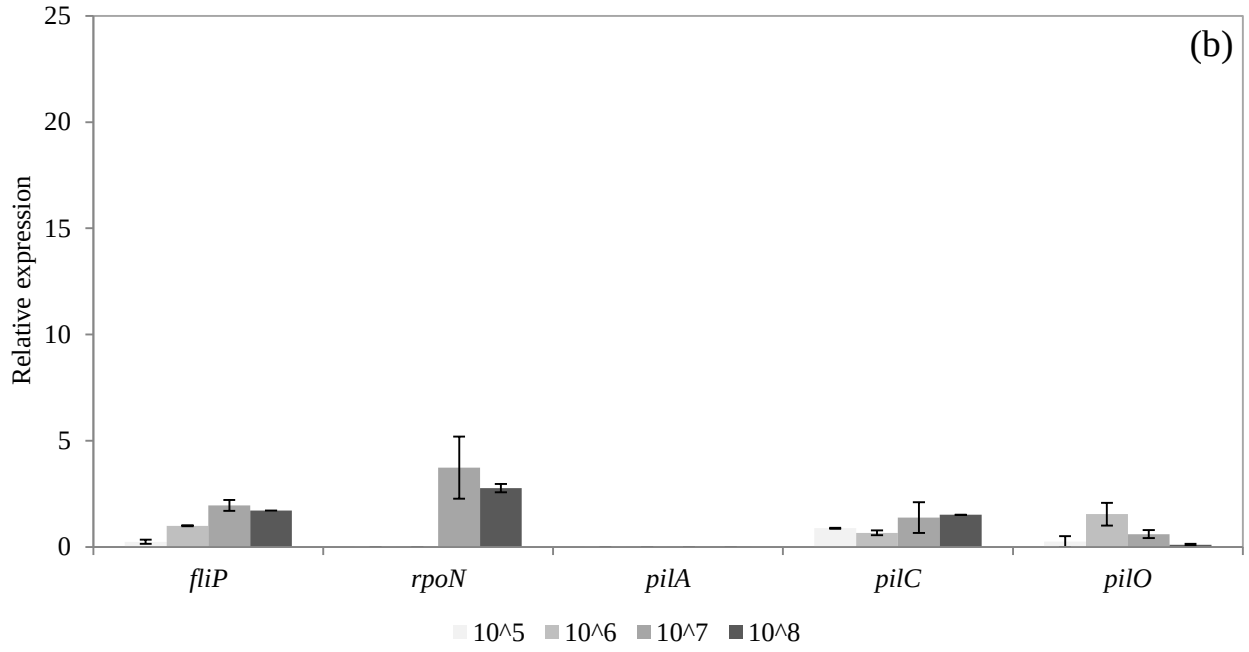
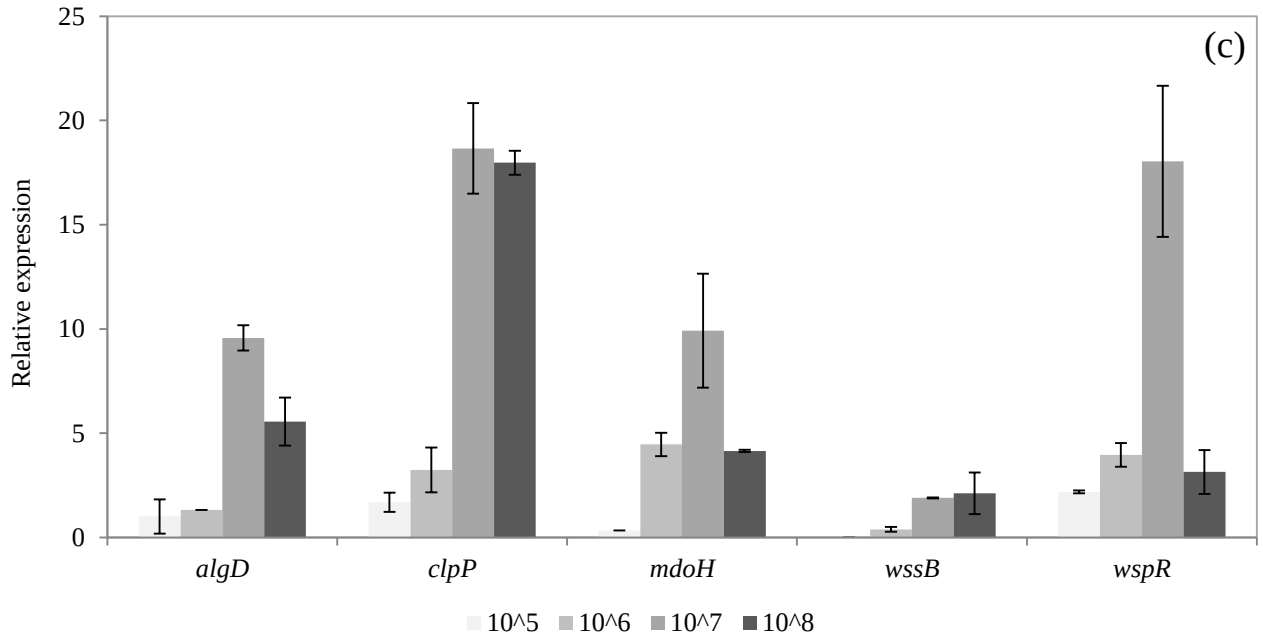


Figure 4. Genes expression in relation to four cell densities in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 cultures (10^5, 10^6, 10^7, 10^8 CFU ml^{-1}). A: density related gens, B: motility, C: biofilm related genes, D: virulence related genes. Gene expression was normalized to th3 of *rpoD* and *recA* genes. Standard error is shown. Bar marked with an asterisk (*) were significantly different according to Student's *t* test ($P < 0.05$).






(c)
Relative expression
25
20
15
10
5
0
algD
clpP
mdoH
wssB
wspR
10^5
10^6
10^7
10^8

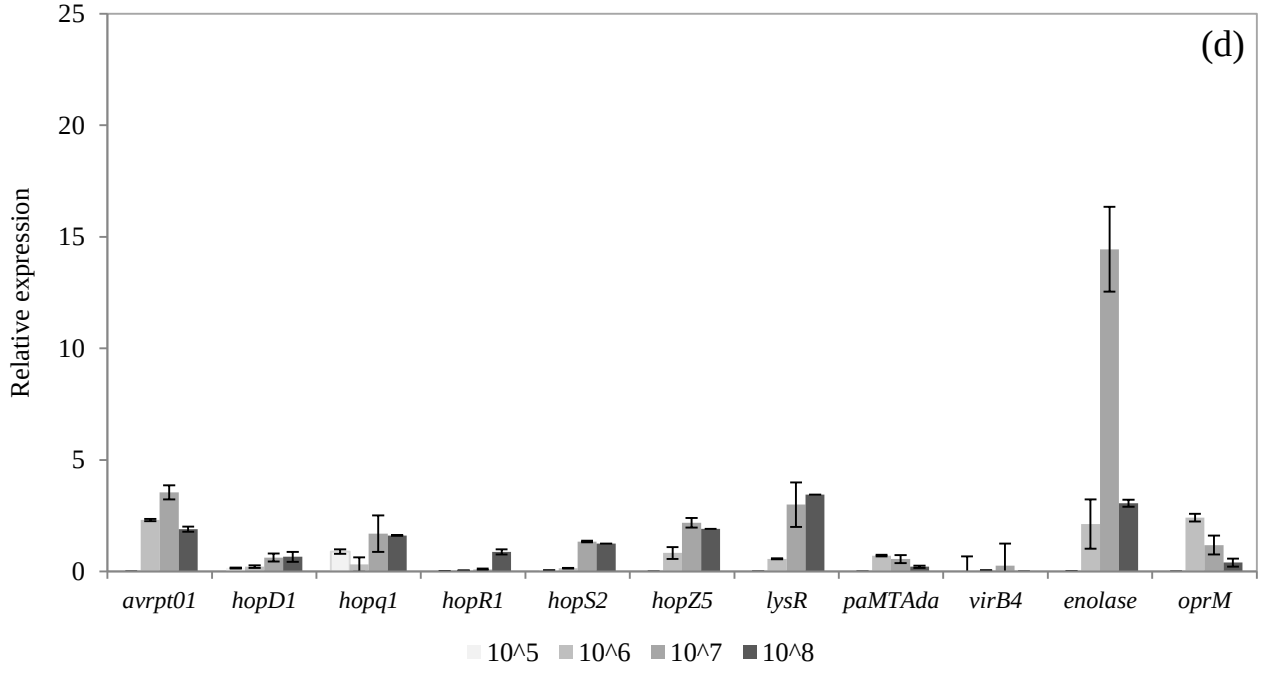

(d)
Relative expression
25
20
15
10
5
0
avrpt01
hopD1
hopq1
hopR1
hopS2
hopZ5
lysR
paMTAda
virB4
enolase
oprM
10^5
10^6
10^7
10^8

Figure 5. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 in the presence of 1 μM hydroxylated acyl-homoserine lactones added (OH-C6, OH-C8, OH-C10, OH-C12). The control (NO AHL) consisted of PsaCFBP7286 biofilm production in plates with no addition of synthetic signals. Biofilm was quantified after staining with crystal violet (0.5% w/v). Standard error bars are shown. Bar marked with an asterisk (*) were significantly different according to Student's *t* test ($P < 0.05$).

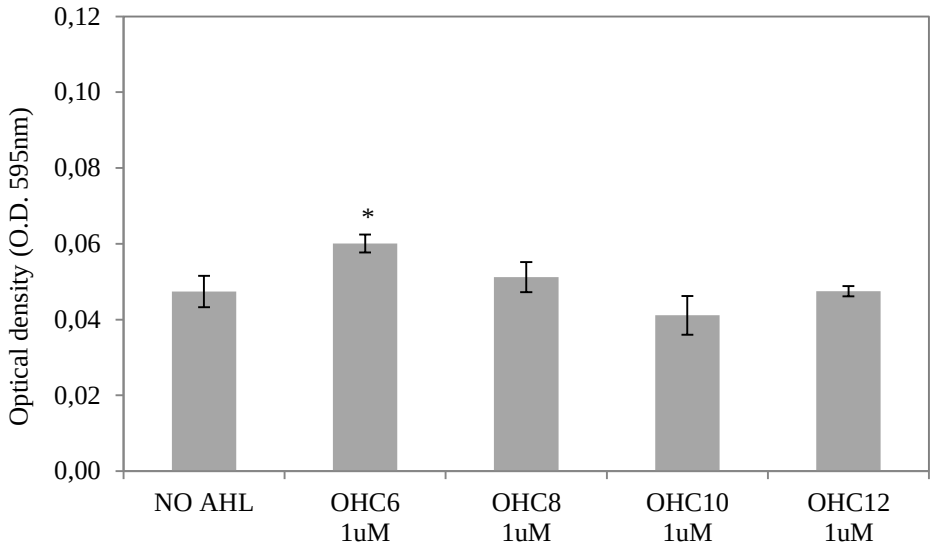
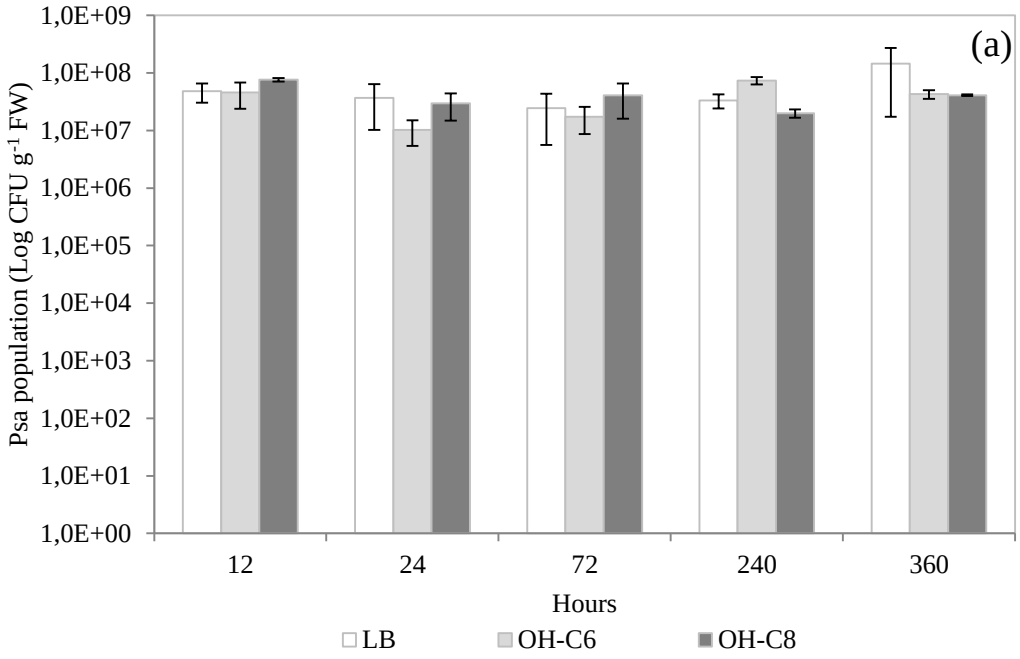


Figure 6. Epiphyte (a) and endophyte (b) populations of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 analysed with qPCR of groups of plantlets inoculated with a high density inoculum grown in LB with the presence of OH-C6- or OH-C8-AHL. Y axis: CFU per gram of the fresh weight of group of plantlets of three replicates. The standard errors are showed. Data marked with an asterisk (*) were significantly different according to the Student's *t* test, with $P < 0.05$.



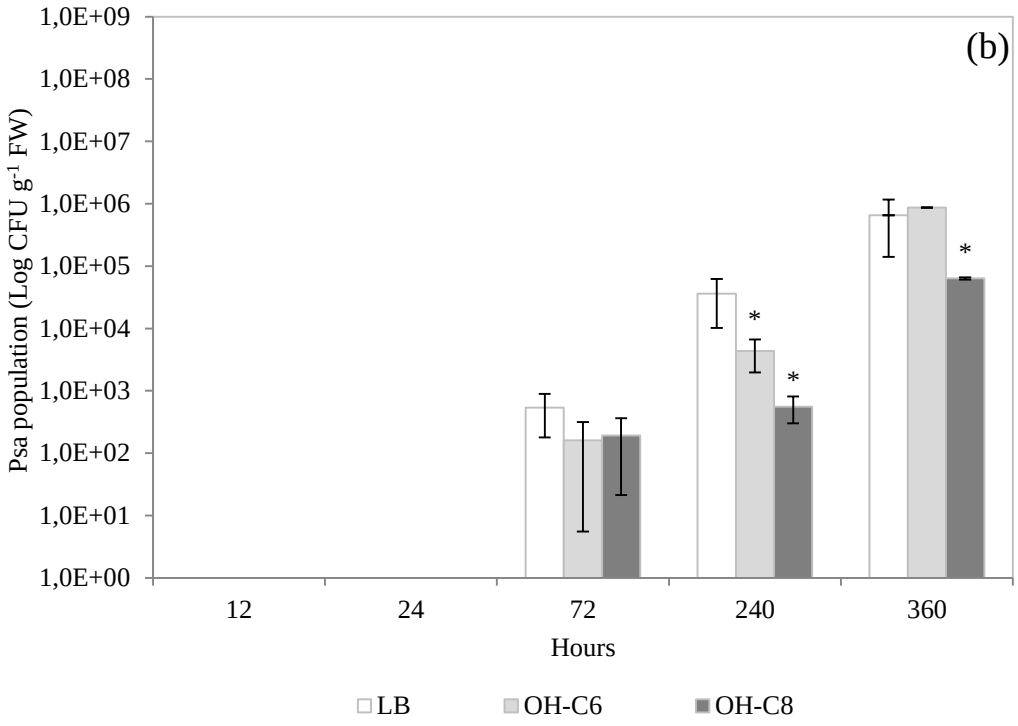


Figure 7. Percentage of swarming colonies of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 after addition of five different concentrations (0.1, 0.25, 0.50, 1.00, 10.00 µM) of synthetic OH-C6- and OH-C8-AHL. The paper disks were inoculated with a 10µl drop of high density bacterial suspension (10^8 CFU ml^{-1}) adjusted to contain 1 x 10^7 cells ml^{-1}. PBS was the solvent used for AHLs, thus it was added as negative control. Left Y axes: percentage of swarming colonies. Right Y axes: percentage of area covered by the development of the swarming colonies over the plate surface. Standard errors bars are showed. Values with different letter are significantly different according to Fisher's LSD test ($P<0.05$).

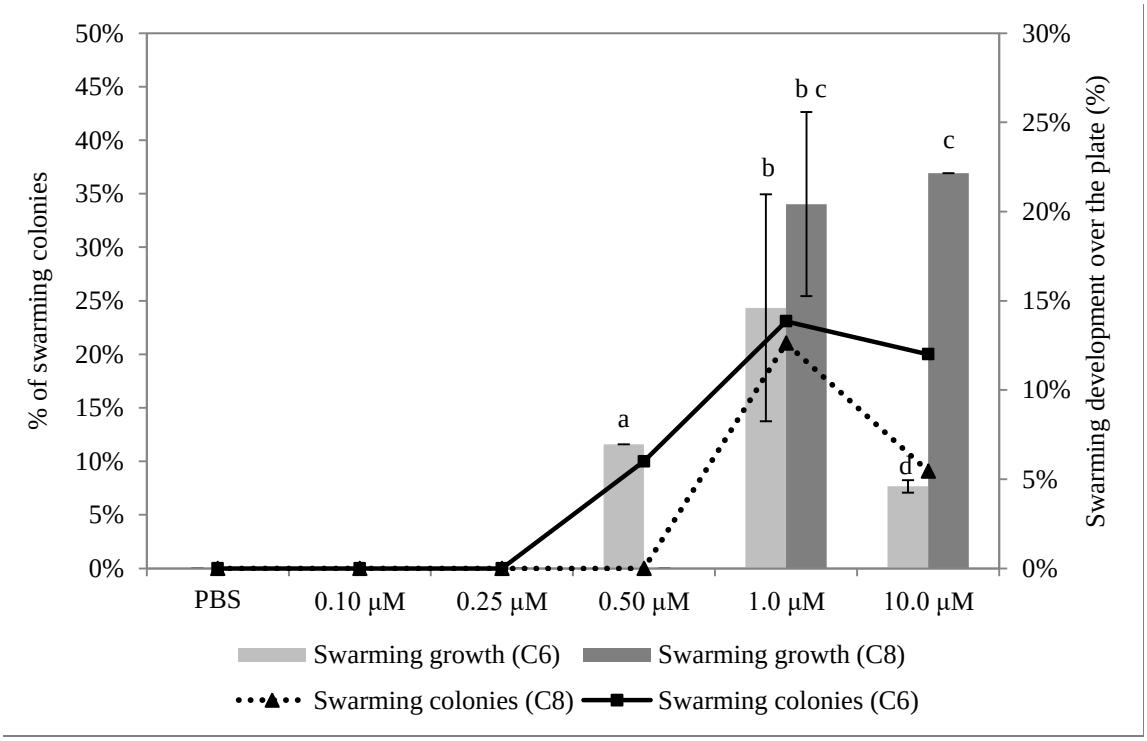
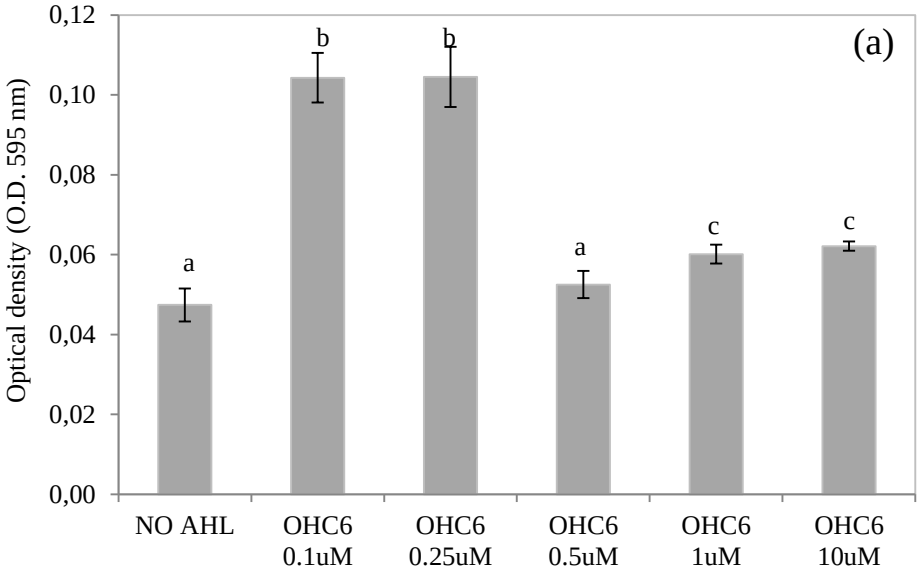


Figure 8. Biofilm quantitation of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 by crystal violet staining (0.5% w/v) after addition in LB of five different concentrations (0.1, 0.25, 0.50, 1.00, 10.00 μM) of (a) OH-C6-AHL and (b) OH-C8-AHL. The control is represented by biofilm formation in LB without signals. Standard error bars are showed. Values with different letter are significantly different according to Fisher's LSD test ($P<0.05$).



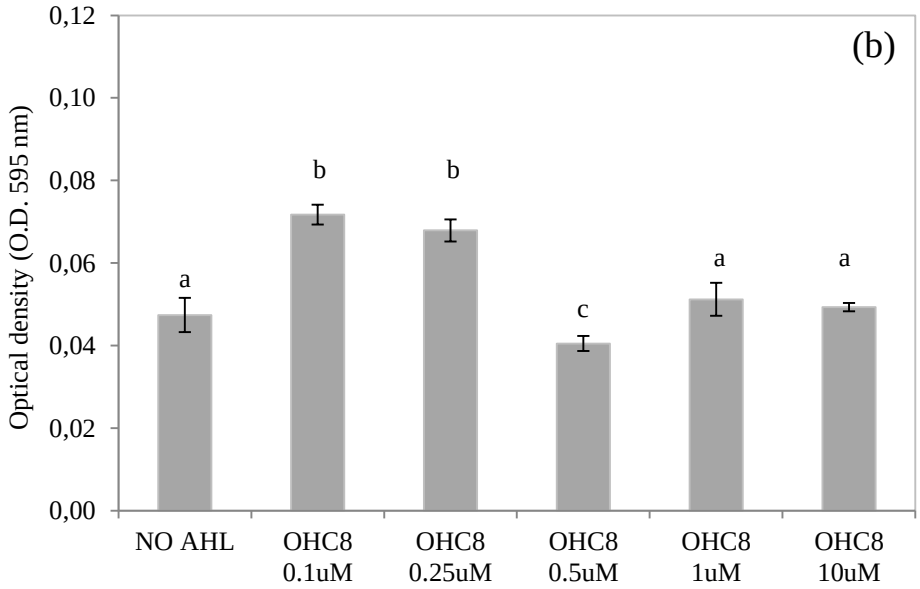
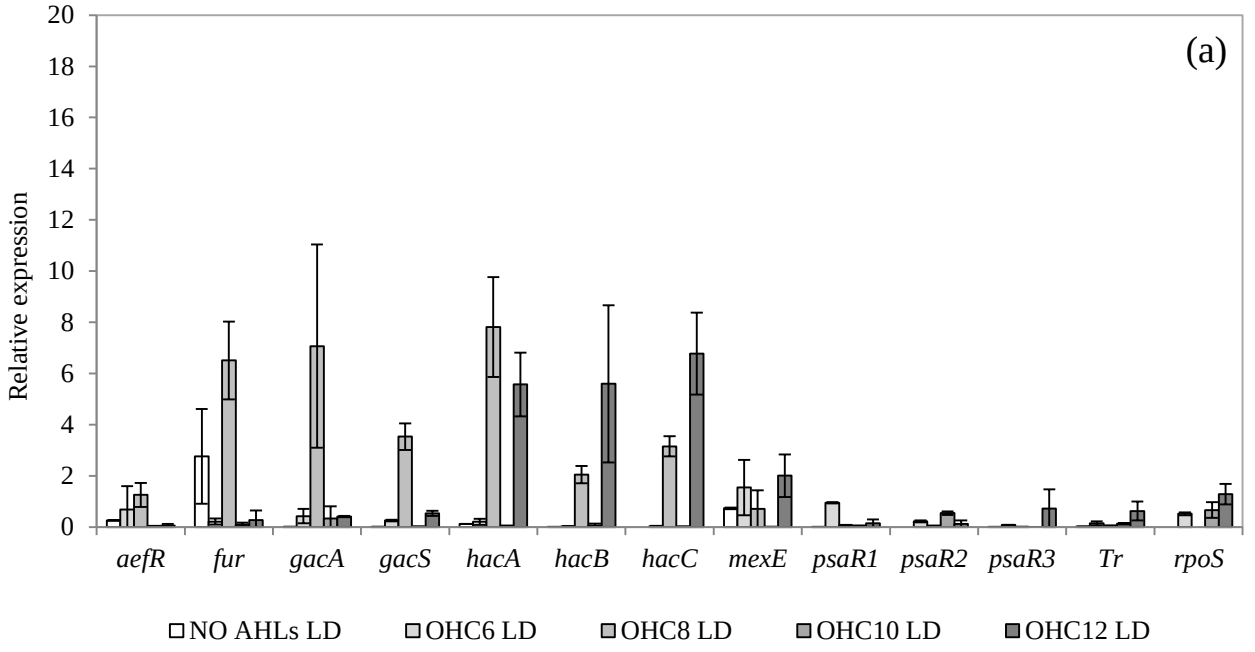


Figure 9. Effect of exogenous application of 4 different AHLs (1 μM) on the expression of genes related to density of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.



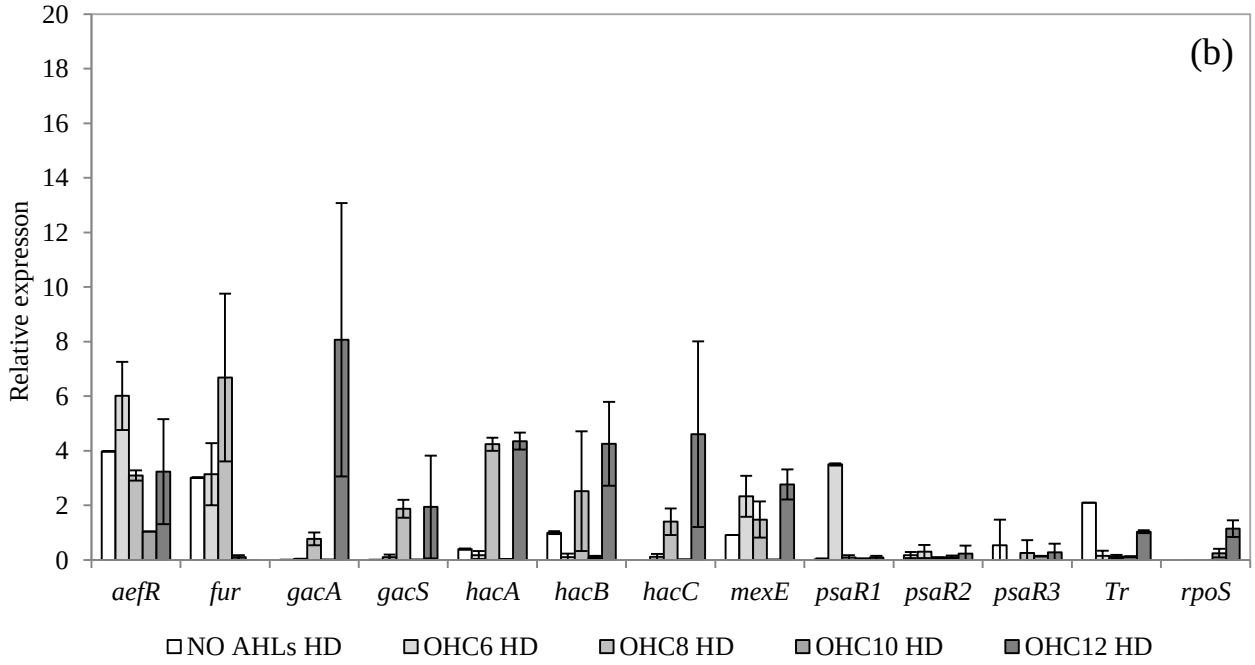
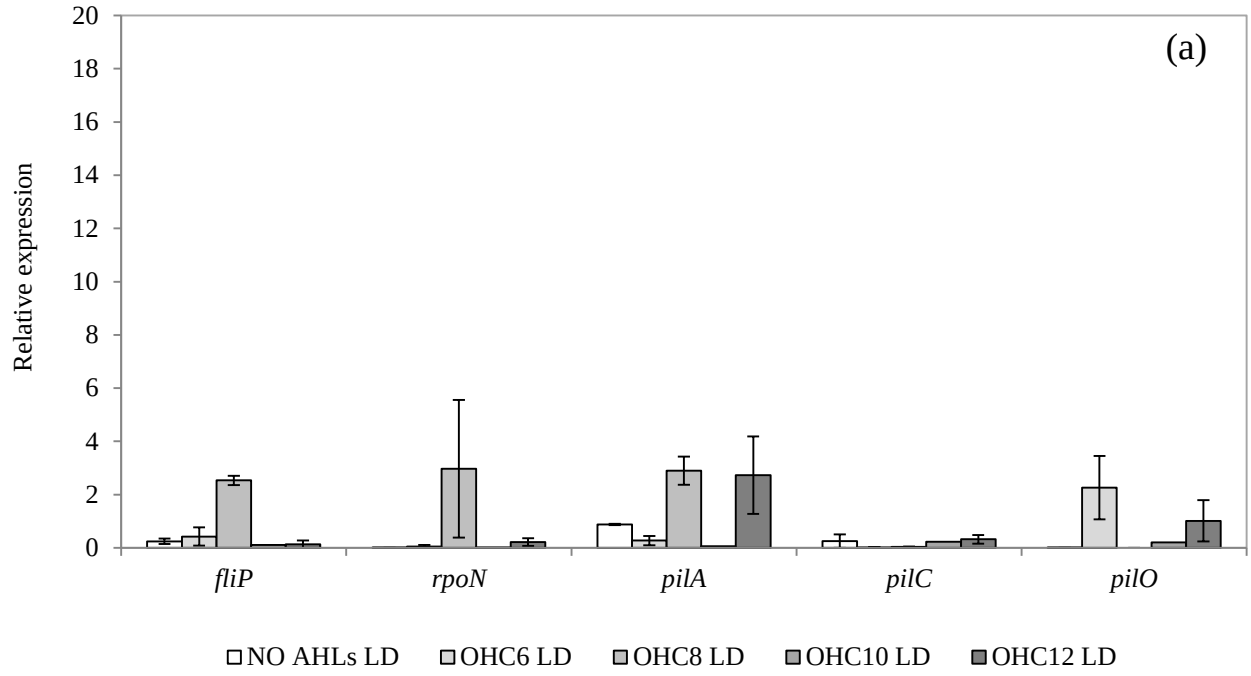


Figure 10. Effect of exogenous application of 4 different AHLs (1 μM) on the expression of genes related to motility of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.



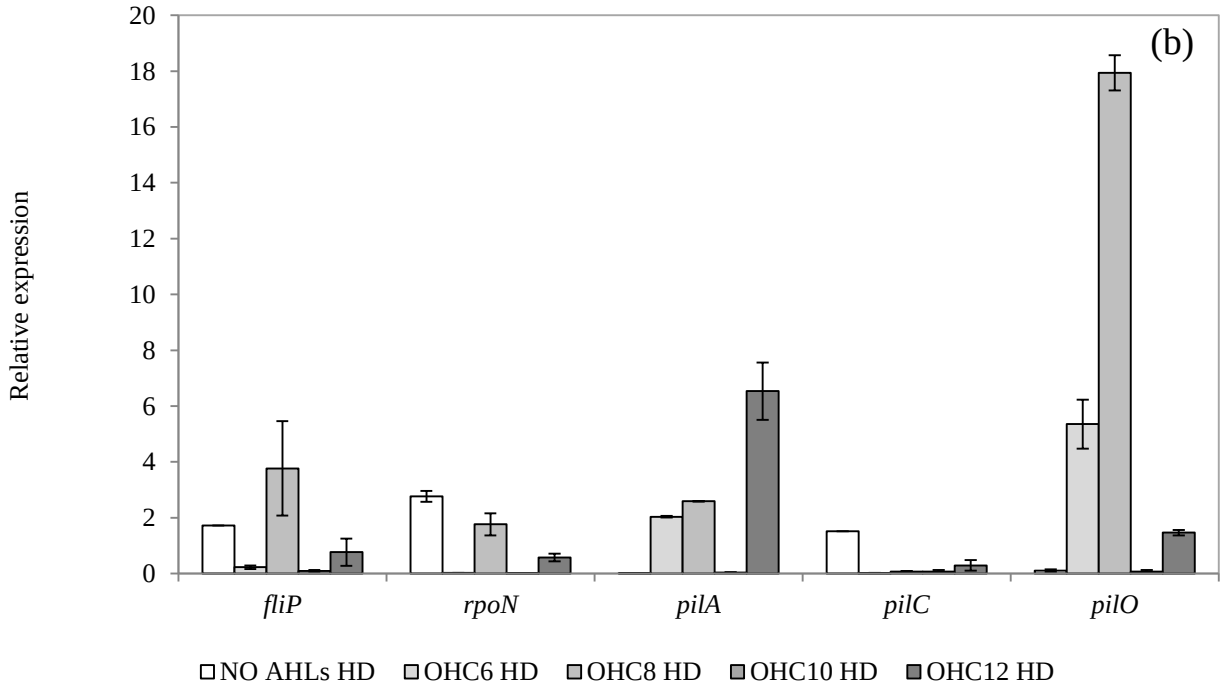


Figure 11. Effect of exogenous application of 4 different AHLs (1 μM) on the expression of genes related to biofilm of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.

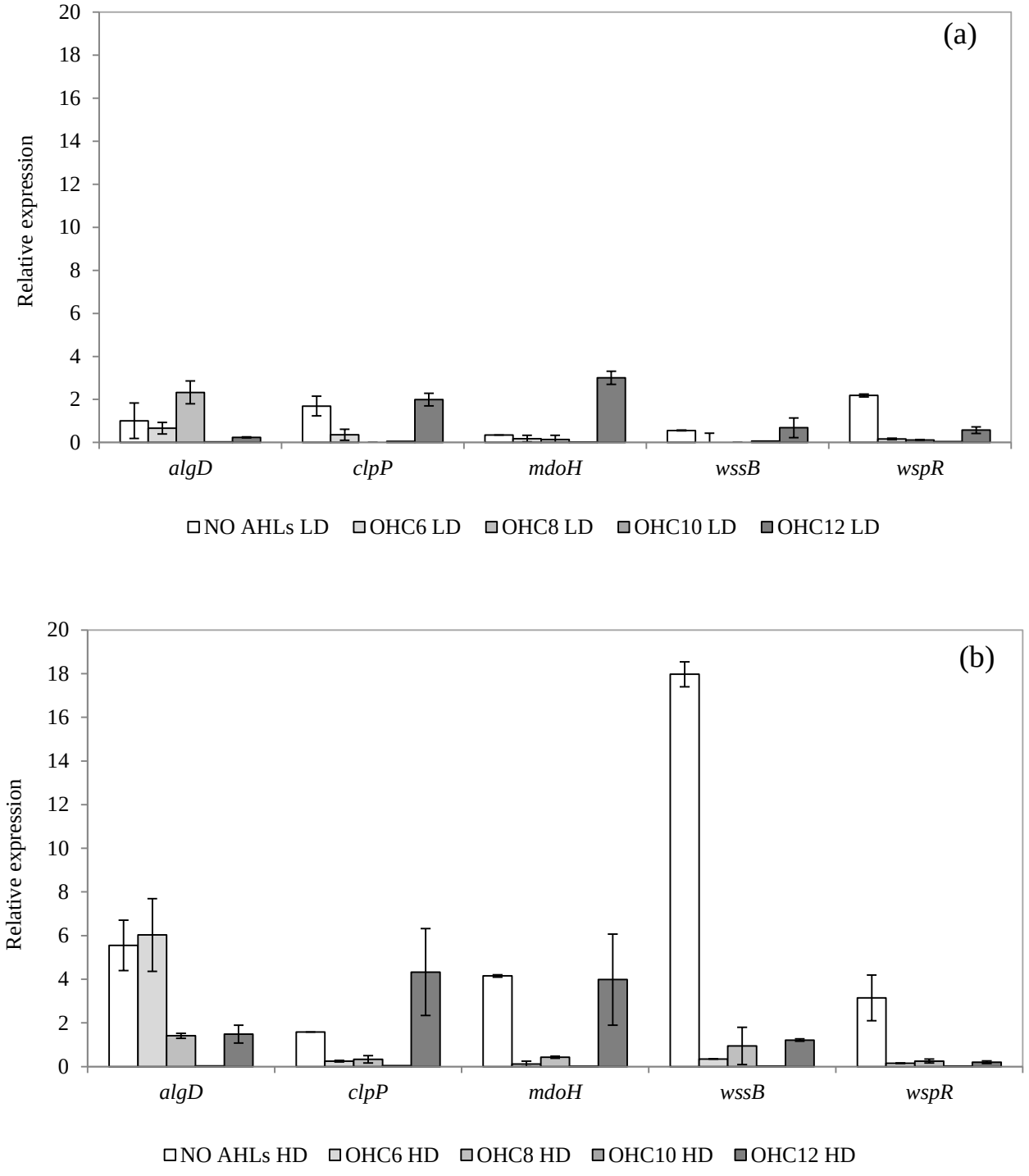
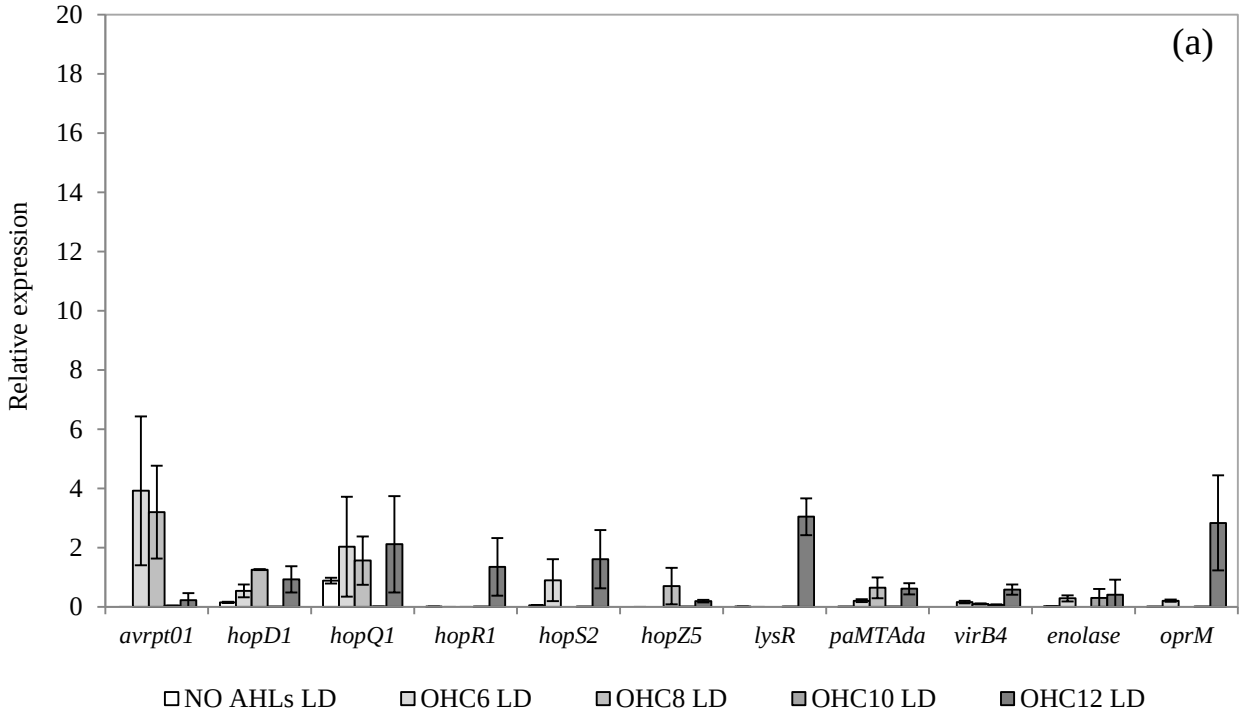


Figure 12. Expression of genes related to virulence of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.



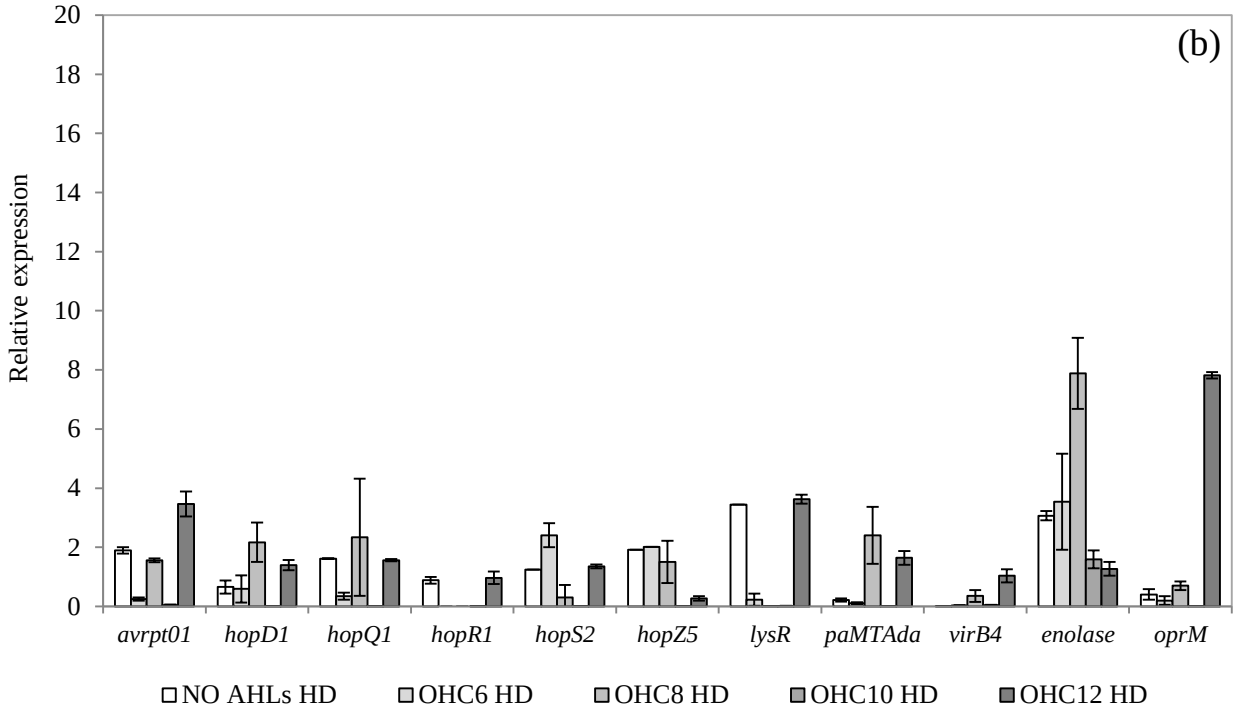


Figure 13. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286, Psa-mR1 and Psa-mR3 in the presence of 0.25µM OH-C6 and OH-C8-AHL. Biofilm was quantified after staining with crystal violet (0.5% w/v). The controls are represented by biofilm formation in LB without signals. Standard error bars are showed. Significance was calculated using Student's *t* test ($P < 0.05$). (*) indicates the difference from the control.

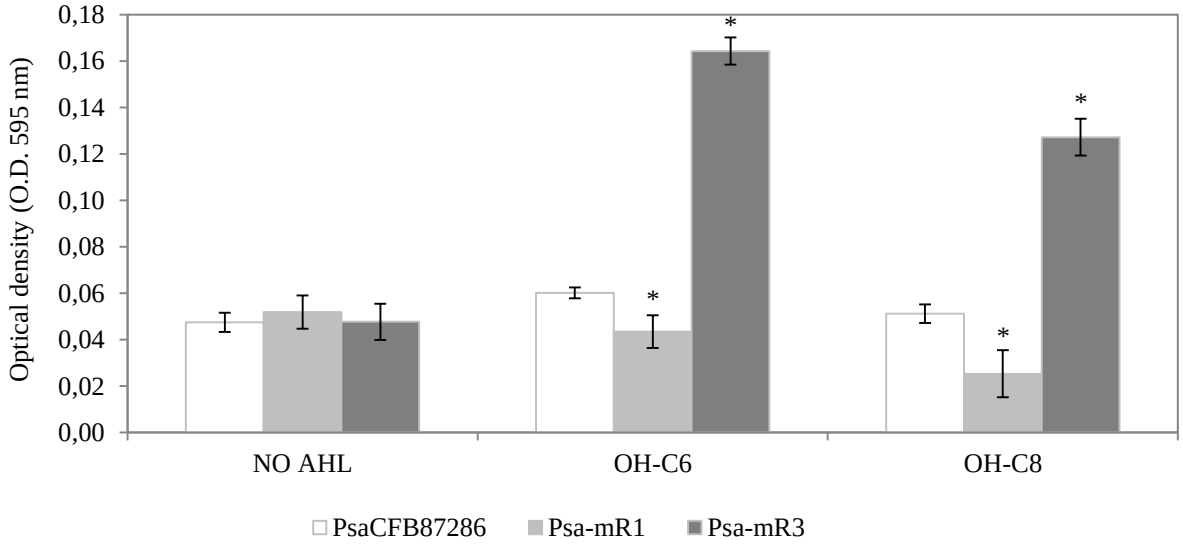


Figure 14. Representative pictures of Psa-AlgDsile swarming motility in Petri dish with IPTG (0.5%) added for active induction of gene silencing. a) PsaCFBP7286 + IPTG. b) Psa-AlgDsile + IPTG. Photographs were taken binocular Nikon SMZ25 fluorescence microscope (Zoom magnification 1x).

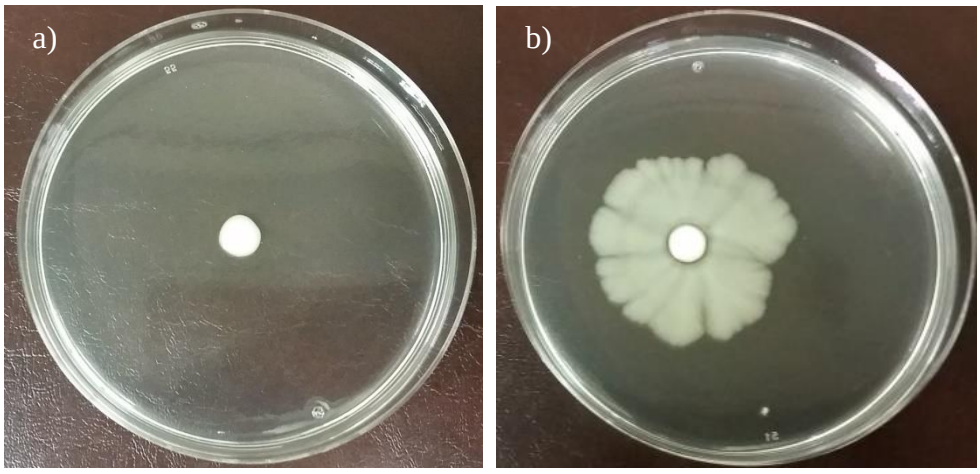
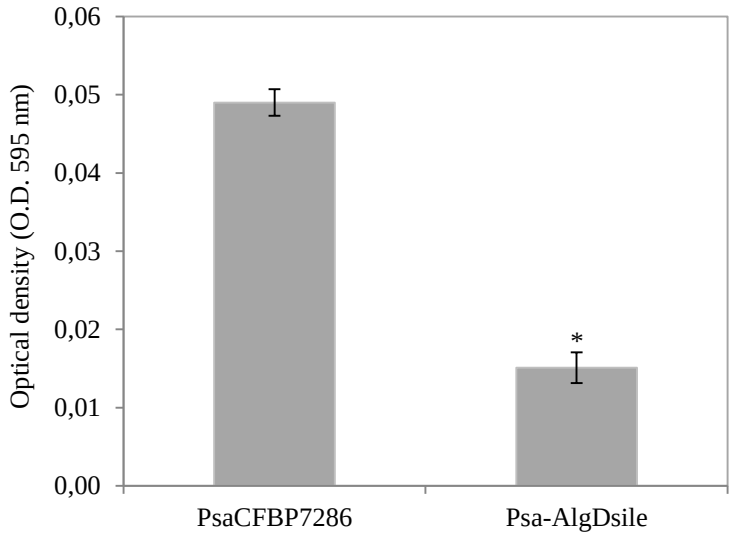


Figure 15. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 and Psa-AlgDsile in LB with IPTG (0.5%) added. Biofilm was quantified after staining with crystal violet (0.5% w/v). Standard error bars are showed. Significance was calculated using Student's *t* test ($P < 0.05$). (*) indicates the difference from the control.



The interspecific signalling in kiwifruit phyllosphere: how *Pseudomonas syringae* pv. *actinidiae* interact with other kiwifruit pathogens

Luca Fiorentini[1], Giampaolo Buriani[1], Joel Vanneste[2], Guglielmo Costa[1] and Francesco Spinelli[1]

[1] Department of Agricultural Sciences, Alma Mater Studiorum -University of Bologna, viale Fanin 46, 40127 Bologna – Italy,

[2] Bioprotection, The New Zealand Institute for Plant & Food Research Ltd.,
Ruakura Research Centre, Hamilton, New Zealand

Abstract

Bacterial pathogens use an array of molecular sensors to perceive and facilitate adaptation to changes in their environment. *Pseudomonas syringae* pv. *actinidiae* (*Psa*), the causal agent of the bacterial canker of kiwifruit plants, shows density dependent behaviours. Biofilm formation, virulence and motility appear to be finely regulated by the PsaR1 and PsaR3 receptors, which belong to the class of LuxR-solos signal receivers. Since those receptors reacts to exogenous AHLs signals that may also be produced by other bacteria of the kiwifruit epiphytic biocoenosis, the aim of this work was to investigate the influence of *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* on the cell density depend behaviours of *Pseudomonas syringae* pv. *actinidiae*. *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* increased motility, biofilm formation and virulence in *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava*. The inhibitory effects of the biocontrol agent *Pantoea agglomerans* were confirmed, but not those of *Pseudomonas fluorescens*. Molecular analysis on gene expression by qPCR confirmed *in vitro* and *in vivo* evidences. Furthermore, a bioinformatics research based on homologies in promoter sequences of several signal synthases led to the finding of a transcriptional regulator (*Tr*) that responded to bacterial extracts. RNA silencing of the *Tr* revealed that it acted as positive regulator of the biofilm and negative of the motility. Eventually, *psaR1* and *psaR3* signal receptors gene knock-out indicated their involvement in intercommunications. These innovative results suggest that the bacterial cross-talk may influence *Psa* ecological fitness and may aid towards its successful adaptation and survival.

Keywords: cross-talk, *Pseudomonas viridiflava, Pantoea agglomerans, Pseudomonas fluorescens*, biofilm, motility, virulence, qPCR.

Introduction

Bacteria have been often studied as isolated cells that act independently by their neighbours. However, in the last decade a large amount of evidence showed that many bacteria species present intra and interspecific signalling systems strongly contributing to their adaptability and metabolic plasticity (Lindow and Brandl, 2003). Bacteria interact with the surroundings by exchanging information with other cells belonging to their own community or other species (Keller and Surett, 2006), thus exploring optimal growth conditions and sensing and responding to environmental stress (Camilli and Bassler, 2006).

Bacterial communities interact with each other through a great variety of signals, mainly small molecules such as butyrolactones, quinolones, oligopeptides and others (Danhorn and Fuqua., 2007). In gram-negative bacteria, The most common type of intercellular signalling molecules are N-acylhomoserine lactones (AHLs), (Holden *et al*., 1999). Many AHLs cross membranes freely and are detected in the cytoplasm by LuxR-type proteins (Miller and Bassler, 2001). Upon ligand binding, the LuxR-AHL complexes bind DNA promoter elements and activate transcription of specific genes (C. Fuqua and P Greenberg, 2002). The specificity of the LuxR-AHL interaction is conferred by an acyl binding pocket in the LuxR protein, which precisely accommodates the acyl chain of its cognate AHL signal. Communication both at cell and at population levels triggers the constitution of complex multispecies communities in which each component module its behaviour in response to continues changes of the environments (Bassler, 2002).

Moreover, phyllosphere and rhizosphere communities have been demonstrated to affect virulence of many plant pathogenic bacteria (Mendes *et al*., 2013; Turner *et al*., 2013). The phyllosphere is a habitat colonized by a variety of microorganisms. It harbours saprophytes, as well as plant-pathogenic bacteria (Wiken Dees *et al*., 2015). Up to now it is not clear how bacteria populations are able to survive and be stable in the phyllosphere in spite of environmental challenges or against the emergence of non-cooperating cheater mutants. Current explanations suggest that specific, species-dependent defence mechanisms or simple mechanical protection present in particular communities (such as seen in biofilms) may be the major factors underlying the stability of multispecies consortia (Venturi et al., 2010). Bacteria mostly grow as microbial consortia in which different species of microorganisms act together as a community and where interspecies signalling through the action of diffusible signal molecules often occurs (Ryan and Dow, 2008; Duan *et al*., 2009). It is widely accepted that phytopathogens needs to interact with biocoenosis in order to attempt pathogenesis. These interactions occurs mainly in three ways: (i) a disease complex where disease is the result of the interaction of several plant pathogens belonging to the same species or phylum; (ii) more rarely disease complex due to different plant pathogens belonging to different phyla (Lamichhane and Venturi, 2015); (iii) phytopathogens can cooperate via quorum sensing-like involved molecules with non-pathogen epiphytes in order to became more aggressive (Buonaurio *et al*., 2015).

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 *Actinidia chinensis*. The pathogen can penetrate through natural openings such as flowers, stomata, lenticels and wounds, and spreads rapidly in the host's tissues, causing the plant death and severe

crop losses (Spinelli *et al*., 2011). Psa features density-related signal receptors, namely PsaR1 and PsaR3, that responded to synthetic homoserine-lactones (Fiorentini 1). These receptors belong to the family of "LuxR-solos" proteins (Subramoni and Venturi, 2009). LuxR-solos" bind exogenous AHLs produced by other neighbouring bacteria (Ahmer 2004), fulfilling, in this way, a key role in the epiphytic fitness of the bacterial communities. Since phenotypes such as biofilm formation and motility are often the result of the production of signals and group coordination (Danhorn and Fuqua, 2007), but also may be the result of the bacterial cross-talk (Holden *et al*., 1999), these typical social behaviours were evaluated during the interaction of Psa with other bacteria which colonizes the same phyllosphere.

The aim of the work was to investigate the role of the other bacteria of the epiphytic biocoenosis on Psa motility, biofilm formation and virulence. Biofilm production and motility, that are two important traits of preliminary host colonization and invasion (Bais et al., 2004). These two phenotypes are strictly interdependent. In fact, motility is involved both in biofilm formation and dispersal, an essential feature for the invasion of new habitats (Sauer et al. 2002). Furthermore, within bacterial biofilms, the cells adhere to surfaces, increasing their fitness during environmental challenges (Costerton et al., 1995; Prakash et al., 2003). Moreover, virulence *in vivo* and gene expression profiles associated to biofilm formation, motility, density and virulence were analysed in order to establish the entity of the interaction between *Psa* and the microbial *biocoenosis*. Ecological implications of the bacterial cross-talk are presented and discussed.

Materials and methods

Bacterial species, culture conditions and bacteria quantification

The bacterial species, strains and plasmids used in this study are listed in table 1. The *Pseudomonas syringae* pv. *actinidiae* (Psa) strains belong to the high virulent biovar 3 which is responsible for the present p7286andemic. *Escherichia coli* and all other strains tested, including PsaCFBP7286 wild type, mutants and silenced strain, were grown on Luria-Bertani (LB) medium (Sambrook *et al*., 1989) at appropriate temperature (37°C *E. coli*; 27°C all the other strains) under moderate shaking (120 rpm). Each actively growing culture was initiated by inoculating the appropriate sterile medium with a two days old single colony grown in LB plates. Low density cultures were obtained by inoculating 100 ml LB or bacterial supernatant LB till bacterial growth reached a turbidity lower than $OD_{600} < 0.010$. High density cultures were obtained in 20 ml LB or bacterial supernatant LB from $OD_{600} > 0.100$-0.200, when Psa reached the late log phase. Optical density was measures in cuvettes at 600 nm wavelength using spectrophotometer (Biochrom Libra, Cambridge UK). Bacterial population was also enumerated by direct plating 3 replicates of each 10-fold serial dilution. Colonies were counted after two days incubation.

Two Psa mutants, Psa-mR1 and Psa-mR3 (courtesy provided by dr. V. Venturi, ICGEB Trieste, IT) were used in order to elucidate the role of PsaR1 and PsaR3 receptors, that responded to homoserine (Fiorentini 1) and are supposed to be involved in the signaling with neighboring bacteria by AHLs eavesdropping (Patel et al., 2014), in the interspecific studies conducted in this work with bacterial supernatant.

Bacterial supernatant

In order to study the influence of microbial phyllosphere on biofilm formation, motility, virulence and gene expression of *Pseudomonas syringae* pv. *actinidiae*, liquid supernatants were obtained. Bacterial strains were grown on LB agar plates and two days old single colonies were stab inoculated in LB liquid medium. After an overnight growth, LB cultures reached the late log phase and were consequently centrifuged (15000 rpm, 10 min, 4°C) and the supernatants were filter-sterilized with a 0.02 μm pore membrane (Millipore, Billerica, Massachusetts, US). LB-supernatant media were promptly used.

Motility assay

Motility was assessed according to (Kinscherf and Willis 1999). A sterile disk of filter paper disk (6 mm Ø) was centrally placed on a LB plates (Sigma-Aldrich, Saint Louis, St. Louis, MO, USA) containing 0.4% agar. Motility was proved to be expressed by Psa colonies from high density *inocula* (Fiorentini 1). Therefore, paper disk was placed in the centre of the plate and inoculated with a 10μl drop of high density bacterial suspension (10^8 CFU ml^{-1}) adjusted to contain 1 x 10^7 cells ml^{-1}. Plates were then incubated at 27°C for 5 days. Bacterial spread and the surface of the plate covered by the colony was evaluated using the MacBiophotonics ImageJ 1.48 software (MacBiophotonics, Hamilton, ON, Canada).

To assess motility in the presence of bacterial supernatants, 1 ml of supernatant-LB was homogenously added on top of the plate and let dried off before inoculating with a 10μl drop of high density bacterial

suspension (10^8 CFU ml^{-1}) adjusted to contain 1 x 10^7 cells ml^{-1}. Microscopic visualization of motility in soft agar was performed using a binocular Nikon SMZ25 fluorescence microscope (Nikon Instruments Corporation, Tokyo, JAPAN) under UV source (excitation wavelength of GFP-B: 460-500 ηm, emission wavelength: 510–560 ηm). The 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. Images were obtained and processed by software Nikon NIS ELEMENTS V. 4.30.02 (Nikon Instruments Corporation, Tokyo, JAPAN)

Biofilm assay

The assay was performed as described by Taguchi *et al*., 2006. A volume of 3 ml of LB medium or filter-sterilized LB-supernatant medium was added into a 35 mm polystyrene Petri dish and inoculated with 50 µl of a 10^8 CFU ml^{-1} fresh liquid culture of Psa. After inoculation, plates were sealed with parafilm and incubated at 27°C with slow shaking at 70 rpm for 5 days. Successively, the bacterial population in each tube was assessed by serial dilutions and plating as previously described. Plates were thoroughly rinsed with distilled sterile water and dried for 45 min under laminar hood at room temperature. Thereafter, 3 ml of a crystal violet water solution (0.5% w/v) (Sigma-Aldrich) were added to each plate. The plates were incubated for 60 minutes at room temperature under shaking (70 rpm) and subsequently washed thoroughly with distilled water to remove nonspecific staining. For quantitative analysis of biofilms, crystal violet was re-solubilized by adding 3 ml of ethanol 95%. The solution was transferred to cuvette and absorbance at 595 nm was measured by using spectrophotometer. Aspecific staining was also quantified by with the same procedure on sterile, non-inoculated LB petri dishes. Unspecific absorbance was subtracted from all the measures. For each species and conditions 10 plates were inoculated with 50 µl of a 10^8 CFU ml^{-1} fresh liquid culture of Psa and let grow to high density for efficient cell adhesion and quantification (Ghods et al. 2015).

Virulence assay

The effect of bacterial supernatant on PsaCFBP7286 virulence was performed *in vivo* by inoculating kiwifruit plants. Three months old micropropagated plants of *Actinidia deliciosa* cv. Hayward. The plants were grown on MS medium (Murashige and Skoog, 1962) 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}), GA3 (0.1 mg l^{-1}), adjusted to pH 5.7 with KOH. The plants were kept in a growing chamber for the whole duration of the experiments (22°C, 70% RH and a light/dark cycle of 16:8 hours). Psa was grown to log phase in filter-sterilized bacterial supernatant-LB or in fresh LB. Before the inoculation, Psa cultures were pelleted by centrifugation at 9000 rpm, 18°C for 10 minutes and re-suspended in sterile 10mM $MgSO_4$ in order to adjust them to the same cell concentration in the suspension (OD_{600} 0.200 corresponding approx. to 10^8 CFU ml^{-1}). The cell concentration in the suspension was successively confirmed by sequential dilutions and plating. Fifteen plants were used for each

treatment and divided into three repeats of three plants. At 12, 24, 72, 240 and 360 hours plant weight, symptoms and endophytic bacterial population was assessed. Each plant was ground 5 ml of sterile $MgSO_4$ (10mM) addition, the suspension was filtered and centrifuged for 5 minutes at 14000 rpm. Bacterial pellets were frozen in liquid N_2 and stored at -80°C for subsequent molecular quantification epiphytic populations. At the same time points, plant material was frozen in liquid N_2 and stored at -80°C for subsequent molecular quantification endophytic populations.

Transcriptional analysis

The effect of bacterial supernatant LB on Psa gene expression was investigated by qPCR. A number of genes putatively responding to cell density and social behavior were selected according to the current knowledge on Psa related bacterial species. The list of selected genes is reported in table 2. The aminoacidic sequences of the reporter genes were blasted in PsaCFBP7286 genome using FASTA sequence similarity searching tool (EMBL-EBI, Cambridge, UK). Standard parameters were maintained. Only identities greater than 60% were accepted (table 2). The corresponding nucleotidic sequences in Psa genome were elaborated with Geneious software (version R8) (Kearse *et al*., 2012).

Gene expression studies were performed on a comparable amount of bacterial cells obtained from two cell densities: 10^5 and 10^8 CFU ml^{-1}. Psa cultures either grown in LB or in bacterial supernatant were centrifuged (14000rpm, 4°C, 10min) and the pellet obtained were suspended with differential amount of $MgSO_4$ (10 mM) in order to obtain the same OD_{600} 0.200 corresponding approx. to 10^8 CFU ml^{-1}. The cell concentration in the suspension was successively confirmed by sequential dilutions and plating.

Total bacterial RNA was extracted form Psa culture using Total RNA Purification kit (Norgen Biotek Corp., Thorold, CA), whereas were extracted Spectrum Plant Total RNA kit (Sigma-Aldrich) was used for Psa suspension obtained from infected plants. RNA purity and quantity was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). An aliquot of 1 μg of purified RNA was reverse-transcripted using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem Life Technologies, Carlsbad USA) according to the manufacturer's recommendations. cDNA samples were used as template for QPCR which was performed with Quick SybrGreen chemistry (Applied Biosystem) in a 96 well spectrofluorometric thermal cycler StepOnePlus (Thermo Fisher Scientific Inc.). Each template was adjusted to a final concentration of 100 ηg of cDNA per reaction and run in triplicate. QPCR cycles were performed as follows: 1 cycle of 50°C 2 min, 1 cycle of 95°C 10 min, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute Melting-curve analysis was performed immediately after completion of the real-time PCR (95°C for 15 seconds, 60°C for 15 seconds). Gene expression was expressed as relative expression to reference housekeeping genes *recA*, *rpoD* that used in *Pseudomonad* (Shi-En Lu et al., 2005; Narusaka et al., 2011; Greenwald et al., 2012) The relative quantification of gene expression was evaluated using the comparative C_t method (Pfaffl, 2001). Prior of raw C_t analysis all primers efficiency was assessed using LingRegPCR software (Ruijter et al. 2009). The qPCR primers developed for each gene are listed in table 3. Beacon Designer V 8,0 (PREMIER Biosoft, Palo Alto CA, USA) and Primer3Plus (Untergasser *et*

al., 2007; Thornton and Basu, 2010) were used to design the primers. All primers pair were checked for specificity by end point PCR (performed as described for qPCR with Psa Genomic DNA as template).

RNA-silencing of *Tr* gene

A Psa *Tr* silenced strain was preliminary tested in biofilm and motility assays. Tr resulted from a bioinformatics research of signal synthase to which PsaR1 and PsaR3 responded (Steindler et al., 2008; Patel *et al*., 2014). In previous studies Tr gene expression was related to density (Fiorentini 1). To silence *Tr* gene the plasmid pHN678, kindly provided by Dr. N. Nakashima was used as scaffold (Nakashima and Tamura, 2009). Following the author's specifications, the plasmid was digested with the appropriate enzymes (NEB, New England Biolabs, Massachusetts, USA) (table 4). *Tr* was amplified with complementary restriction site at 5' and 3' ends (primers table 2), followed by amplicons purification using the QIAquick Gel Extraction Kit (Qiagen, Redwood City, USA) according to the manufacturer's instructions. The purified amplicon was digested as described above. Linearized vector and the purified amplicon were checked for purity and digestion on a 1% agarose gel stained with GelRED (Biotium Inc., Hayward, USA). The linearized vector and the amplicon (1:6 ratio) were ligated in a volume of 15 µl with Quick Ligation Kit (NEB). An aliquot of 2 µl was used to transform electrocompetent *E.coli* DH5α cells. Transformed cells were plated on selective LB amended with chloramphenicol (25µg/ml). The presence of the plasmid inside the growing colonies was confirmed by sequencing. The plasmid pHN678-TR was extracted and purified from the selected colonies by using a QIAprep minipre kit (Qiagen). Plasmid quantity and purity was verified by NanoDrop spectrophotometer and agarose gel analysis. The purified plasmid was used to transform by electroporation competent Psa-CFBP7286 cells as described above. The efficacy of the silencing was tested on transformed Psa-TRsile cultures grown on liquid LB amended with 0.5% IPTG for plasmid expression. From these cultures, RNA was (i) extracted, (ii) purified using total RNA extraction KIT (Sigma-Aldrich), (iii) reverse-transcripted to cDNA by using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.) (iv) and amplified with gene specific primers.

Flower sampling

Flowers of *Actinidia chinensis* cv. JinTao were collected in commercial orchards located in Faenza region (Emilia Romagna, Italy). Prior to sampling, plants were divided in three distinct disease classes according to symptomatology of the bacterial canker on flowers and leaves: (i) heavy, (ii) medium and (iii) light. Flowers were sealed in a sterile bag and placed in ice immediately after sampling. For every disease class were randomly collected from different plants 60 flowers divided into three bags.

Flowers were washed in 5 ml of sterile 10 mM $MgSO_4$ in gentle agitation for 15 minutes. Washing solutions were pelleted by centrifugation at maximiun speed for 20 minutes. Then supernatant was discarded and pellets were frozen in liquid N_2 and stored a -80°C. From frozen pellet, genomic DNA were extracted and purified using NucleoSpin Soil kit (MACHEREY-NAGEL) following manufacturer's instruction. Purified gDNA was checked for concentration and purity using NanoDrop 1000 spectrophotometer

(Thermo Fisher Scientific Inc.) and used as template for qPCR populations determination. Primers used for identification and quantification of *Psa*, *Pss* and *P. viridiflava* are listed in table 5. Results are showed in supplementary information.

Statistical analysis

Each experiment was independently repeated at least twice. Where indicted, difference between data are calculated with the Student's *t* test. ANOVA and SNK test were applied to gene expression analysis data. Statistically significant differences were assumed for $P \leq 0.05$.

Results

Experiments with bacterial supernatant media

Growth curve of *Psa* in different supernatant-LB media

The effect of supernatants obtained by the cultures of other bacterial species on Psa growth was assessed in order to exclude possible detrimental effect due to the depletion of nutrients or the accumulation of inhibiting catabolites. The addition of the supernatants did not affect Psa growth till stationary phase (fig. 1a-b). At stationary phase, all the supernatants caused a comparable reduction of Psa population. Only the supernatant derived by *Pseudomonas fluorescens* strain A506 have an inhibitory effect on Psa (fig. 1a).

Motility of Psa on supernatant LB supernatants

The addition of filter-sterilized supernatants obtained from *Pseudomonas syringae* pv. *syringae* strain 4364, *Pseudomonas viridiflava*, *P. putida* strain IBE3 and *P. fluorescens* strain A506 stimulated Psa swarming motility. On the other hand, generally, the addition of the supernatants inhibited Psa swimming motility (tab. 7). The supernatants obtained by *E. coli* and *P. putida* IBE2 which do not produce AHLs did not affect Psa swarming motility. In comparison with the others, these two supernatants were also the less inhibitory of swimming motility

Biofilm quantitation of Psa in bacterial supernatant-LB media

The supernatant obtained from *Pseudomonas syringae* pv. *syringae* 4364, *Pseudomonas viridiflava*, *P. putida* IBE3 and *P. fluorescens* A506 enhanced biofilm formation in Psa (fig. 2). In the other hand, *P. agglomerans* strain P10c, *E. coli* DH5α and *P. putida* IBE2 inhibited biofilm production.

Gene expression of planktonic populations in bacterial supernatant-LB media

We previously demonstrated that *Psa* modulates phenotypes following the administration of signal molecules such as homoserine-lactones and, in part, this response was reflected on gene expression as a function of cell density. For this reason it was supposed that external signals produced by other bacteria can be perceived and therefore trigger in *Psa* important traits of host colonization and invasion (Fiorentini 1). Furthermore, from our preliminary experiments emerged that swarming and biofilm are influenced by microbial interactions. We wanted to investigate the existence and the influence of the bacterial cross-talk also at a molecular level, estimating the gene expression of planktonic populations of *Psa* cultivated in bacterial supernatant LB media. In order to appreciate if the priming effect of the intercommunication occurred dependently by population density, gene expression was evaluated both at low (LD) and high (HD) cell density. Since we estimated that the effect of *P. putida* strain IBE2 and *E. coli* strain DH5α on biofilm and motility was comparable, we decided to adopt as negative species *E.coli* strain DH5α only.

Density-related genes. These genes showed an induction only at high cell densities (fig.3 a-b). In particular, at HD the substrates of *P. syringae* pv. *syringae* strain 4364, *P. viridiflava*, *P. putida* strain IBE3 increased the gene expression of *hacA*, *psaR1* and the *Tr*.

Motility-related genes. Significative induction of the genes occurred at the high densities only (fig.4 a-b), where the greatest expression of the flagella gene *fliP* and pila gene *pilC* was induced by *P. putida* strain IBE3. However, also *P. viridiflava*, *P. fluorescens* strain A506 and *P. syringae* pv. *syringae* strain 4364 contributed to a slight increase of *fliP*. Moreover, *Psa* gene expression in supernatant of *P. agglomerans* strain P10c was significantly reduced.

Biofilm-related genes. At LD, apart from *wspR* in LB-P.VIR, gene expression was comparabile to the control (fig. 5a). In HD, *wspR* continued to be expressed more than the control in LB-P.VIR, but also in *P. syringae* pv. *syringae* strain 4364 and *P. fluorescens* strain A506 (fig. 5b). Interestingly, *clpP* in HD was inhibited in all the supernatant (*clpP* is a gene involved in the formation of the biofilm from glucose metabolism). Considering *Psa* espression in LB-IBE3 at HD, that particular supernatant stimulated *algD* (therefore the synthesis of alginate) and *wssB* (belongs to an operon appointed to cell adhesion).

Virulence-related genes. At LD gene expression was comparabile to the control (fig. 6a). Interestingly, at HD, many virulence-related genes resulted to be affected by the supernatant in which *Psa* was cultured (fig. 6b). The largest number of genes stimulated were found in all the media of the "neighbouring bacteria" group. Whereas gene expression in LB-P10C was significantly inhibited.

Results grouped in table 8 design a clearer picture about the induction of *Psa* genes, releatively to the HD condition in which occurred the most significative gene induction.

Virulence of Psa cultures derived from bacterial supernatants

Since the analysis of the gene expression profiles showed a response of *Psa* to some co-culture media, revealing that *Psa* would modulate its gene expression relatively to cellular aggregation, motility and virulence, we examined whether the interaction with the secretions of other bacterial specie could influence *Psa* ability to colonise *Actinidia deliciosa* plants. The epiphytic fitness and phyllosphere colonization did not seem to be influenced by the treatments (fig. 7a). Despite no visive symptoms could be ever noted on leaves of none of the treatments, molecular quantifications of *Psa* populations derived from inoculums grown in supernatant LB of Pss4364 and *P. viridiflava* revealed that the two treatments, compared to the control (growth in LB), anticipated the endophytic invasion (fig. 7b). In particular, only the treatment with *P. viridiflava* after 15 days was significantly higher than the control condition. The two treatments with the BCAs similarly reduced the endophytic colonization in the first 10 days. Finally, the growth in supernatant of *P. putida* strain IBE3 reduced consistently the endophytic populations.

In table 8, the *in vivo* results are compared with the relative gene expression obtained from the previous analysis of the gene expression of planktonic populations in bacterial supernatant-LB media.

Bioassays with Psa mutants

PsaR1 and PsaR3 are two receptors that responded to homoserine (Patel *et al*., 2014). Being LuxR-like type receptors, they may be involved in regulation of QS-dependent phenotypes. For this reason, we used two mutants (courtesy provided by dr. V. Venturi, ICGEB Trieste, IT) in order to elucidate their potential involvement in the intraspecific signalling with other bacteria. Supernatants derived from the culture of other bacteria were provided to these mutants. Biofilm formation and motility which are, in many species, density-dependent behaviours were evaluated after the application of the supernatants.

Moreover, the transcriptional regulator named Tr (Fiorentini 1) showed an enhanced expression when the supernatant from supernatant Pss4364 and *P. viridiflava,* and PfA506 was added (fig 3a-b). Therefore, *Tr* was silenced with RNA silencing. Here we report our preliminary results on motility and biofilm formation in order to outline the importance of this gene in *Psa*.

Testing Psa-mR1 and Psa-mR3 strains in bacterial supernatant supernatants

Mutants Psa-mR1 and Psa-mR3 were tested in the presence of LB supernatant and compared to PsaCFBP7286 and to the condition with fresh LB.

Motility. supernatants obtained from *Pseudomonas syringae* pv. *syringae* strain 4364, *Pseudomonas viridiflava*, *P. putida* strain IBE3 and *P. fluorescens* strain A506 induced biofilm formation in the wild time strain an in the two mutants supernatant (fig. 8). In particular, LB-IBE3 added on top of the plates determined a consistent swarming phenotype of Psa-mR1. A significant effect was also noticed after addition of LB-PSS and LB-A506, for which 75% of the petri showed a swarming phenotype. Psa-mR3 in general resulted less or equal to PsaCFBP7286.

Biofilm. Even though the biofilm produced by PsaCFBP7286 was found to be stimulated by LB-PSS and LB-IBE3, this stimulation was however greater in Psa-mR3 (fig. 9). Compared to PsaCFBP7286, Psa-mR1 produced less biofilm in supernatant LB of Pss4364 and *P. viridiflava*. The stimulative effect of LB-A506 registered in PsaCFBP7286 was compromised in both mutants for the deletion of *psaR1* and *psaR3*.

Psa-TRsile mutant phenotyping bioassays

Motility. Psa-TRsile mutant swarmed a greater distance from the point of inoculation, spreading on large areas over the plates. Compared to PsaCFBP7286, the *Tr* silenced mutant growth in control conditions resulted in compact dendritic formation typical of the swarming motility. Moreover, in preliminary evaluations, bacterial supernatant of Pss4364, PfA506, *P. viridiflava* and *P. putida* strain IBE3, had a similar effect (data not shown).

Biofilm. Compared to PsaCFBP7286, the effect of *Tr* silencing resulted in a severe reduction of biofilm production either in fresh LB and bacterial supernatants (fig. 10).

Microbial consortia in *A. chinensis* flowers

The relative presence of the three main bacterial pathogens of kiwifruit (*P. syringae* pv. *actindiae*, *P. syringae syringae*, *P. viridiflava*) was determined by qPCR from gDNA extracted from the bacterial pellets collected from washing solutions of *A. chinensis* flowers (see material and methods). The flowers were divided into three classes of disease severity (light, medium and heavy symptoms), and their analysis revealed that the pathogens were detected in all samples (figure S1, supplementary information). Moreover, while *Psa* populations varied according to disease severity, *Pss* and *P. viridiflava* epiphytic and endophitic populations remained constant, despite the major or minor presence of *Psa*. This result provides evidence of an effective bacterial consortium *in planta*.

Discussion

Motility and biofilm are influenced by microbial *biocoenosis*

Our results demonstrated that bacterial supernatants, since have had any particular effect on growth and the trend of *Psa* growth curves is the same compared to the control, could be used for biofilm, gene expression and motility applications. Only for *Pseudomonas fluorescens* strain A506 supernatants, *Psa* growth appeared to be influenced perhaps by the typical vigorous growth of PfA506 that usually is better sustained by the addition of glucose (Pereira *et al*., 2002) and therefore could have partially impoverished the media.

Psa swarming motility was stimulated by the presence of bacterial supernatant media derived from the neighbouring bacterial species and AHLs signal-producers Pss4364, PfA506, and *P. putida* strain IBE3. In addition, *Psa* on the other media tested showed no difference compared with the standard LB, thus demonstrating the reliability of the method when bacteria that do not produce signals (i.e. *P. putida* strain IBE2, *E. coli* strain DH5α) or effective antagonists (i.e. *P. agglomerans* strain P10c) are used. Interestingly, also the supernatant of *P. viridiflava* enhanced the swarming phenotype. This species share with *Psa* the same environment and was found on *Actinidia chinensis* flowers together with *P. syringae* pv. *syringae* (see supplementary information). Moreover, it is not clear in literature whether *P. viridiflava* produces AHLs. Interestingly, LuxR-solos have been shown to respond to endogenous signals which are not AHLs (Brachmann *et al*., 2013) and *Psa* displays at least of two (PsaR1 and PsaR3). Therefore, the influence of *P. viridiflava* on *Psa* swarming results even more interesting as it highlights the possibility that there could also be a signalling system not mediated by homoserine-lactones. However, we also have to consider the possibility that *Psa* motility could have been the result not only of signal perception but also of molecules that would act as stimulants of the movement such as biosurfactants. In fact, AHL-dependent gene regulation is required for swarming motility in *Pseudomonas aeruginosa* and *Serratia liquefaciens* by promoting the synthesis of biosurfactants that facilitate the spreading of bacteria on the surface (Köhler *et al*. 2000; Lindum *et al*. 1998). *P. syringae* pv. *syringae* swarming motility was demonstrated to rely on flagella and surfactant production (Kearns, 2010) and in particular on syringafactin and 3-(3-hydroxyalkanoyloxy) alkanoic acid, whose biosynthesis is ruled respectively by *syfA* and by *fleQ* and *rhlA* and where also the global regulator of secondary metabolites and extracellular enzymes *gacS* is involved (Burch *et al*., 2012). These genes are shared by *Psa* (*syfA* 84%, *fleQ* 98%, *rhlA* 48% of identity), indicating that swarming motility also in *Psa* can be mediated by the signalling and rely on perception and production of biosurfactants. The effect of bacterial supernatant was also confirmed by the expression of the gene *fliP*, responsible for the biosynthesis of flagellar proteins. In fact, *fliP* was clearly induced by the supernatant of Pss4364, PfA506, *P. putida* strain IBE3 and *P. viridiflava*. In addition, *E. coli* induced a significant expression of *rpoN* probably due to the impoverishment of the media, associated to the perception of a "hostile" environment from which it was necessary to escape, being *rpoN* involved in flagellar formation and nitrogen assimilation in limiting conditions (Lardi *et al*., 2015).

As regards biofilm production in liquid cultures of *Psa*, the supernatant media of *E.coli* strain DH5α and *P. putida* strain IBE2 led to a decrease of biofilm, comparable to that of the biocontrol agent *P. agglomerans* strain P10c. This result could be due to the fact that in those substrates have been released substances that in some way contrasted the cell attachment to the surfaces. For this reason, further investigations on peculiar composition of those supernatant will be necessary. However, more importantly, the biofilm quantitated was found to be increased by bacterial supernatant LB of neighbouring bacterial species or AHLs signal-producers PfA506, *P. viridiflava*, *P. putida* strain IBE3, and above all by Pss4364. This is of little surprise, because *Psa* biofilm formation is supported by AHLs (Fiorentini 1) and one of the roles of biosurfactants production in bacteria includes not only motility but also biofilm structure maintenance (Raaijmakers *et al*., 2010). Gene expression analysis allowed to go further in understanding these mechanisms. *wspR*, that codes for the regulator of cellular aggregation was induced by the supernatant of *P. viridiflava*, both at high and low cell densities. Therefore, *P. viridiflava* could potentially favour, under natural conditions, the colonization of small populations of *Psa*. Moreover, *wspR* was induced by Pss4364 and PfA506, but not by *P. putida* strain IBE3 in high cell density populations of *Psa*. However, IBE3, which is a *P. putida* strain that synthetize consistent amounts of different homoserine-lactones, stimulated *algD* and *wssB*. Interestingly, these two genes, that are related to alginate synthesis and surface adhesion, were not induced by administration of AHLs in previous experiments (Fiorentini 1). Therefore, *P. putida* strain IBE3 effect on *algD* and *wssB* or it is not attributable to AHLs production, or it may depend either on AHLs concentration or on the synthesis of signals, different from the synthetic AHLs, that determined that response in gene expression.

An ecological interpretation of what has been here presented consists in that *Psa* and the species with which it has co-evolved work together to efficiently colonize the environment. This effect is taken to an extreme when *Psa* instead perceives efficient antagonist that inhibits *Psa* from gene expression up to phenotypes manifestations.

Bacterial cross-talk plays a crucial role in *in vivo* virulence

The growth in exhausted media leads *Psa* to an indirect contact with other bacteria. However, this method is useful in assessing the effect that substances released and secreted by other bacteria can have on this pathogen. When we analysed the virulence of *Psa* grown in different bacterial supernatant LB, the treatment with the biocontrol agent *P. agglomerans* strain P10c reduced *Psa* endophyte populations. This evidence was strongly confirmed by the significant inhibition of pathogen-related genes of *Psa* cultured in LB-P10c. In contrast, Pss4364 and *P. viridiflava* anticipated the endophytic invasion of the host. In fact, three days after inoculation of the plantlets, these media magnified the endophyte growth of two and three orders respectively, compared to the standard LB. The analysis of the genes linked to the pathogenesis revealed that there was not an induction in low cell densities by any bacterial supernatant, but in high cell density conditions only. The major induction occurred in the media of the neighbouring bacteria, where each of them activated differentiated target genes. This result is very interesting, because *Psa* was demonstrated to be able to perceive those bacterial species with whom it evolved on the plant-host (*P. viridiflava* and *P. syringae*) and consequently activate ecological fitness mechanisms that favour the adaptation. Moreover, *P. viridiflava* and Pss4364 significantly magnified *oprM* expression. This gene codes for proteins that constitute the outer part of the multi-compomnent efflux transporter that in Gram-negative bacteria catalyze the active efflux of antibiotics and virulence factors in the outer spaces (Phan *et al*., 2010). This would in part explain why *P. viridiflava* and Pss4364 treatments anticipated the *in vivo* invasion of *Psa* into the host tissues. Very interesting it is the case represented by knot disease of the olive tree caused by *Pseudomonas savastanoi* pv. *savastanoi* (Psv). It has been experimentally demonstrated that the virulence of Psv AHL synthase mutant is restored by co-inoculations with the bacterial species that are part of its bacterial consortium on olive plants (*P. agglomerans* and *Erwinia toletana*), that are also signal producers (Hosni *et al*., 2011). Likely to mutant of Psv, the wild type of *Psa* has not the synthase and its virulence was increased by bacterial consortia. This demonstrates the importance of bacterial consortia in the regulation of *Psa* epiphytic fitness.

In addition, the fact that most of the virulence genes were mildly induced would appear in contradiction with *in vivo* assay. Therefore, we can speculate that *Psa* virulence in strict sense relies mostly on mechanisms of recognition between the plant and the pathogen, whereas in a broad sense it can be the result of the bacterial cross-talk and its influence on the ecological-fitness. The fact that the effect of the treatment with synthetic AHLs resulted in a lack of induction of *in vivo* virulence and pathogenesis related genes of *Psa* (Fiorentini 1) would support this hypothesis. We can also suppose that most likely AHLs alone are not sufficient for virulence, but it is a complex of bacterial molecules perceived that provides *Psa* the necessary stimuli.

Gene expression reveals that bacterial cross-talk may influence ecological fitness of *Psa*

The study of the relative expression of density-related genes produced relevant information on the interspecific cross-talk. First of all, gene expression was significant only at high cell densities. Secondly, the negative control that was used, *E. coli*, determined a relative expression that was comparable to the standard condition in fresh LB. This indicates that the methodology adopted allowed to highlight the effect of the bacterial supernatant on *Psa* gene expression unspoiled of false inductions.

The expression of the AHLs acylase *hacA* indicated that the production of this long-chain AHLs degradative enzyme was stimulated in *P. putida* strain IBE3 and *P. viridiflava* bacterial supernatant. *Psa* possesses three AHLs acylases (*hacA, hacB, hacC*) that were induced also by AHLs (Fiorentini 1). AHLs acylases are produced by bacteria to influence antibiotic production of other strains via quorum-sensing interference (Morello *et al*., 2004) or with the aim of exploiting AHLs breakdown products as carbon sources (Huang *et al*., 2003). This could mean that *Psa* perceived in the supernatant LB the presence of AHLs and proceeded with their degradation. To confirm this, the expression of the AHLs receptor *psaR1* resulted in a similar trend to that of *hacA*.

PsaR1 and PsaR3 are LuxR-solos regulators and signal receptors intended for bacterial intercommunication (Patel *et al*., 2014) and their genes responded selectively to the bacterial supernatant tested. In particular, *psaR3* was expressed only in *P. putida* strain IBE3 and with the same intensity of *psaR1*. Interestingly, *psaR1* was greatly induced by *P. viridiflava* and moderately by Pss4364 and *P. agglomerans* strain P10c. These results indicate that *Psa* is an active signal eavesdropper and that the activation of the signal receptors can be responsible of the regulation related to bacterial cross-talk. This hypothesis seems to be corroborated by the induction of *gacS*/*gacA* two components system which occurred in Pss4364, *P. viridiflava, P. putida* strain IBE3 and *P. agglomerans* strain P10c, that in general are the bacterial supernatant that determined the greatest (positive or negative) effects on the phenotypes assayed in this work. *gacS*/*gacA* system is related to many mechanisms, such as AHLs metabolism, ecological fitness and to the regulation of surfactant production (Kinscherf and Willis, 1999). Consequently, when the interaction with neighbouring bacteria occurs, this interaction can trigger in *Psa* responses related to group behaviours. In addition, *aefR* and *fur* expression resulted less or comparable to the control condition, indicating that these regulators would operate independently from the relationships of *Psa* with the microbial *biocoenosis*. Furthermore, the putative *Tr* is overexpressed in *Psa* cultures grown in supernatant of Pss4364 and P. viridiflava, suggesting that it could function as a pivotal regulator during the intercommunications occurring with the bacterial *biocoenosis* and, in particular, with two species of bacteria that are hosted by kiwifruit plants. Moreover, the silencing of *Tr* showed that this regulator should act as a positive regulator of the biofilm and negative of the motility. These preliminary results will be supported by further investigations about virulence *in vivo* and qPCR analysis with the aim to elucidate the role of *Tr* in the manifestation of such phenotypes.

Role of PsaR1 and PsaR3 receptors

When bacterial supernatant were tested with Psa-mR1 and Psa-mR3 mutants, swarming motility was increased in Psa-mR1, whereas Psa-mR3 swarmed less or similarly to the wild type. This result is comparable to previous observations in the presence of AHLs (Fiorentini 1). Psa-mR1motility was particularly increased by the supernatant of *P. putida* strain IBE3, Pss4364 and PfA506, three AHLs producing bacteria. Thus there is a parallel between the two motility experiments in AHLs and the bacterial supernatant. Biofilm formation of the mutants in bacterial supernatant-LB showed that the media caused a significant difference between the two mutants. In fact, deletion of *psaR1* determined a lower production of biofilm in Pss4364, *P. viridiflava* and PfA506, not only compared to the mutant Psa-mR3, but also compared to the wild type. These evidence confirmed that PsaR1 regulator would act as a repressor of the motility, and at the same time as an activator of the biofilm formation, perhaps through interference with the signal transduction operated by PsaR3.

Conclusions

From the experiments conducted *in vitro* we evaluated an influence of some bacterial substrates on swarming and biofilm formation of *Psa*. These results were partially elucidated by the analysis of gene expression of *Psa* cultivated in bacterial supernatant media. In general, all gene expressions at low cell densities resulted very little. Only few targets demonstrated an evident induction and that is the case of motility-related genes. For the majority of the genes to express, a bigger community was needed. Thus *Psa* showed a double typology of control of gene regulation: one that is related to the perception of its own community, maybe due to cell-to-cell contact (Fiorentini 1), and another one that is dedicated to the recognition of external stimuli such as signals from the neighbouring bacteria, and presumably from the plant-host, thanks to which *Psa* obtains information about the conditions of the environment. We also assume that some regulators are key points in interspecific regulations. In fact, the AHLs signal receptors PsaR1 and PsaR3 and a novel transcriptional regulator Tr were demonstrated to be involved in biofilm production and motility regulation, two fundamental traits of the ecological fitness. It has also emerged that the virulence genes were poorly expressed in bacterial co-cultures. However, the *in vivo* tests showed a positive effect of two neighbouring bacteria (in particular *P. syringae* pv. *syringae* and *P. viridiflava*) and a negative effect of a biocontrol agent (*P. agglomerans*) on *Psa* endophyte colonization of *A. deliciosa* plantlets. Supposedly, a direct effect on virulence genes and pathogenesis mechanisms it is likely to be stimulated by the interaction and recognition processes that occur between *Psa* and the host-plant. Therefore, bacterial cross-talk would act more on the epiphytic fitness, as well as on colonization skills. Even though we conducted the experiments *in vitro* with an artifice, it was mimed a scenario that could occur in natural conditions, for example on plant surfaces, such as the leaves, that can be rich in nutrients and therefore sustain epiphytic growth and movement. For this reason, our results demonstrated a very important implication of the interspecific communication in *Psa* pathosystem. The putative phytopathogenic association composed by *Psa*, *P. syringae* pv. *syringae* and *P. viridiflava* showed that synergisms among the different bacterial species can take place either *in vitro* and in field conditions, indicating that the bacterial canker of kiwifruit can potentially take advantage of a poly-bacterial consortium. However, most likely also the other protagonists of the microbial consortium draw in turn benefits from *Psa*.

In conclusion, our experiments were conducted analysing singular strains synergism. Therefore next step of the research will be to evaluate the effect of *Psa* ecological fitness in multi-species interactions.

Reference

Ahmer, B. M. M. (2004). Cell-to-cell signalling in Escherichia coli and Salmonella enterica. *Molecular Microbiology*, *52*(4), 933–945. http://doi.org/10.1111/j.1365-2958.2004.04054.x

Appleby, J. L., Parkinson, J. S., & Bourret, R. B. (1996). Signal transduction via the multi-step phosphorelay: Not necessarily a road less traveled. *Cell*, *86*(6), 845–848. http://doi.org/10.1016/S0092-8674(00)80158-0

Argenio, D. a D., Argenio, D. a D., Calfee, M. W., Calfee, M. W., Rainey, P. B., Rainey, P. B., … Pesci, E. C. (2002). Autolysis and Autoaggregation in. *Microbiology*, *184*(23), 6481–6489. http://doi.org/10.1128/JB.184.23.6481

Bais, H. P., Fall, R., & Vivanco, J. M. (2004). Biocontrol of. *Society*, *134*(January), 307–319. http://doi.org/10.1104/pp.103.028712.zobacterium

Bassler, B. L. (2002). Small talk: Cell-to-cell communication in bacteria. *Cell*, *109*(4), 421–424. http://doi.org/10.1016/S0092-8674(02)00749-3

Brachmann, A. O., Brameyer, S., Kresovic, D., Hitkova, I., Kopp, Y., Manske, C., … Heermann, R. (2013). Pyrones as bacterial signaling molecules. *Nature Chemical Biology*, *9*(9), 573–8. http://doi.org/10.1038/nchembio.1295

Buonaurio, R., Moretti, C., da Silva, D. P., Cortese, C., Ramos, C., & Venturi, V. (2015). The olive knot disease as a model to study the role of interspecies bacterial communities in plant disease. *Frontiers in Plant Science*, *6*(June), 434. http://doi.org/10.3389/fpls.2015.00434

Burch, A. Y., Shimada, B. K., Mullin, S. W. A., Dunlap, C. A., Bowman, M. J., & Lindow, S. E. (2012). Pseudomonas syringae coordinates production of a motility-enabling surfactant with flagellar assembly. *Journal of Bacteriology*, *194*(6), 1287–1298. http://doi.org/10.1128/JB.06058-11

Camilli, A., & Bassler, B. L. (2006). Bacterial Small-Molecule Signaling Pathways. *Nature Reviews. Microbiology*, *311*(5764), 1113–1116. http://doi.org/10.1126/science.1121357.Bacterial

Cha, J. Y., Lee, J. S., Oh, J. Il, Choi, J. W., & Baik, H. S. (2008). Functional analysis of the role of Fur in the virulence of Pseudomonas syringae pv. tabaci 11528: Fur controls expression of genes involved in quorum-sensing. *Biochemical and Biophysical Research Communications*, *366*(2), 281–287. http://doi.org/10.1016/j.bbrc.2007.11.021

Chancey, S. T., & Wood, D. W. (1999). Two-Component Transcriptional Regulation of N -Acyl-Homoserine Lactone Production in Pseudomonas aureofaciens, *65*(6), 2294–2299.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Review of Microbiology*, *49*, 711–45. http://doi.org/10.1146/annurev.mi.49.100195.003431

Danhorn, T., & Fuqua, C. (2007). Biofilm Formation by Plant-Associated Bacteria. *Annual Review of Microbiology*, *61*(1), 401–422. http://doi.org/10.1146/annurev.micro.61.080706.093316

De Souza, A. A., Takita, M. A., Coletta-Filho, H. D., Caldana, C., Yanai, G. M., Muto, N. H., … Machado, M. A. (2004). Gene expression profile of the plant pathogen Xylella fastidiosa during biofilm formation in vitro. *FEMS Microbiology Letters*, *237*(2), 341–353. http://doi.org/10.1016/j.femsle.2004.06.055

Dees, M. W., Lys??e, E., Nordskog, B., & Brurberg, M. B. (2015). Bacterial communities associated with surfaces of leafy greens: Shift in composition and decrease in richness over time. *Applied and Environmental Microbiology*, *81*(4), 1530–1539. http://doi.org/10.1128/AEM.03470-14

Donati, I., Buriani, G., Cellini, A., Mauri, S., Costa, G., & Spinelli, F. (2014). New insights on the bacterial

canker of kiwifruit (Pseudomonas syringae pv. actinidiae). *Journal of Berry Research*, *4*(2), 53–67. http://doi.org/10.3233/JBR-140073

Duan, Y., Zhou, L., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., … Gottwald, T. (2009). Complete genome sequence of citrus huanglongbing bacterium, "Candidatus Liberibacter asiaticus" obtained through metagenomics. *Molecular Plant-Microbe Interactions : MPMI*, *22*(8), 1011–1020. http://doi.org/10.1094/MPMI-22-8-1011

Fuqua, C., & Greenberg, E. P. (2002). Listening in on bacteria: acyl-homoserine lactone signalling. *Nature Reviews in Molecular Cell Biology*, *3*(September), 685–695. http://doi.org/10.1038/nrm907

Ghods, S., Sims, I. M., Moradali, M. F., & Rehma, B. H. A. (2015). Bactericidal compounds controlling growth of the plant pathogen pseudomonas syringae pv. actinidiae, which forms biofilms composed of a novel exopolysaccharide. *Applied and Environmental Microbiology*, *81*(12), 4026–4036. http://doi.org/10.1128/AEM.00194-15

Greenwald, J. W., Greenwald, C. J., Philmus, B. J., Begley, T. P., & Gross, D. C. (2012). RNA-seq analysis reveals that an ECF ?? factor, AcsS, regulates achromobactin biosynthesis in pseudomonas syringae pv. syringae B728a. *PLoS ONE*, *7*(4). http://doi.org/10.1371/journal.pone.0034804

Guan, R., Ho, M. C., Fr??hlich, R. F. G., Tyler, P. C., Almo, S. C., & Schramm, V. L. (2012). Methylthioadenosine deaminase in an alternative quorum sensing pathway in Pseudomonas aeruginosa. *Biochemistry*, *51*(45), 9094–9103. http://doi.org/10.1021/bi301062y

Heeb, S., & Haas, D. (2001). Regulatory Roles of the GacS / GacA Two-Component System in Plant-Associated and Other Gram-Negative Bacteria. *Molecular Plant-Microbe Interactions*, *14*(12), 1351–1363. http://doi.org/10.1094/MPMI.2001.14.12.1351

Holden, M. T. G., Chhabra, S. R., De Nys, R., Stead, P., Bainton, N. J., Hill, P. J., … Williams, P. (1999). Quorum-sensing cross talk: Isolation and chemical characterization of cyclic dipeptides from Pseudomonas aeruginosa and other Gram-negative bacteria. *Molecular Microbiology*, *33*(6), 1254–1266. http://doi.org/10.1046/j.1365-2958.1999.01577.x

Hosni, T., Moretti, C., Devescovi, G., Suarez-Moreno, Z. R., Fatmi, M. B., Guarnaccia, C., … Venturi, V. (2011). Sharing of quorum-sensing signals and role of interspecies communities in a bacterial plant disease. *The ISME Journal*, *5*(12), 1857–1870. http://doi.org/10.1038/ismej.2011.65

Huang, J. J., Han, J., Zhang, L., & Leadbetter, J. R. (2003). Utilization of Acyl-Homoserine Lactone Quorum Signals for Growth by a Soil Pseudomonad and Pseudomonas aeruginosa PAO1 Utilization of Acyl-Homoserine Lactone Quorum Signals for Growth by a Soil Pseudomonad and Pseudomonas aeruginosa PAO1. *Applied and Environmental Microbiology*, *69*(10), 5941–5949. http://doi.org/10.1128/AEM.69.10.5941

Kalia, V. C. (2013). Quorum sensing inhibitors: An overview. *Biotechnology Advances*, *31*(2), 224–245. http://doi.org/10.1016/j.biotechadv.2012.10.004

Kawakita, Y., Taguchi, F., Inagaki, Y., Toyoda, K., Shiraishi, T., & Ichinose, Y. (2012). Characterization of each aefR and mexT mutant in Pseudomonas syringae pv. tabaci 6605. *Molecular Genetics and Genomics*, *287*(6), 473–484. http://doi.org/10.1007/s00438-012-0693-9

Kearns, D. B. (2010). A field guide to bacterial swarming motility. *Nat Rev Microbiol.*, *8*(9), 634–644. http://doi.org/10.1038/nrmicro2405.A

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649. http://doi.org/10.1093/bioinformatics/bts199

Keller, L., & Surette, M. G. (2006). Communication in bacteria: an ecological and evolutionary perspective. *Nature Reviews. Microbiology*, *4*(4), 249–58. http://doi.org/10.1038/nrmicro1383

Khöler, T., Curty, L. K., Barja, F., Van Delden, C., & Pechere, J.-C. (2000). Swarming of Pseudomonas aeruginosa Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili, *182*(21), 5990–5996. Retrieved from C:\Users\alo13\Documents\Citavi 5\Projects\Pseudomonas2\Citavi Attachments\11029417.pdf

Kinscherf, T. G., & Willis, D. K. (1999). Swarming by Pseudomonas syringae B728a requires gacS (lemA) and gacA but not the acyl-homoserine lactone biosynthetic gene ahlI. *Journal of Bacteriology*, *181*(13), 4133–4136.

Lamichhane, J. R., & Venturi, V. (2015). Synergisms between microbial pathogens in plant disease complexes: a growing trend. *Front Plant Sci*, *6*(May), 1–12. http://doi.org/10.3389/fpls.2015.00385

Lardi, M., Aguilar, C., Pedrioli, A., Omasits, U., Suppiger, A., C??rcamo-Oyarce, G., … Pessi, G. (2015). ??^{54}-Dependent response to nitrogen limitation and virulence in Burkholderia cenocepacia strain H111. *Applied and Environmental Microbiology*, *81*(12), 4077–4089. http://doi.org/10.1128/AEM.00694-15

Lindow, S. E., & Brandl, M. T. (2003). Microbiology of the phyllosphere. *Applied and Environmental Microbiology*. http://doi.org/10.1128/AEM.69.4.1875-1883.2003

Lindum, P. W., Molin, J. B., Givskov, M., Anthoni, U., Christophersen, C., & Eberl, L. (1998). MG1 liquefaciens Serratia Required for Swarming Motility of Extracellular Lipopeptide Biosurfactant Autoinducers Control Production of an N -Acyl-l-Homoserine Lactone, *180*(23), 6384–6388. Retrieved from http://jb.asm.org/content/180/23/6384\nhttp://jb.asm.org/content/180/23/6384#ref-list-1

Lu, S.-E., Wang, N., Wang, J., Chen, Z. J., & Gross, D. C. (2005). Oligonucleotide microarray analysis of the salA regulon controlling phytotoxin production by Pseudomonas syringae pv. syringae. *Molecular Plant-Microbe Interactions : MPMI*, *18*(4), 324–333. http://doi.org/10.1094/MPMI-18-0324

Marcelletti, S., Ferrante, P., Petriccione, M., Firrao, G., & Scortichini, M. (2011). Pseudomonas syringae pv. actinidiae draft genomes comparison reveal strain-specific features involved in adaptation and virulence to Actinidia species. *PLoS ONE*, *6*(11). http://doi.org/10.1371/journal.pone.0027297

Martin, P. R., Watson, A. A., McCaul, T. F., & Mattick, J. S. (1995). Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in Pseudomonas aeruginosa. *Molecular Microbiology*, *16*(3), 497–508. http://doi.org/10.1111/j.1365-2958.1995.tb02414.x

McCann, H. C., Rikkerink, E. H. A., Bertels, F., Fiers, M., Lu, A., Rees-George, J., … Templeton, M. D. (2013). Genomic Analysis of the Kiwifruit Pathogen Pseudomonas syringae pv. actinidiae Provides Insight into the Origins of an Emergent Plant Disease. *PLoS Pathogens*, *9*(7). http://doi.org/10.1371/journal.ppat.1003503

Mendes, R., Garbeva, P., & Raaijmakers, J. M. (2013). The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews*, *37*(5), 634–663. http://doi.org/10.1111/1574-6976.12028

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Review of Microbiology*.

Morello, J. E., Pierson, E. A., & Pierson, L. S. (2004). Negative cross-communication among wheat rhizosphere bacteria: Effect on antibiotic production by the biological control bacterium Pseudomonas aureofaciens 30-84. *Applied and Environmental Microbiology*, *70*(5), 3103–3109. http://doi.org/10.1128/AEM.70.5.3103-3109.2004

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. http://doi.org/10.1021/jf9040386

Nakashima, N., & Tamura, T. (2009). Conditional gene silencing of multiple genes with antisense RNAs and

generation of a mutator strain of Escherichia coli. *Nucleic Acids Research*, *37*(15). http://doi.org/10.1093/nar/gkp498

Narusaka, M., Shiraishi, T., Iwabuchi, M., & Narusaka, Y. (2011). rpoD gene expression as an indicator of bacterial pathogens in host plants. *Journal of General Plant Pathology*, *77*(2), 75–80. http://doi.org/10.1007/s10327-011-0298-x

O'Toole, G. A., & Kolter, R. (1998). Initation of biofilm formation in Pseudomans fluorescens WC365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology*, *28*, 449–461.

Patel, H. K., Ferrante, P., Covaceuszach, S., Lamba, D., Scortichini, M., & Venturi, V. (2014). The kiwifruit emerging pathogen Pseudomonas syringae pv. actinidiae does not produce AHLs but possesses three luxR solos. *PLoS ONE*, *9*(1). http://doi.org/10.1371/journal.pone.0087862

Penaloza-Vazquez, A., Sreedharan, A., & Bender, C. L. (2010). Transcriptional studies of the hrpM/opgH gene in Pseudomonas syringae during biofilm formation and in response to different environmental challenges. *Environmental Microbiology*, *12*(6), 1452–1467. http://doi.org/10.1111/j.1462-2920.2010.02160.x

Pereira, M. O., Kuehn, M., Wuertz, S., Neu, T., & Melo, L. F. (2002). Effect of flow regime on the architecture of a Pseudomonas fluorescens biofilm. *Biotechnology and Bioengineering*, *78*(2), 164–171. http://doi.org/10.1002/bit.10189

Pernestig, A. K., Melefors, Ö., & Georgellis, D. (2001). Identification of UvrY as the cognate response regulator for the BarA sensor kinase in Escherichia coli. *Journal of Biological Chemistry*, *276*(1), 225–231. http://doi.org/10.1074/jbc.M001550200

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), e45. http://doi.org/10.1093/nar/29.9.e45

Phan, G., Benabdelhak, H., Lascombe, M. B., Benas, P., Rety, S., Picard, M., … Broutin, I. (2010). Structural and Dynamical Insights into the Opening Mechanism of P. aeruginosa OprM Channel. *Structure*, *18*(4), 507–517. http://doi.org/10.1016/j.str.2010.01.018

Prakash, B., Veeregowda, B. M., & Krishnappa, G. (2003). Biofilms -A survival strategy of bacteria, *85*(9).

Quiñones, B., Dulla, G., & Lindow, S. E. (2005). Quorum sensing regulates exopolysaccharide production, motility, and virulence in Pseudomonas syringae. *Molecular Plant-Microbe Interactions : MPMI*, *18*(7), 682–693. http://doi.org/10.1094/MPMI-18-0682

Raaijmakers, J. M., de Bruijn, I., Nybroe, O., & Ongena, M. (2010). Natural functions of lipopeptides from Bacillus and Pseudomonas: More than surfactants and antibiotics. *FEMS Microbiology Reviews*. http://doi.org/10.1111/j.1574-6976.2010.00221.x

Ruijter, J. M., van der Velden, S., & Ilgun, A. (2011). LinRegPCR (11.0) - Analysis of quantitative RT-PCR data. *Heart Failure Research Center*, 1–29. Retrieved from papers://476b58c2-a19a-46f3-a8ef-897be4da28ce/Paper/p17039

Ryan, R. P., & Dow, J. M. (2008). Diffusible signals and interspecies communication in bacteria. *Microbiology*, *154*(7), 1845–1858. http://doi.org/10.1099/mic.0.2008/017871-0

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press*.

Sauer, K., Sauer, K., Camper, A. K., Camper, A. K., Ehrlich, G. D., Ehrlich, G. D., … Davies, D. G. (2002). Pseudomonas aeruginosa. *Society*, *184*(4), 1140–1154. http://doi.org/10.1128/JB.184.4.1140

Segura, A., Duque, E., Hurtado, A., & Ramos, J. L. (2001). Mutations in genes involved in the flagellar

export apparatus of the solvent-tolerant Pseudomonas putida DOT-T1E strain impair motility and lead to hypersensitivity to toluene shocks. *Journal of Bacteriology*, *183*(14), 4127–4133. http://doi.org/10.1128/JB.183.14.4127-4133.2001

Sheng, L., Gu, D., Wang, Q., Liu, Q., & Zhang, Y. (2012). Quorum sensing and alternative sigma factor RpoN regulate type VI secretion system i (T6SSVA1) in Wsh pathogen Vibrio alginolyticus. *Archives of Microbiology*, *194*(5), 379–390. http://doi.org/10.1007/s00203-011-0780-z

Shepherd, R. W., & Lindow, S. E. (2009). Two dissimilar N-aeyl-homoserine lactone acylases of pseudomonas syringae influence colony and biofilm Morphology. *Applied and Environmental Microbiology*, *75*(1), 45–53. http://doi.org/10.1128/AEM.01723-08

Spiers, A. J., Deeni, Y. Y., Folorunso, A. O., Koza, A., Moshynets, O., & Zawadzki, K. (2013). Cellulose Expression in Pseudomonas fluorescens SBW25 and Other Environmental Pseudomonads. *Cellulose - Medical, Pharmaceutical and Electronic Applications*, 1–26. http://doi.org/10.5772/53736

Spinelli, F., Donati, I., Vanneste, I. L., Costa, M., & Costa, G. (2011). Real time monitoring of the interactions between Pseudomonas syringae pv. actinidiae and Actinidia species. *Acta Horticolture*, *913*, 461–465. Retrieved from http://www.actahort.org/members/showpdf?booknrarnr=913_61

Steindler, L., Bertani, I., De Sordi, L., Bigirimana, J., & Venturi, V. (2008). The presence, type and role of N-acyl homoserine lactone quorum sensing in fluorescent Pseudomonas originally isolated from rice rhizospheres are unpredictable. *FEMS Microbiology Letters*, *288*(1), 102–111. http://doi.org/10.1111/j.1574-6968.2008.01344.x

Subramoni, S., & Venturi, V. (2009). LuxR-family “solos”: Bachelor sensors/regulators of signalling molecules. *Microbiology*, *155*(5), 1377–1385. http://doi.org/10.1099/mic.0.026849-0

Taguchi, F., Takeuchi, K., Katoh, E., Murata, K., Suzuki, T., Marutani, M., … Ichinose, Y. (2006). Identification of glycosylation genes and glycosylated amino acids of flagellin in Pseudomonas syringae pv. tabaci. *Cellular Microbiology*, *8*(6), 923–938. http://doi.org/10.1111/j.1462-5822.2005.00674.x

Thornton, B., & Basu, C. (2011). Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education*, *39*(2), 145–154. http://doi.org/10.1002/bmb.20461

Tian, Y., Wang, Q., Liu, Q., Ma, Y., Cao, X., & Zhang, Y. (2008). Role of RpoS in stress survival, synthesis of extracellular autoinducer 2, and virulence in Vibrio alginolyticus. *Archives of Microbiology*, *190*(5), 585–594. http://doi.org/10.1007/s00203-008-0410-6

Tran, H., Ficke, A., Asiimwe, T., Höfte, M., & Raaijmakers, J. M. J. M. (2007). Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. *New Phytologist*, *175*(4), 731–742. http://doi.org/10.1111/j.1469-8137.2007.02138.x

Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome, 1–10.

Ude, S., Arnold, D. L., Moon, C. D., Timms-Wilson, T., & Spiers, A. J. (2006). Biofilm formation and cellulose expression among diverse environmental Pseudomonas isolates. *Environmental Microbiology*, *8*(11), 1997–2011. http://doi.org/10.1111/j.1462-2920.2006.01080.x

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J. A. M. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research*, *35*(SUPPL.2), 71–74. http://doi.org/10.1093/nar/gkm306

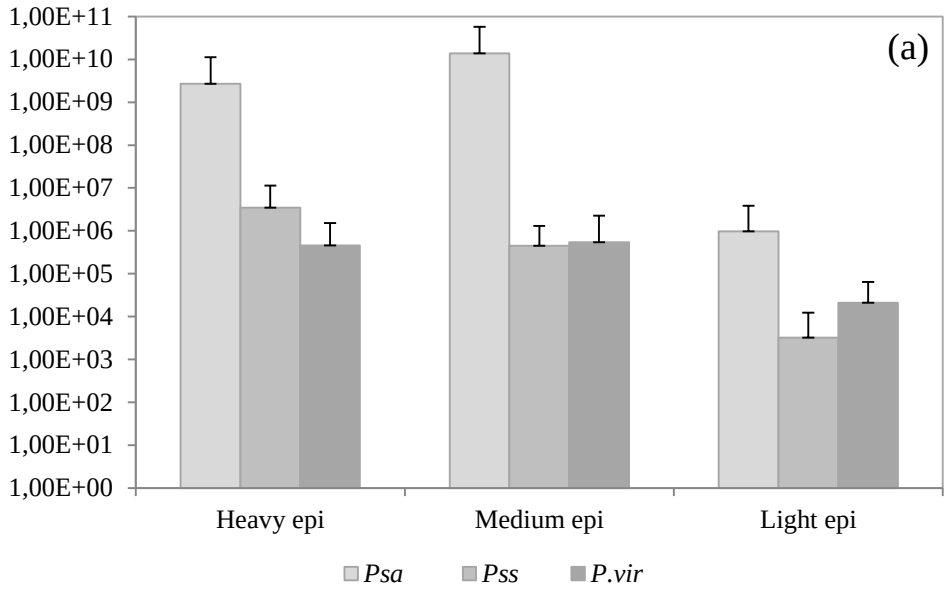
Vanneste, J. L., Cornish, D. A., Spinelli, F., & Yu, J. (2004). Colonisation of Apple and Pear Leaves By Different Strains of Biological Control Agents of Fire Blight. *Society*, *53*, 49–53.

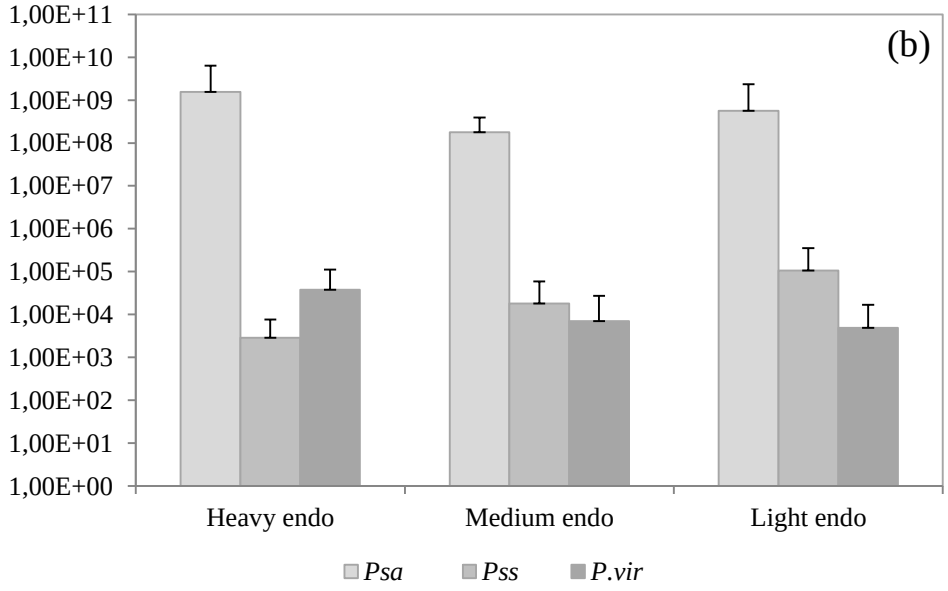
Venturi, V., Kerényi, A., Reiz, B., Bihary, D., & Pongor, S. (2010). Locality versus globality in bacterial

signalling: can local communication stabilize bacterial communities? *Biology Direct*, *5*, 30. http://doi.org/10.1186/1745-6150-5-30

Supplementary information

Figure S1. (a) Epiphyte and (b) endophyte populations found in *A. chinensis* flowers. Standard error bars are indicated.





Tables and figures

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference/Source
Pseudomonas syringae pv. *actinidiae* (Psa)		
Psa wild type strain CFBP7286	Cultivated in LB at 27°C	Spinelli et al 2011
Psa-mR1 (psaR1::pKNOCK); derivative of wild type	Cultivated in LB at 27°C Km^r (100 μg/ml)	Venturi (ICGEB)
Psa-mR3 (psaR3:: in-frame deletion mutant generated by pEX19Gm plasmid); derivative of wild type	Cultivated in LB at 27°C Nf^r (150 μg/ml)	Venturi (ICGEB)
Psa-TRsile (Psa harbouring pSileTR)	Cultivated in LB at 27°C; gene silencing is induced with IPTG 0.5%; Chl^r (25 μg/ml)	This work.
Pseudomonas syringae pv. *syringae* (*Pss*) Pss wild type strain 4364	Cohexist with Psa in kiwifruit plants, but it is not pathogenic. Cultivated in LB at 27°C.	This lab.
Pseduomonas fluorescens (*Pf*) Pf wild type strain A506	Biocontrol agent isolated in New Zealand. Cultivated in LB at 27°C.	Vanneste et al., 2004
Pseudomonas viridiflava (Pv)	Causative agent of bacterial leaf spots, leads to kiwifruit flowers necrosis. Strain isolated from *Actinidia chinensis* in Italy Cultivated in LB at 27°C.	This lab.
Pantoea agglomerans wild type strain P10c	Biological control agent isolated in New Zealand. Cultivated in LB at 27°C.	Vanneste et al., 2004
Pseudomonas putida		
strain IBE2	AHLs non-producing strain; Km^r Cultivated in LB at 27°C	Bertani-Venturi 2004
strain IBE3	AHLs over-producing strain; Km^r Cultivated in LB at 27°C	Bertani-Venturi 2004
Escherichia coli (*E. coli*) strain DH5α	Cultivated in LB at 37°C; electrocompetent cells are used for multiplication of pHN678-AlgD	Sigma-Aldrich
pSile Tr	Derived from pHN678, expressing the N terminal part and putative Shine-Delgarno sequence of Tr gene; Chl^r (25 μg/ml)	Nakashima-Tamura 2009

Table 2. Genes selected from gene expression studies. The genes were divided into 4 classes according to their function. The gene function/s, the source of the original sequence used to screen Psa genome and the homology are also reported. Reference for each gene is indicated.

	Gene	Identity	Organism	Strain	Function/s	Reference
Biofilm	*algD*	99,3	*Pseudomonas syringae* pv. *tomato*	DC3000	*algD* is involved in biofilm formation both *in vitro* and *in planta*. First gene to be transcribed during the biosynthesis of alginate, a virulence factor in *Ps. syringae*.	Penaloza-Vazquez et al., 2010
	clpP	87,6	*Pseudomonas fluorescens*	SBW25	Codes for a protein required for biofilm formation of *P.fluorescens*.	O'Toole and Kolter, 1998
	mdoH	99,5	*Pseudomonas syringae* pv. *tomato*	DC3000	Membrane derived oligosaccharides, a family of glucans present in periplasmic space of Gram negative bacteria. Required for biofilm formation.	Penaloza-Vazquez et al., 2010
	wspR	85,2	*Pseudomonas fluorescens*	SBW25	Is involved in colony morphology and cellular aggregation; regulator of genes that encode a putative fimbrial adhesin required for biofilm formation	D'Argenio et al., 2002 Ude et al., 2006
	wssB	68,3	*Pseudomonas fluorescens*	SBW25	Cellulose synthase catalytic subunit. Cellulose is used by *P. fluorescens* SBW25 in the colonization of plant surfaces.	Spiers et al., 2013

	Gene	Identity	Organism	Strain	Function/s	Reference
Motility	*fliP*	81,9	*Pseudomonas putida*	W619	Flagellar biosynthetic protein FliP	Segura et al., 2001
	pilA	92,4	*Pseudomonas syringae* pv. *tabaci*		involved in the attachment of the bacteria and/or biofilm formation	A. de Souza et al., 2004
	pilC	90,4	*Pseudomonas syringae* pv. *tomato*	DC3000	type IV pilus biogenesis protein PilC	A. de Souza et al., 2004
	pilO	74,9	*Pseudomonas aeruginosa*	PAO1	type 4 fimbrial biogenesis protein PilO	Martin et al., 1995
	rpoN	60,0	*Vibrio alginolyticus*		Involved in polar flagellar formation.	Sheng et al., 2012

	Gene	Identity	Organism	Strain	Function/s	Reference
Density-related genes	*aefR*	91,6	*Pseudomonas syringae* pv. *syringae*	B728a	Potential regulator of mexEF/oprN in *Ps. tabaci*; Synthesis of 3OC6-HSL. Gene product: "AHL and epiphytic fitness regulator"	Quinones et al., 2005; Kawakita et al., 2012
	fur	100,0	*Pseudomonas syringae* pv. *tabaci*		Ferric uptake regulator. Role in the control of genes involved in QS.	Cha et al., 2007
	gacA	99,1	*Pseudomonas syringae* pv. *syringae*	B728a	The GacS/GacA system interacts positively with the PhzI/PhzR quorum sensing system by regulating the synthesis of N-acyl-homoserine lactone	Chancey et al.,1999
	gacS	100,0	*Pseudomonas syringae* pv. *syringae*	B728a	Membrane-bound sensor kinase protein that recognizes specific environmental stimuli and activates GacA	Appleby et al ., 1996 Heeb and Haas, 2001 Pernestig et al., 2001
	hacA	88,4	*Pseudomonas syringae* pv. *syringae*	B728a	AHL acylase; Peptidase S45, penicillin amidase	Shepherd and Lindow, 2009
	hacB	94,3	*Pseudomonas syringae* pv. *syringae*	B728a	AHL acylase; Penicillin amidase. Can inactivate 3OC6HSL	Shepherd and Lindow, 2009
	hacC	97,0	*Pseudomonas syringae* pv. *syringae*	B728a	Aspartate transaminase: it is an AHL acylase that degrades AHL signal	Kalia, 2013
	mexE	93,7	*Pseudomonas syringae* pv. *phaseolicola*		Related with resistance to antimicrobial agents component of multidrug efflux system; multidrug efflux RND transporter, membrane fusion protein MexE.	Kawakita et al., 2012
	oprM	73,1	*Pseudomonas aeruginosa*	PAO1	Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM precursor	Phan et al., 2010
	psaR1	-	*Pseudomonas syringae* pv. *actindiae*		Highly similar to LuxRs which bind AHLs and are part of the canonical LuxI/R AHL QS systemslo that bind to AHLs	Patel et al., 2014
	psaR2	-	*Pseudomonas syringae* pv. *actindiae*		Binds and responds to yet unknown plant signal molecules.	Patel et al., 2014
	psaR3	-	*Pseudomonas syringae* pv. *actindiae*		Highly similar to LuxRs which bind AHLs and are part of the canonical LuxI/R AHL QS systemslo that bind to AHLs	Patel et al., 2014
	rpoS	69,4	*Vibrio alginolyticus*		Part of the regulatory networks of virulence and LuxS quorum sensing system; RpoS is cell density related; RpoS positively regulates AHL levels.	Tian et al., 2008
	Tr	-	*Pseudomonas syringae* pv. *actindiae*		Transcriptional regulator derived from promoter blast analysis. Potentially involved in signaling transduction and biofilm regulation.	This work

	Gene	Identity	Organism	Strain	Function/s	Reference
Virulence	*avrpt01*	-	*Pseudomonas syringae* pv. *actindiae*		Unknown	This work
	enolase	-	*Pseudomonas syringae* pv. *actindiae*		Pathogenesis related genes	McCann et al., 2013
	hopD1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	McCann et al., 2013
	hopQ1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopR1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopS2	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopZ5	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	McCann et al., 2013
	lysR	93,5	*Pseudomonas syringae* pv. *tabaci*		LysR-type transcriptional regulators (LTTRs) regulate a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility.	Kawakita et al., 2012
	pamTada	74,7	*Pseudomonas aeruginosa*		Potential target for QS in most Gram-negative bacteria.	Guan et al., 2012
	virB4	-	*Pseudomonas syringae* pv. *actindiae*		Secretion system	Marcelletti et al., 2011

Table 3. Primers used for PCR and qPCR. The genes are grouped in 4 classes according to their function. The source of primer sequences is also reported.

	Gene	Forward primer	Reverse primer	Source
Biofilm				
	algD	GACCTGGAACTGGACTACATC	TGCTGCGAACCACGATAG	This work
	clpP	CTTATATTCAGCAGAACTCT	GCGAATAGATGTCATAGG	This work
	mdoH	ACGGTAACCTTGAACTTGC	CACCATCGTTCTGCTGTT	This work
	wspR	ACGACTATCTGGTCAAACTG	ATAGGCTTCATCACGCTG	This work
	wssB	CGCTGGTGATGATGATGGT	CTGACGCTCAACGCTGTG	This work
Motility				
	fliP	TCAAGACGGCGTTTCAGA	CGGCGAGAGCATCATCAT	This work
	pilA	GCCATTCCTTCCTATCAA	GTAAGACCATTGCTCCAG	This work
	pilC	CGCTGGACATCGCATTCT	GCACCTTCGGCAATGATG	This work
	pilO	CCTACAGAAAGCAGATGGA	GTGATGTCTTCAAGCAGTC	This work
	rpoN	GCACCGACTCCTGATTGA	GAATCCACAGAAGCCGAATAC	This work
Density-related				
	aefR	AACTGCTGGAATTGCTCTG	TGTATCGTGGCACCTACC	This work
	fur	TGAAAATAGCGAACTACGAAAAGC	TGTAAACATCCTCGGCACTC	This work
	gacA	GATGACCATGACCTTGTTC	TCTTCAGCGATTCCTCAC	This work
	gacS	AGAACCTGGAAACCATCG	ATCTCGTGGCTCATGTTG	This work
	hacA	AGTTCACTGAAGCCTTTGC	CCAGTTGTAGCGCCTGAA	This work
	hacB	ACGGCATCAACCAGTATC	ATGCTGACCGTGTCCTCT	This work
	hacC	GACCTTCTTCGCCTCCAG	TTCCTTCGATTCGGTGATGA	This work
	mexE	TGTACGCACGGCTGAAACTG	TCCTTGTCCATCACCAGCAC	This work
	oprM	CGCTGGACATCGCATTCT	GCACCTTCGGCAATGATG	This work
	psaR1	ATACCTGGTCAGTAGTCTCA	GCAGCACTTCAAGTTCAC	This work
	psaR2	ACTGTTTGACCAGAAGATG	CTGAACGGTTGAGTTGAT	This work
	psaR3	GGTTCGCTCATTATCTGAT	GCAATGCTTGAGGATAGG	This work
	rpoS	CGTCGCTCAAACAACACAAAT	GAGACAGCAGAGGGGAAAAC	This work
	Tr	ACATTTACCATGTCACCCGCC	TGATGGCTTCTGCGTCGTTC	This work
Virulence				
	avrpt01	GGAGCGAATCTTGCCATT	GGAGCGATATGCGTGAAG	This work
	enolase	CATCGCCAACCTCAATGG	CCTGGATGTCGATGTTGTTAT	This work
	hopD1	CAGTAGACAGCAGTAGCC	CGGGTTATCGGAAACAAG	This work
	hopQ1	GGCATTCCACTTCGTATAG	CAACGCACTCTTCTGAAC	This work
	hopR1	GACATAACTGCCGATGCT	TCCAGATAGGCTCGATCA	This work
	hopS2	CCTTAAACGGCTGGCAGAG	CGAAGTGATGCTTGAGGTGAA	This work
	hopZ5	TCAGGCTACAATACTTACGCATCA	CAGGAATAGAACGGAACTCAGGAT	This work
	lysR	TGCGGAAGTTGAAGCGGATTACG	ACCGAAATGTTGCTGCCTCCC	This work
	pamTada	ACACATGACCCAGATCAG	CAGCTTGAGGTTGGATTC	This work
	virB4	TTTGAAGACACCACTGTTTC	CTGCGTCACCTACTACTC	This work
Reference				
	rpoD	CCGAGATCAAGGACATCAAC	GAGATCACCAGACGCAAGTT	Narusaka et *al.*, 2011
	recA	CGCACTTGATCCTGAATACG	CATGTCGGTGATTTCCAGTG	This work

Table 4. Enzymes for *Tr* cohesive-ends digestion. The cleavage site for each enzyme are underlined.

Primer_For	CG	GGATCC BamHI	GGCTTTCCATATTTGTTCGAG
Primer Rev	CG	GAATTC EcoRI	TTGAGCCGTGGACCTATCC

Table 5. Primers used for determination of *Psa*, *Pss* and *P.viridiflava* populations species by qPCR from bacterial pellets obtained from flowers.

***Pseudomonas syringae* pv. *actinidiae* (*Psa*)**			
Gene	**Forward primer**	**Reverse primer**	**Source**
rpoD	CCGAGATCAAGGACATCAAC	GAGATCACCAGACGCAAGTT	Narusaka *et al.*, 2010
recA	CGCACTTGATCCTGAATACG	CATGTCGGTGATTTCCAGTG	This study
16S rRNA	ACACCGCCCGTCACACCA	GTTCCCCTACGGCTCCT	This study
***Pseudomonas syringae* pv. *syringae* (*Pss*)**			
syrB	TCCTTATCGATCTGCAACTGGCGA	ATGGTTGCCTGCAGTTCATTCCC	Pour and Taghavi, 2011
Pseudomonas viridiflava			
16S rRNA	GTAGGTGGTTTGTTAAGTTGAA	GTAGGTGGTTTGTTAAGTTGAA	Alimi *et al.*, 2011

Table 6. Classification of the bacteria used for the co-colture experiments described in this work

Group characteristics	Species	Strain	Description	Signals produced	Abbreviations used
Bacterial species living on kiwifruit plants	*Pseudomonas syringae* pv. *syringae*	4364	Kiwifruit pathogen Producer of communication signals	O-C6-AHL	PSS; Pss4364
	Pseudomonas viridiflava		Kiwifruit pathogen Neighbour bacteria of *Psa*	unknown	P.VIR
	Pseudomonas fluorescens	A506	Used as BCA against fire blight Producer of communication signals	OH-C8-AHL, OH-C10-AHL	A506; PfA506
	Pantoea agglomerans	P10c	Used as BCA against fire blight Antagonistic bacteria of *Psa*	O-C3-AHL	P10C
Distant bacteria with which *Psa* does not come in contact and do or do not produce AHLs (here used as controls)	*Escherichia coli*	DH5α	Here used as negative control	non-producing AHLs strain	E.Coli
	Pseudomonas putida	IBE2	Here used as negative control	non-producing AHLs strain	IBE2
	Pseudomonas putida	IBE3	Here used as positive control since overproduces several AHLs molecules	OH-C6-8-10-12-AHL	IBE3

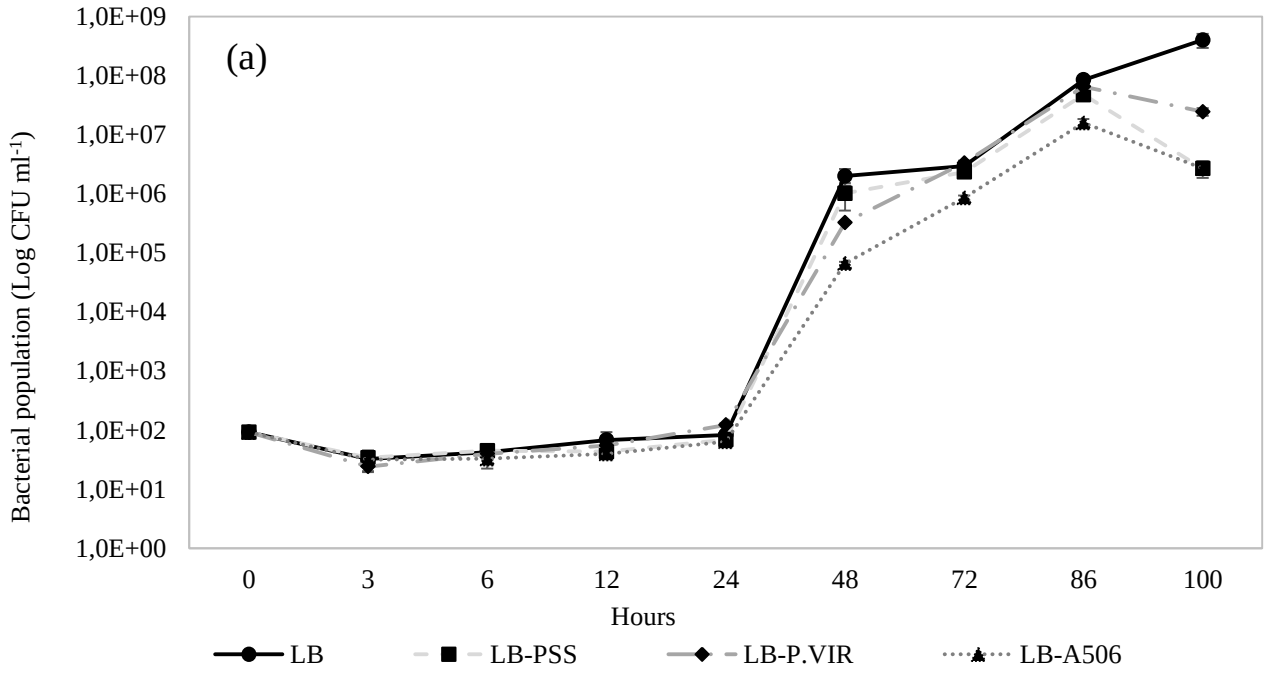
Table 7. Contributions of swarming and swimming motilities to *Psa* total motility and evaluation of the effect of the supernatant supernatants (Stimulation: +, ++, +++. No effect =. Inhibition: -, - -, - - -).

Thesis	% of swarming petri		% of swimming petri	
		Effect		*Effect*
LB	15,2%		36,4%	
LB-PSS	43,8%	+ +	12,5%	- -
LB-P.VIR	29,4%	+ +	0,0%	- - -
LB-IBE3	72,4%	+ + +	0,0%	- - -
LB-A506	52,4%	+ + +	17,2%	- -
LB-P10C	10,0%	-	0,0%	- - -
LB-IBE2	22,2%	+	27,8%	-
LB-E.Coli	17,6%	=	20,0%	-

Table 8. Summary of the effect of the supernatant supernatants on gene induction and in *vivo virulence* that helps to interpret figures 3-7. (Stimulation: +, ++, +++. No effect =. Inhibition: -).

		LB-PSS	LB.P-VIR	LB-A506	LB-IBE3	LB-P10c	LB-COLI
Biofilm	*algD*	=	+	=	+	-	=
	clpP	-	-	-	-	-	-
	mdoH	=	=	=	=	-	-
	wssB	+	+	-	+	-	-
	wspR	++	++	+++	-	-	-
Motility	*fliP*	=	+	+	++	=	-
	rpoN	-	=	-	=	-	+
	pilA	=	=	=	=	=	=
	pilC	+	=	-	+	-	-
	pilO	+	+	=	=	=	=
Density-related genes	*aefR*	-	-	-	=	=	-
	fur	+	-	+	=	-	-
	gacA	+	+	=	+	+	=
	gacS	=	+	=	+	+	-
	hacA	+	+	++	=	-	-
	hacB	-	-	-	-	=	-
	hacC	=	=	=	=	=	=
	mexE	+	-	=	-	-	-
	psaR1	++	+++	+	++	+	=
	psaR2	=	=	=	=	=	=
	psaR3	=	=	=	+	=	=
	Tr	++	+++	+	=	-	-
	rpoS	+++	+	+	++	=	=
Virulence	*avrpt01*	=	+	+	++	=	-
	hopD1	-	=	+	=	-	-
	hopQ1	+	+	++	=	-	-
	hopR1	-	-	-	-	-	-
	hopS2	++	=	++	+++	-	-
	hopZ5	=	=	=	-	=	-
	lysR	+	=	+	=	-	-
	paMTAda	=	+++	=	=	=	=
	virB4	=	+++	=	+++	=	=
	enolase	+	+	+	+	-	-
	oprM	++	++	=	=	=	-
Virulence *in vivo*		++	+++	-	- -	-	-

Figure 1. Growth curves of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 in different bacterial supernatant LB media, up to 100 hours from the moment of inoculation. (a) Growth curve in supernatant of Pss4364, *P. viridiflava*, and PfA506 compared to standard LB. (b) Growth curve in supernatant of *P. putida* IBE3, *P. agglomerans* P10c and *E. coli* DH5α compared to standard LB. Standard error bars are indicated.



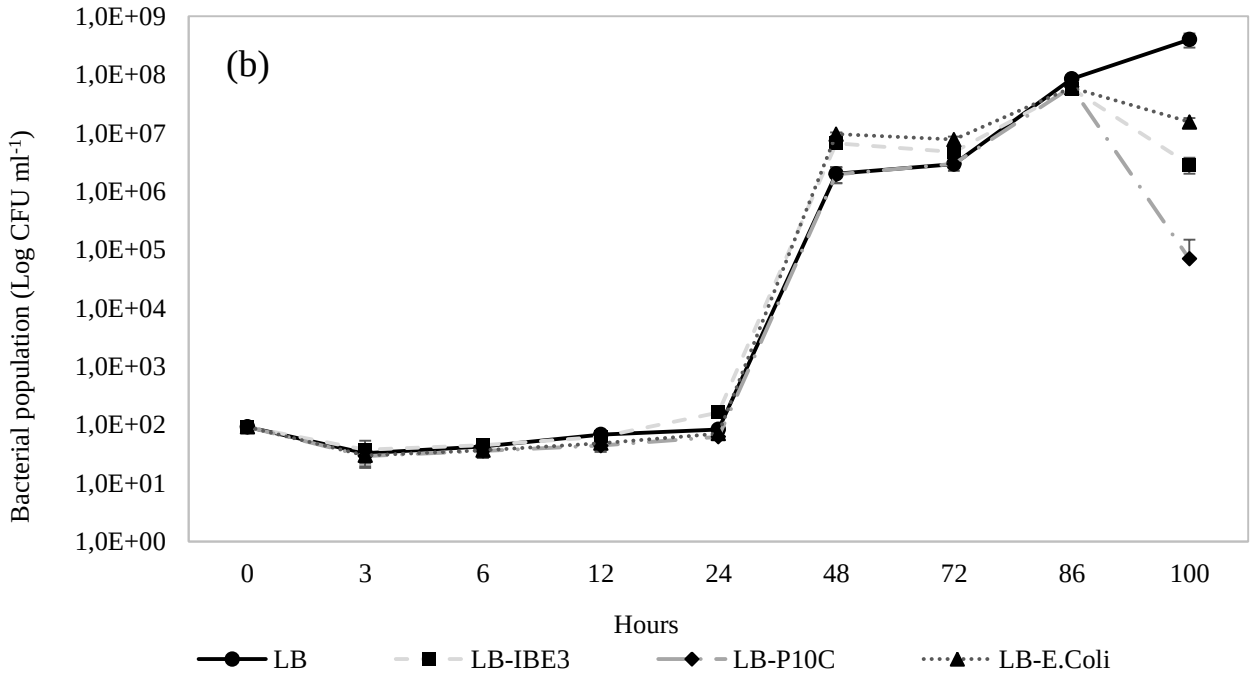


Figure 2. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 grown in the bacterial supernatants. The control consisted of PsaCFBP7286 biofilm production in fresh LB. Biofilm was quantified after staining with crystal violet (0.5% w/v). Standard error bars are shown. Data were significantly different according to Student's *t* test ($P < 0.05$).

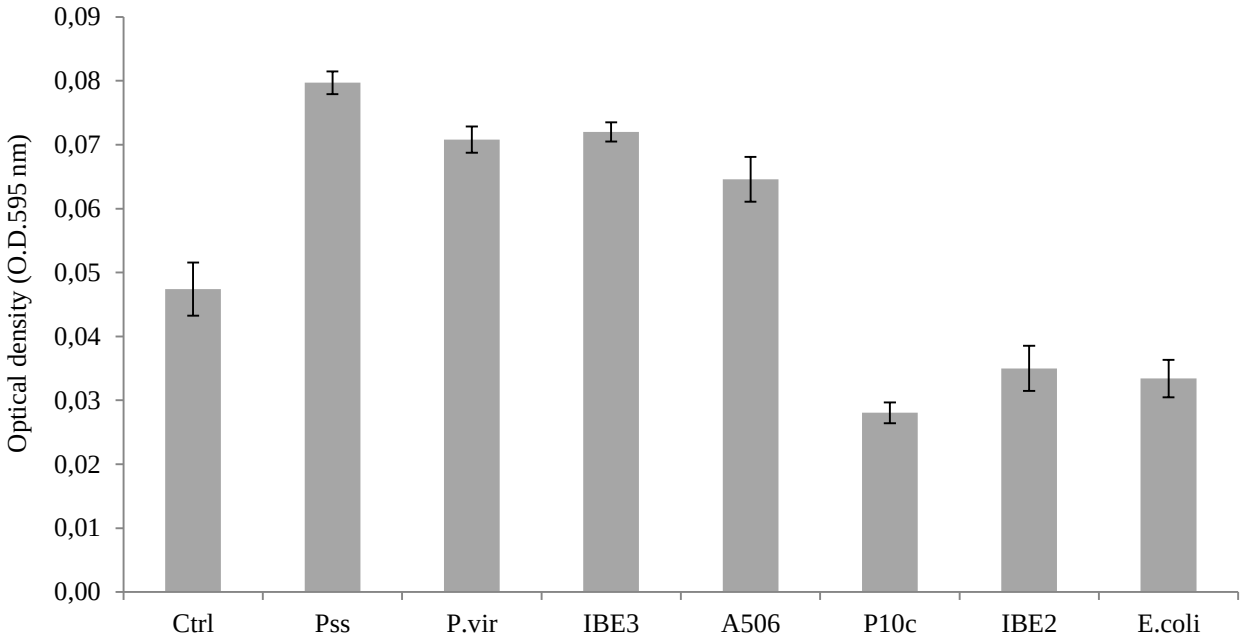


Figure 3. Effect of bacterial supernatants on the expression of genes related to density of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.

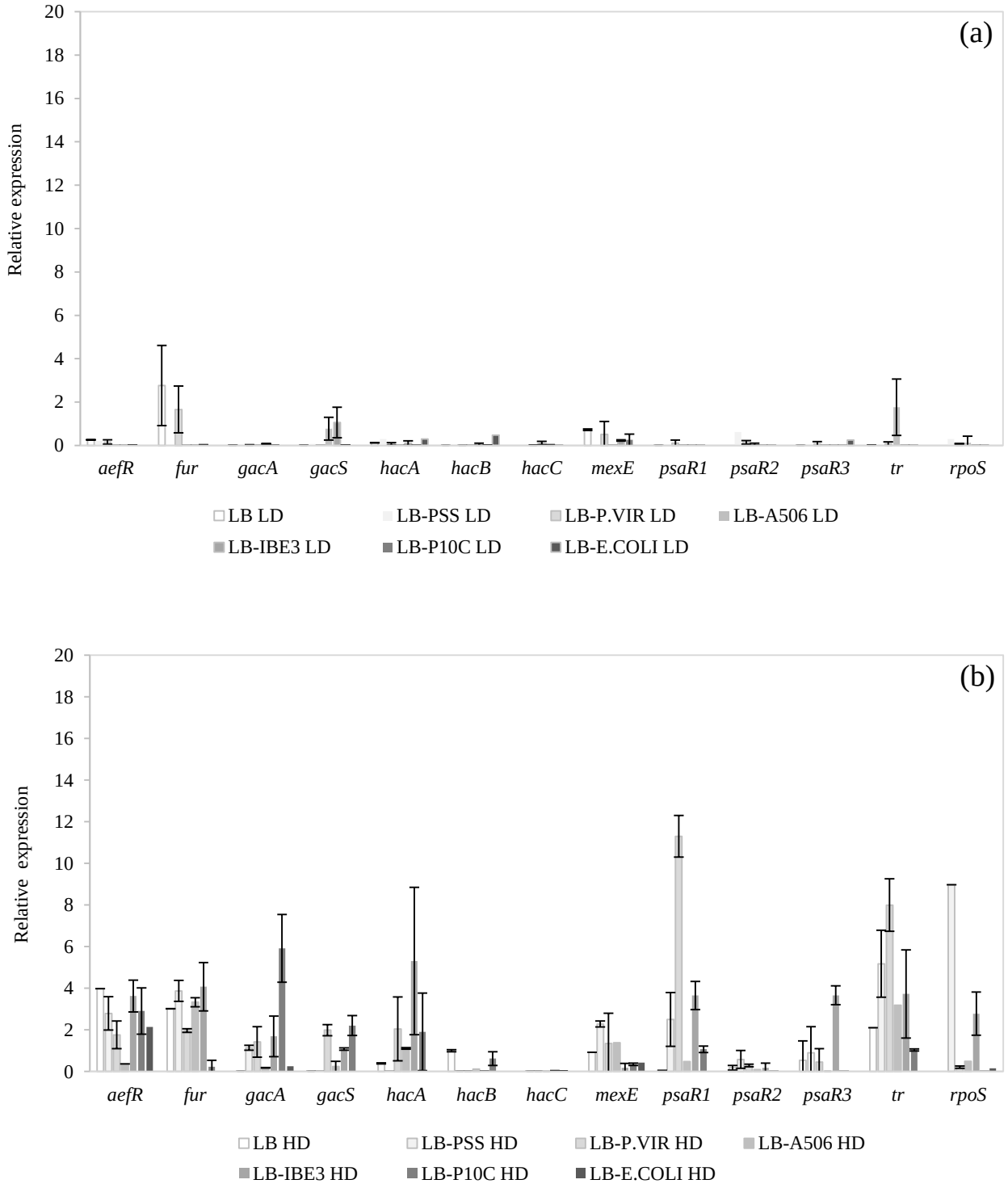
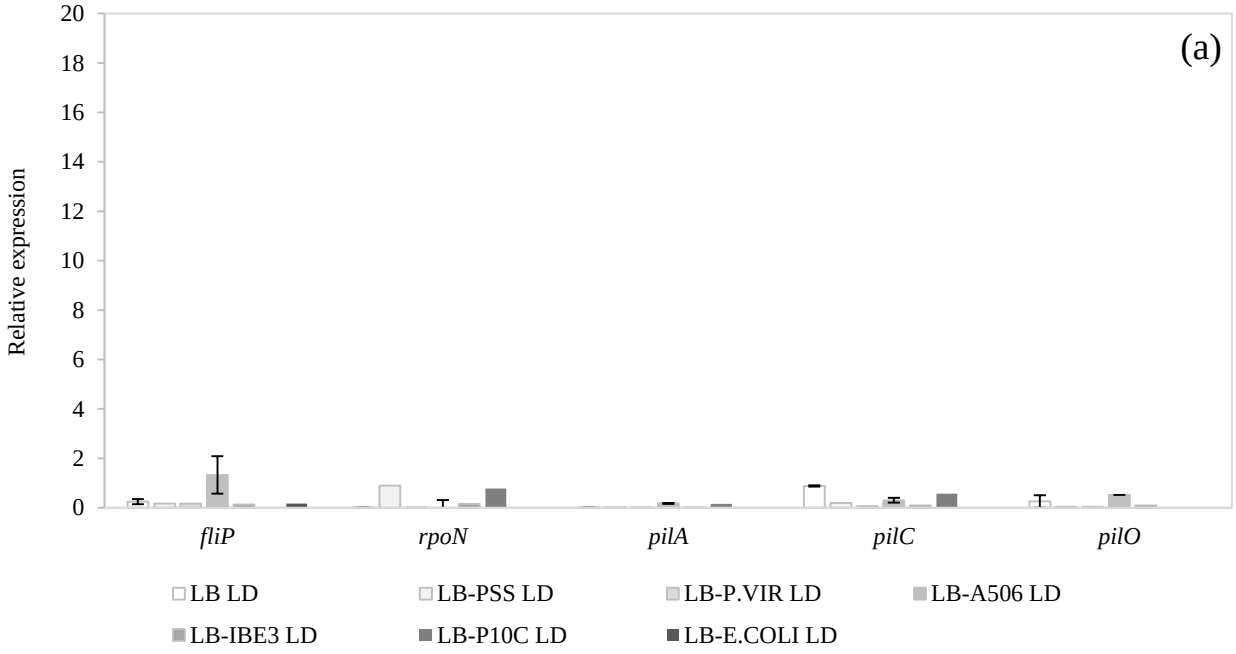


Figure 4. Effect of bacterial supernatants on the expression of genes related to motility of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.



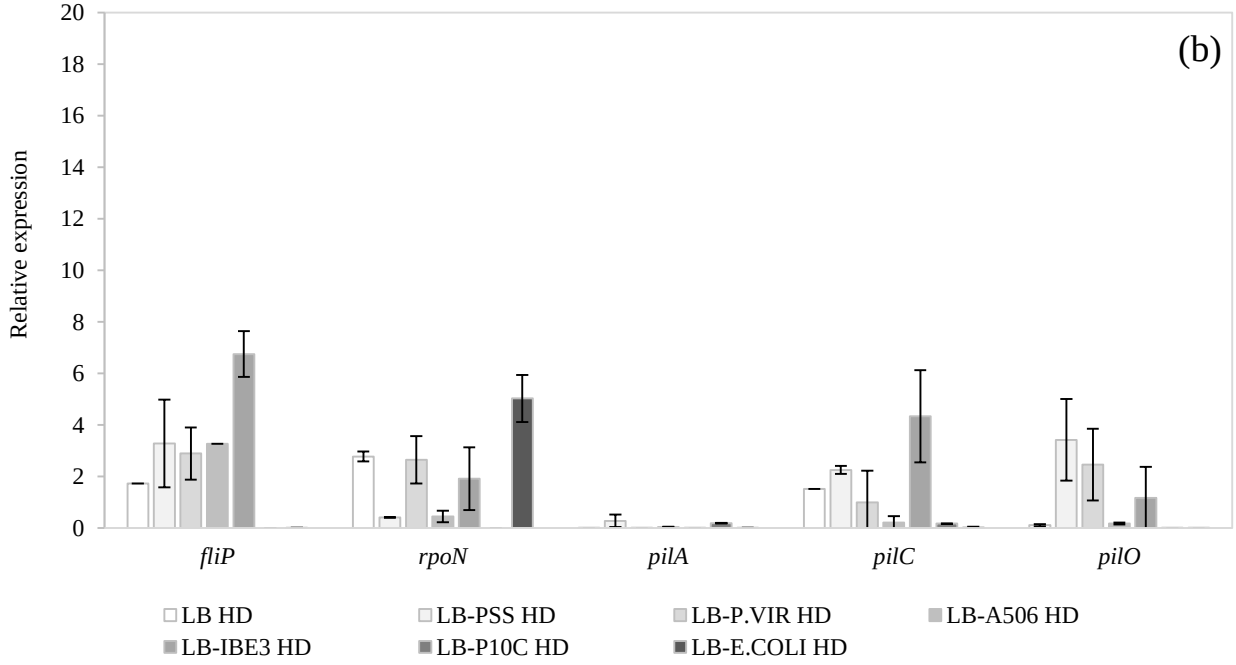


Figure 5. Effect of bacterial supernatants on the expression of genes related to biofilm of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.

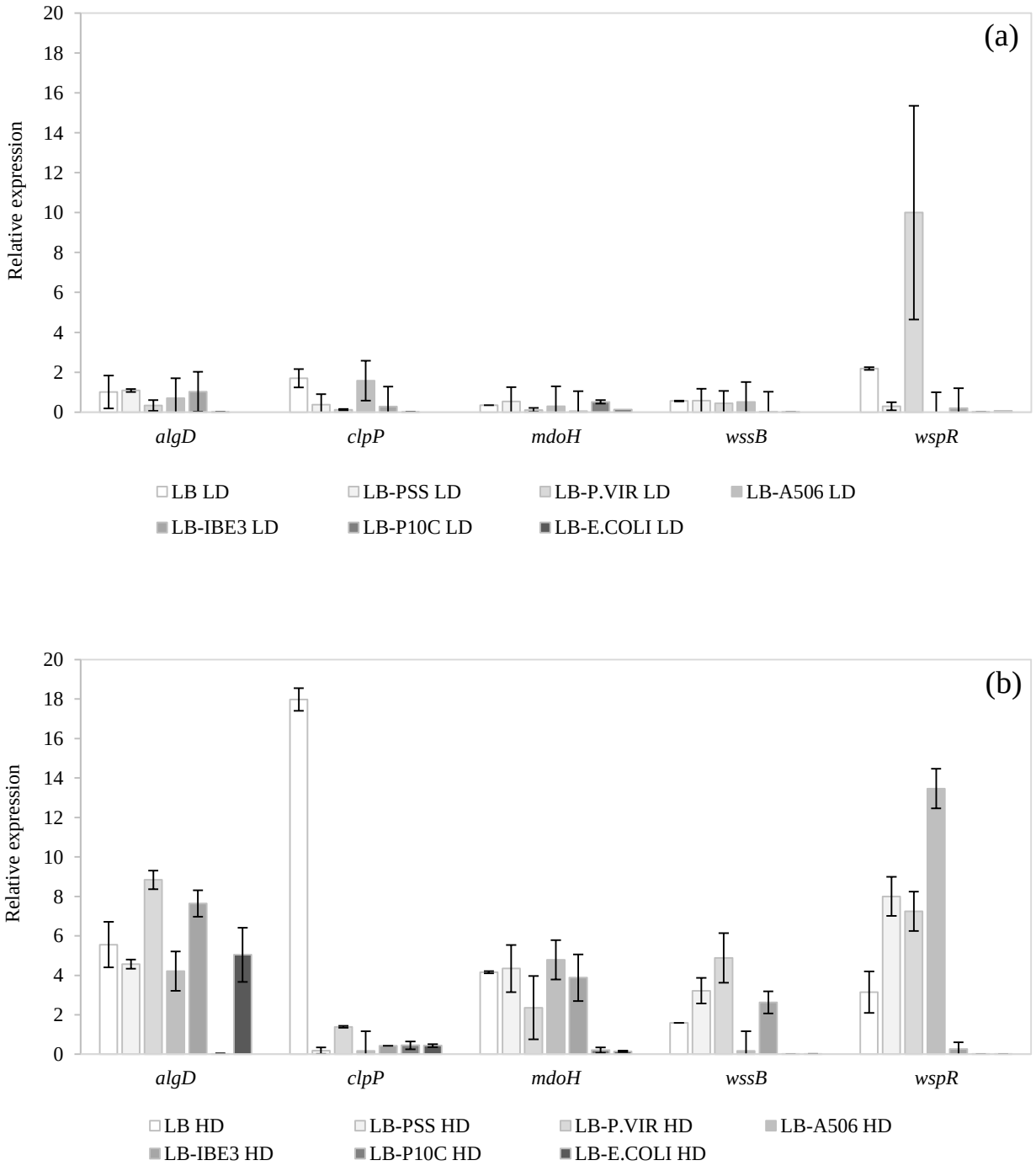
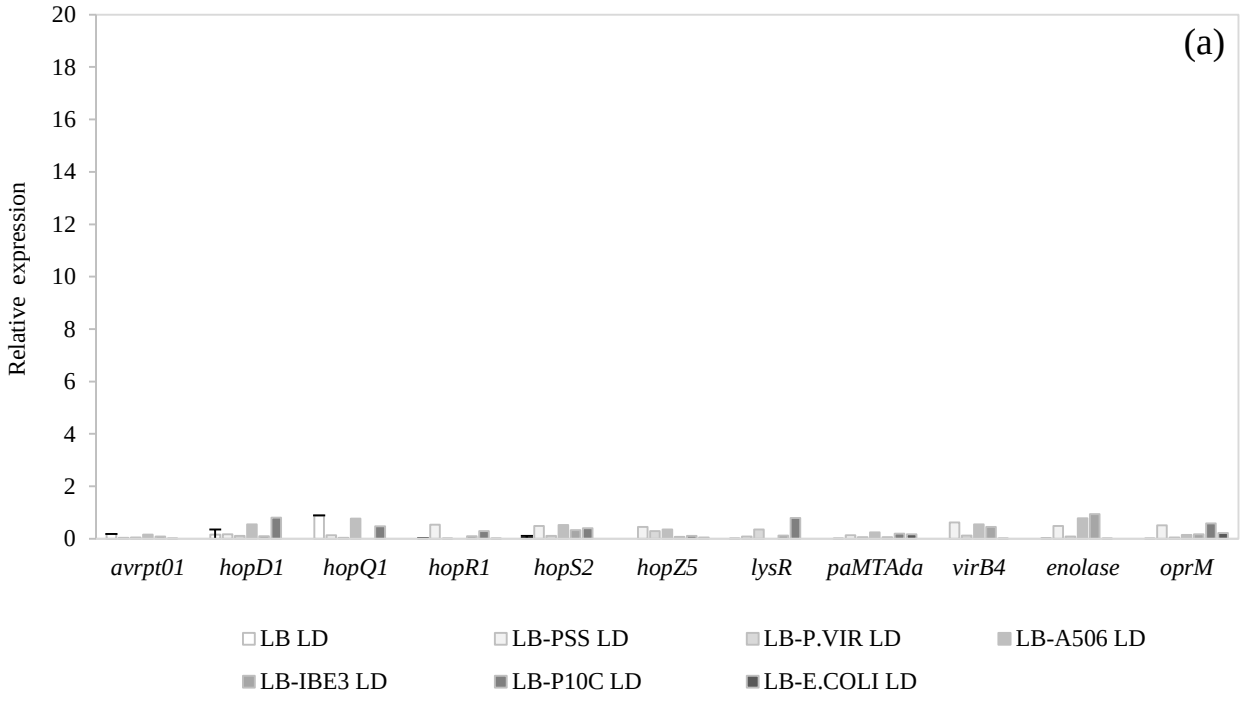


Figure 6. Effect of bacterial supernatants on the expression of genes related to virulence of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density. Standard error bars are showed.



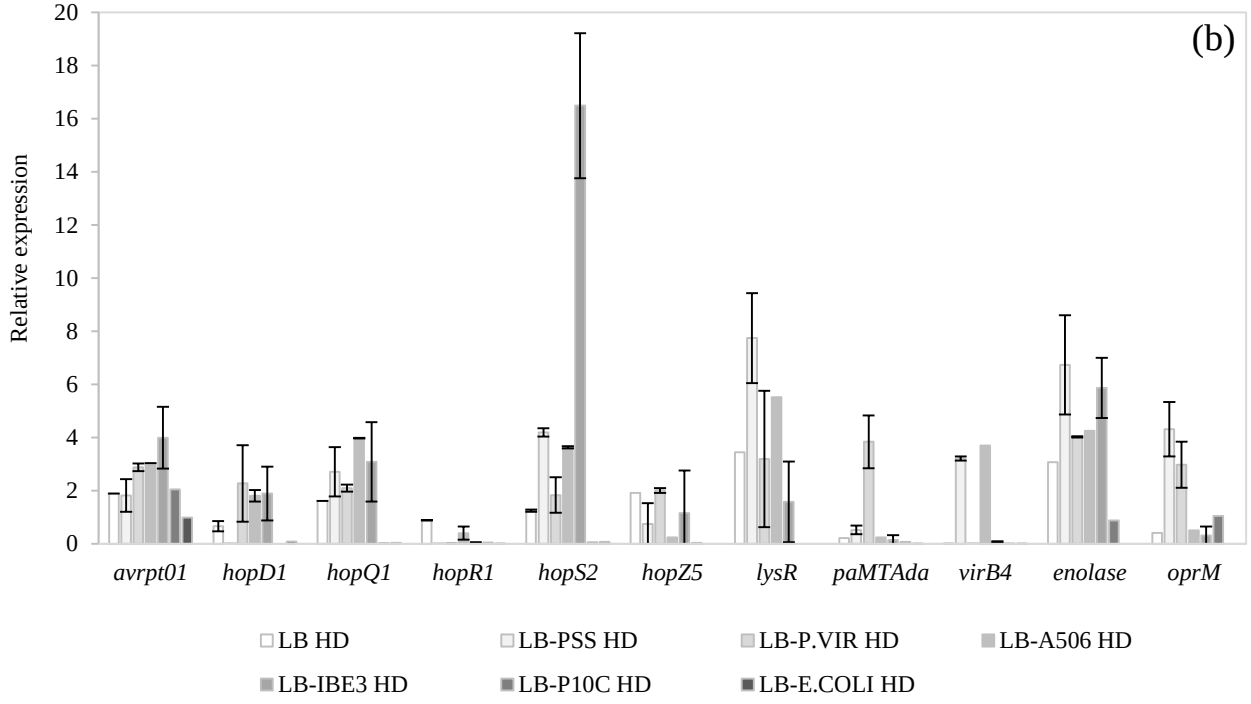
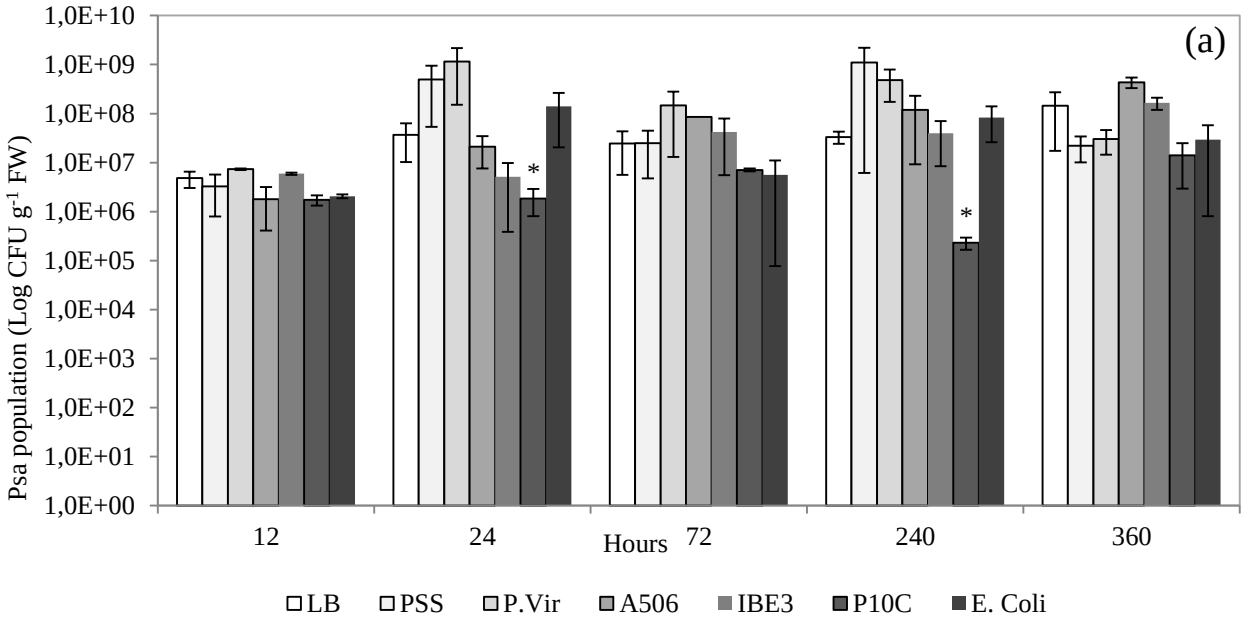


Figure 7. Epiphyte (a) and endophyte (b) populations of *Actinidia deliciosa* cv. Hayward plantlets inoculated with *Pseudomonas syringae* pv. *actinidiae* CFBP7286 cells (10^8 CFU ml^{-1}) cultures grown in LB and bacterial supernatants. Bacterial population was estimated by qPCR. Data are the average of three independent replicates of three plants each. The standard error is shown. Data marked with an asterisk (*) were significantly different from the control according to the Student's *t* test, with $P < 0.05$.



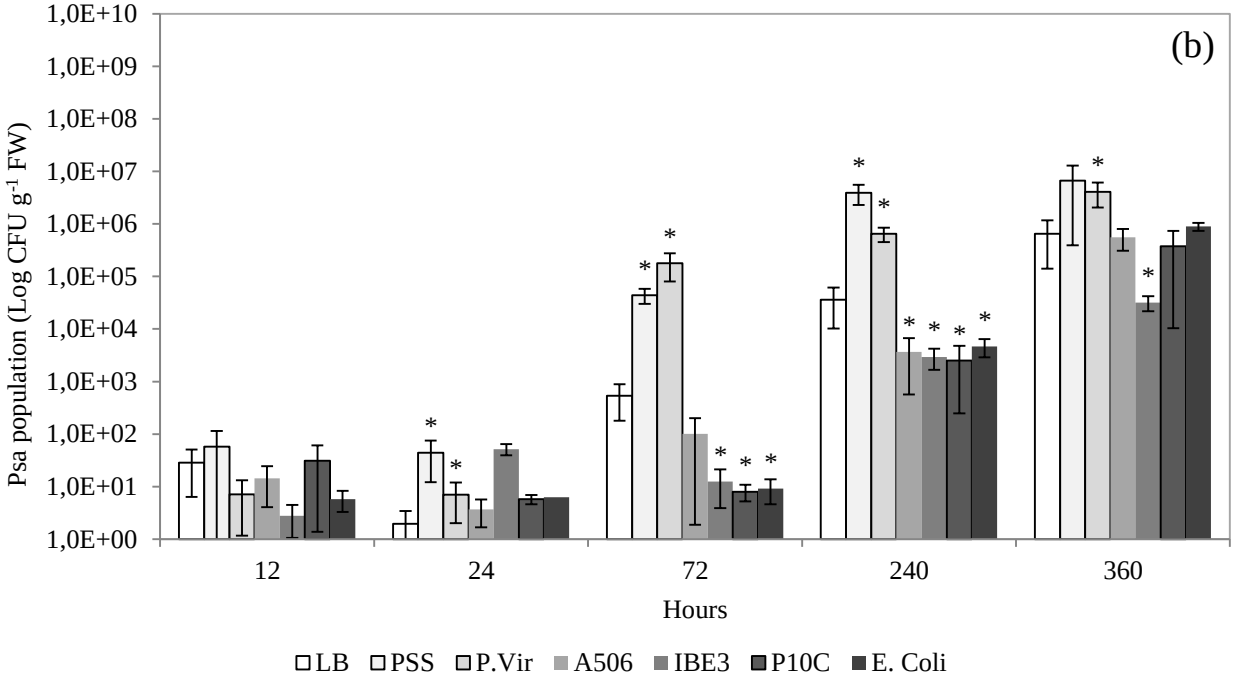


Figure 8. Motility of Psa-mR1 and Psa-mR3 mutants on bacterial supernatant LB and fresh LB compared to PsaCFBP7286. The graph represents the total plates showing swarming motility (in percentage). The control consisted of motility on plates with no addition of bacterial supernatant.

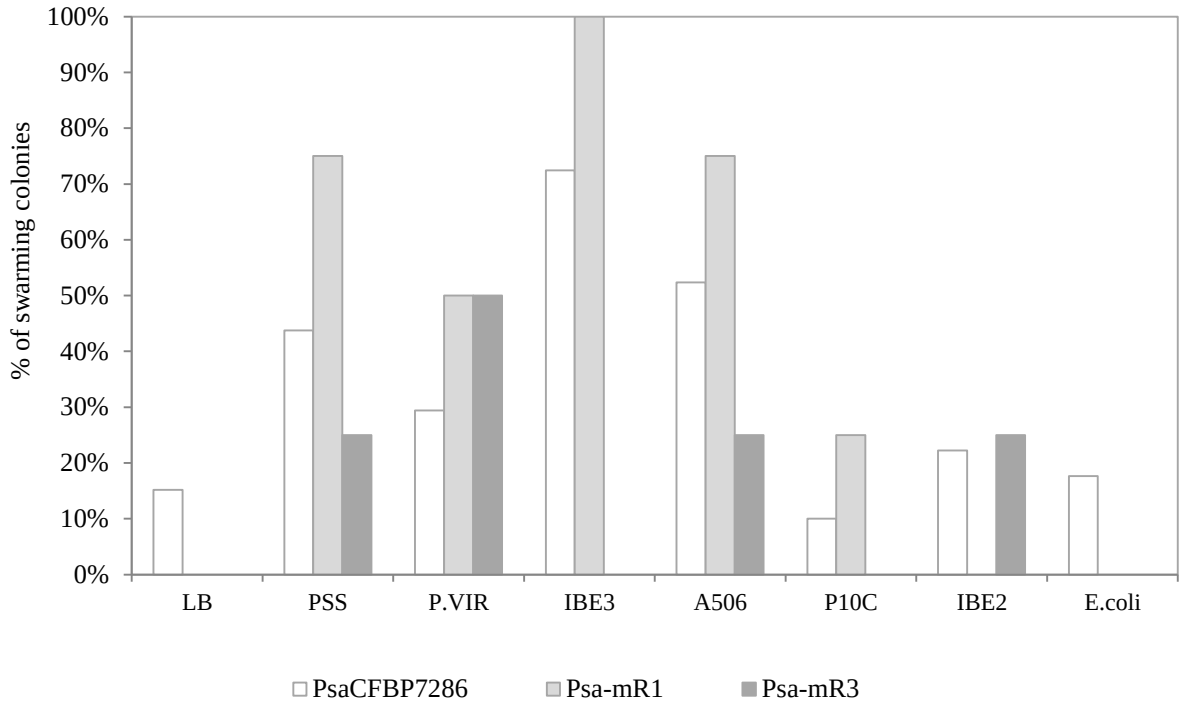


Figure 9. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286, Psa-mR1 and Psa-mR3 in bacterial supernatants. Biofilm was quantified after staining with crystal violet (0.5% w/v). The controls are represented by biofilm formation in LB without signals. Standard error bars are showed. Significance was calculated using Student's *t* test ($P < 0.05$). (*) indicates the difference from the control.

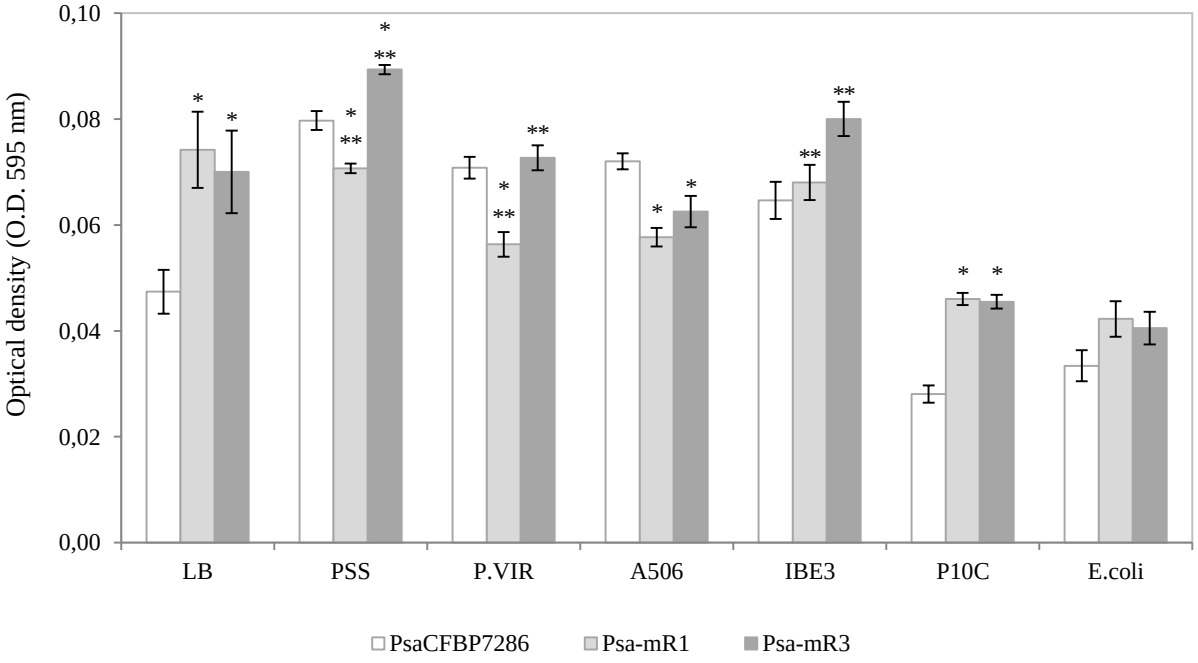


Figure 10. Representative pictures of Psa-TRsile mutant swarming motility in Petri dish with IPTG (0.5%) added for active induction of gene silencing. a) PsaCFBP7286 + IPTG. b) Psa-TRsile + IPTG. Photographs were taken binocular Nikon SMZ25 fluorescence microscope (Zoom magnification 1x).

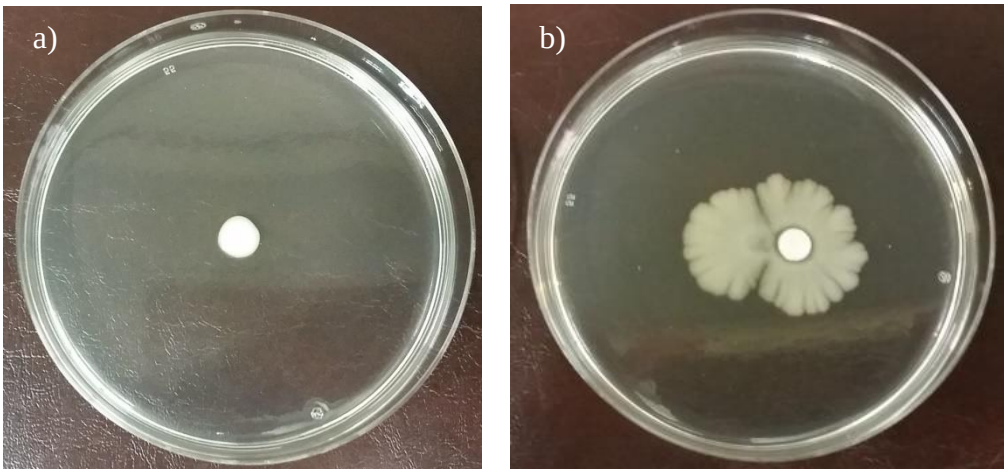


Figure 11. Motility of Psa-TRsile on soft agar (0.4%). The graph represents the development in % of the swarming extension over the total surface of the plates. IPTG (0.5%) was provided for active induction of gene silencing. To exclude aspecific effects, IPTG was also applied in the plates of the control (PsaCFBP7286). Standard error bars are indicated.

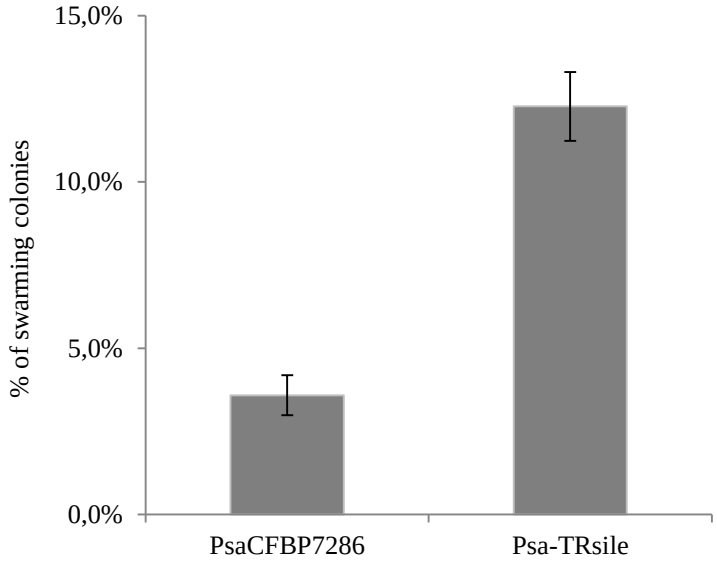
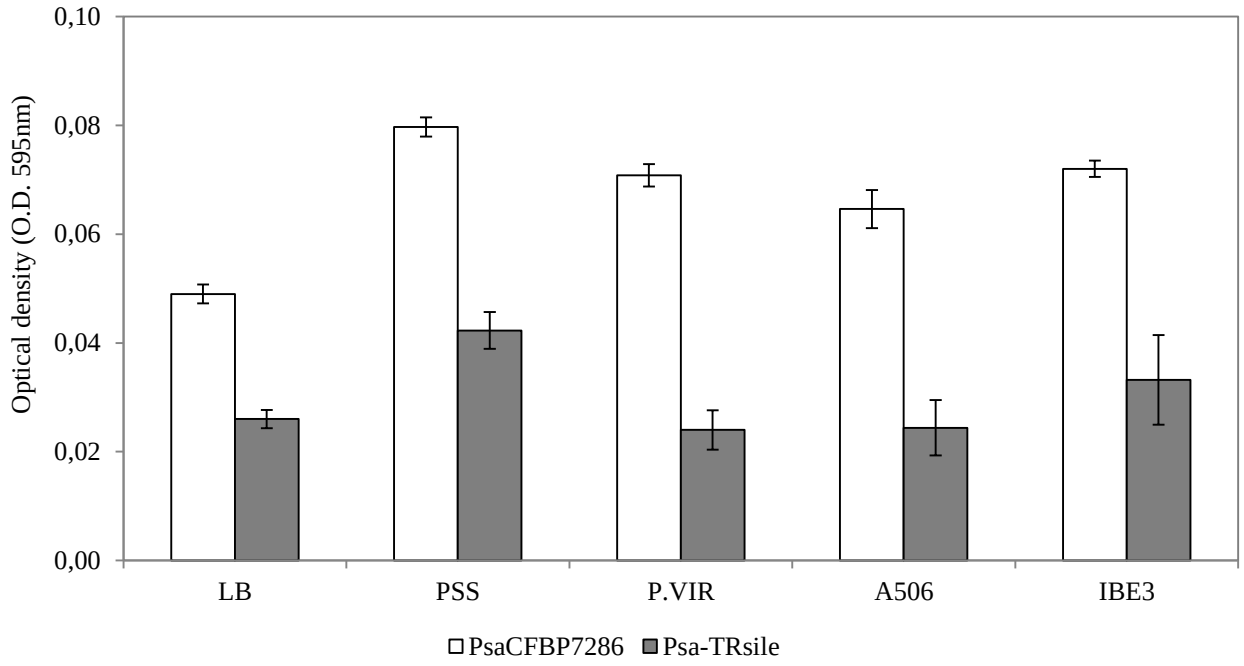


Figure 12. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 and Psa-TRsile grown in fresh LB and bacterial supernatants with IPTG (0.5%). Biofilm was quantified after staining with crystal violet (0.5% w/v). Standard error bars are shown. Data were significantly different according to Student's *t* test ($P < 0.05$).



The interkingdom signalling communication system of *Pseudomonas syringae* pv. *actinidiae*

Luca Fiorentini[1], Giampaolo Buriani[1], Joel Vanneste[2], Guglielmo Costa[1] and Francesco Spinelli[1]

[1] Department of Agricultural Sciences, Alma Mater Studiorum -University of Bologna, viale Fanin 46, 40127 Bologna – Italy,

[2] Bioprotection, The New Zealand Institute for Plant & Food Research Ltd.,
Ruakura Research Centre, Hamilton, New Zealand

Abstract

Interkingdom signaling via chemical signals was investigated for the bacterial canker of kiwifruit plants *Pseudomonas syringae* pv. *actinidiae*. To this end, the influence of plant extracts and xylem saps of plant-host (*Actinidia deliciosa*, *Actinidia chinensis*) and non-host (*Actinidia arguta*, *Camellia sinensis*, *Corylus avellana* and *Nicotiana tabacum*) was evaluated on those phenotypes that are related to bacterial epiphytic fitness and host colonization, such as are biofilm production, motility and virulence. Our results revealed that biofilm and motility were increased by xylem saps and the plant extracts derived from host plant species. Molecular analysis of the gene expression in *Psa* revealed that the plant extracts, especially those of *A. deliciosa*, induced a greater expression compared to the xylem saps. *Psa* in non-host plant extracts lacked of biofilm formation, swarming motility and significative gene induction, thus it was demonstrated that host-pathogen recognition is the result of species-specific interkingdom reporting systems. *In vivo* infections with micropropagated material established that the effect of the priming of the virulence of *Pseudomonas syringae* pv. *actinidiae* by plant substrates can lead to detrimental consequences for plant survival since the infections took over the whole plants within few days. Two promising signalling regulator genes, namely *psaR2* that would be delegated to the receipt of yet unknown plant signals in interkingdom communication, and *Tr* that responded to *Psa* neighbouring bacteria, were also investigated either describing their expression in plant material of *Actinidia* spp. with qPCR, and also via gene knock-out and RNA silencing techniques. The ecological implications of the interkingdom signaling between *Psa* and plant-host and the work in progress for the identification of the plant signal that would bind to PsaR2, are presented and discussed.

Keywords: biofilm formation, motility, virulence, *Actinidia deliciosa*, *Actinidia chinensis*, *Actinidia arguta*, qPCR

Introduction

The close association for millions of years has allowed bacteria and plants to develop mechanisms of production and response for many signaling molecules released by the host plant and its bacterial community (Venturi and Fuqua, 2013). In the past years, interkingdom signaling via chemical signals has been extensively studied in the rhizobia-legume symbiosis and between agrobacterial pathogens and their host (Brencic and Winans, 2005). Recently, three main ways of communication between plant and epiphytic microbioma have been elucidated: (1) regulation of gene expression of virulence-associated genes in pathogenic bacteria by plant phenolic compounds; (2) the role of bacterial quorum-sensing (QS) signals in regulating plant gene expression; (c) the role of low-molecular weight plant compounds in the interference of bacterial QS. (Venturi and Fuqua, 2013). Genomic studies focused on molecular mechanisms of bacterial QS systems mediated by acyl homoserine lactones (AHLs) have also revealed the presence of proteins closely related to AHL receptors LuxR that specifically respond to plant signals and not to AHLs (Subramoni and Venturi, 2009), but the chemical identity of these hypothetical class of compounds is still not yet characterized. In addition it was also revealed that many bacteria showed the presence of only the receptors of molecular signals, but not the cognate synthase genes *Lux I* these LuxR proteins that lack a genetically linked LuxI have been termed "solos" (Subramoni and Venturi, 2009). This kind of signaling is not isolate, but quite spread in proteobacteria: an analysis of 265 proteobacterial genomes showed that 45 genomes contained QS LuxRs, and not the cognate AHL LuxI synthase (Case *et al*., 2008). Up to now, it is determined that Lux-solos are able to regulate target genes via interkingdom signaling when responding to eukaryotic signals (Gonzalez *et al*., 2013; Zhang *et al*., 2007). The subgroup of LuxR harboured by plant-associated bacteria (PAB) that bind to plant-produced compounds showed differences in one or two of the conserved residues in the AHL-binding domain. It is likely that the evolution of these changes corresponds with the ability of these proteins to bind low-molecular weight compounds produced by plants rather than AHLs (Ferluga and Venturi, 2007).

Interestingly, very recently it was demonstrated that *Photorhabdus luminiscens* possesses a LuxR-solo receptor able to respond to bacterial molecules that are not AHLs (Brachmann *et al*., 2013). Therefore, these molecules are probably involved in binding other signalling molecules which have not yet been identified. Putting together these findings, a very complex scenario is outlined, in which the interkingdom signaling between bacteria and plants play a pivotal role.

The major issue to clarify this peculiar system is to identify the structure of the plant molecules to which PAB LuxR-family proteins responds to, and how these molecules are able to prime bacterial behavior. This will be a major challenge, as plants produce a very large number of low molecular weight secondary metabolites (Patel et al., 2013)

Pseudomonas syringae pv. actinidiae (*Psa*) a rod-shaped, Gram-negative, strictly aerobic and mobile bacterium (Takikawa *et al.,* 1989) is the causal agent of the bacterial canker of kiwifruit. *Psa* is able to affect both *Actinidia chinensis* and *Actinidia deliciosa* (Donati *et al.,* 2014*)* penetrating 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* possesses three *luxR* solos genes, two of which were predicted to bind AHLs (PsaR1, PsaR3) and one belongs to the sub-family of LuxR solos (PsaR2) predicted to bind a molecular signal (Patel *et al*., 2014). In particular, it was demonstrated that PsaR1 and PsaR3 respond to homoserine-lactones (Fiorentini 1) and resulted to be involved in bacterial comunity cross-talk (Fiorentini 2). Therefore, in our work we detect the existence of specific interaction between *A. deliciosa* and *A. chinensis* and Psa. In particular we demonstrated that both plant derived medium and xylem sap from both *Actinidia* species were able to induce both "social" motility (Fujishige *et al*. 2006), biofilm formation and extremely enhance virulence. Molecular studies regarding the expression profile of a subset of 30 genes representative of different phenotype tested, confirmed the results. Moreover, the role of PsaR2 and its potential ecological implication will be presented and discussed through the bioassays using a knock out strain. *Psa* gene expression profile.

Materials and methods

Bacterial species, culture conditions and bacteria quantification

The bacterial species, strains and plasmids used in this study are listed in table 1. The *Pseudomonas syringae* pv. *actinidiae* (Psa) strains belong to the high virulent biovar 3 which is responsible for the present pandemic. *Escherichia coli* and all other strains tested, including Psa wild type, mutants and silenced strain, were grown on Luria-Bertani (LB) medium (Sambrook *et al*., 1989) at appropriate temperature (37°C *E. coli*; 27°C all the other strains) under moderate shaking (120 rpm). Each actively growing culture was initiated by inoculating the appropriate sterile medium with a two days old single colony grown in LB plates. Low density cultures were obtained by inoculating 100 ml LB of LB or plant extracts/xylem saps till bacterial growth reached a turbidity lower than $OD_{600} < 0.010$. High density cultures were obtained in 20 ml LB or plant extracts/xylem saps from $OD_{600} > 0.100$-0.200, when Psa reached the late log phase. Optical density was measures in cuvettes at 600 nm wavelength using spectrophotometer (Biochrom Libra, Cambridge UK). Bacterial population was also enumerated by direct plating 3 replicates of each 10-fold serial dilution. Colonies were counted after two days incubation.

Psa-mR2 mutant (courtesy provided by dr. V. Venturi, ICGEB Trieste, IT) were used in order to elucidate the role of PsaR2 receptors that belongs to the sub-family of LuxR solos predicted to bind a molecular signal (Patel *et al*., 2014) in the regulation of QS-dependent phenotypes such as biofilm and motility. *In vivo* virulence was not assessed since it was already proved to be impaired by the mutation (Patel *et al*., 2014).

Collection of xylem and plant extracts for *in vitro* bioassays

Xylem was harvested from adult *Actinidia deliciosa* cv. Hayward and *Actinidia chinensis* cv. JinTao plants in *Psa*-free orchard at early spring directly from pruning cuts just before Leaf unfurling period (Nardozza et al., 2013), immediately after sampling, xylem was put on ice until sterilization by micro-filtration with filtration unit with a 0.02 μm pore membrane (Merck Millipore Billerica, Massachusetts, USA) and stored at -80°C.

Plant extracts were obtained from leaves of microcuttings *A. deliciosa* cv. Hayward, *A. chinensis* cv. JinTao and from full expanded leaves of 1 year old plants of *Actinidia arguta*, *Camellia sinensis*, *Corylus avellana* and *Nicotiana tabacum*. Briefly, 2/3 grams of green plant tissue (leaves and stems deriving from *in vitro* plant, only leaves from other) were grinded in 15 ml of sterile PBS (Phosphate Buffered Saline, pH 7.2) and 1% PVPP (Sigma-Aldrich, Saint Louis, St. Louis, MO, USA) at room temperature in mortar and pastle . The homogenized was filtered with a sterile gauze and centrifuged for two times (12000 rpm, 20 min, 7°C). Supernatant was filter-sterilized with a 0.02 μm pore membrane And store for 24 hours maximum before use at 4°C.

Motility assay

Motility was assessed according to (Kinscherf and Willis 1999). A sterile disk of filter paper disk (6 mm Ø) was centrally placed on a LB plates (Sigma-Aldrich) containing 0.4% agar. The paper disk was inoculated with a 10µl drop of low (10^5 CFU ml^{-1}) or high density bacterial suspension (10^8 CFU ml^{-1}) adjusted to contain 1 x 10^7 cells ml^{-1} . Plates were then incubated at 27°C for 5 days. Bacterial spread and the surface of the plate covered by the colony was evaluated using the MacBiophotonics ImageJ 1.48 software (MacBiophotonics, Hamilton, ON, Canada).

Motility was assessed in the presence of plant extracts and xylem saps by homogenously adding 1 ml of plant extracts/xylem on top of the LB plates. Plates were dried off before inoculating with a 10µl drop of high density bacterial suspension (10^8 CFU ml^{-1}) adjusted to contain 1 x 10^7 cells ml^{-1}. Microscopic visualization of motility in soft agar was performed using a binocular Nikon SMZ25 fluorescence microscope (Nikon Instruments Corporation, Tokyo, JAPAN) under UV source (excitation wavelength of GFP-B: 460-500 ηm, emission wavelength: 510–560 nm). The 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. Images were obtained and processed by software Nikon NIS ELEMENTS V. 4.30.02 (Nikon Instruments Corporation, Tokyo, JAPAN)

Biofilm assay

The assay was performed as described by Taguchi *et al*., 2006. A volume of 3 ml of LB medium or filter-sterilized plant extracts or xylem saps was added into a 35 mm polystyrene Petri dish and inoculated with 50 µl of a 10^8 CFU ml^{-1} fresh liquid culture of Psa. After inoculation, plates were sealed with parafilm and incubated at 27°C with slow shaking at 70 rpm for 5 days. Successively, the bacterial population in each tube was assessed by serial dilutions and plating as previously described. Plates were thoroughly rinsed with distilled sterile water and dried for 45 min under laminar hood at room temperature. Thereafter, 3 ml of a crystal violet water solution (0.5% w/v) (Sigma-Aldrich) were added to each plate. The plates were incubated for 60 minutes at room temperature under shaking (70 rpm) and subsequently washed thoroughly with distilled water to remove nonspecific staining. For quantitative analysis of biofilms, crystal violet was re-solubilized by adding 3 ml of ethanol 95%. The solution was transferred to cuvette and absorbance at 595 nm was measured by using spectrophotometer. Aspecific staining was also quantified by with the same procedure on sterile, non-inoculated LB petri dishes. Unspecific absorbance was subtracted from all the measures. 10 plates were inoculated with 50 µl of a 10^8 CFU ml^{-1} fresh liquid culture of Psa and let grow to high density for efficient cell adhesion and quantification (Ghods et al. 2015).

Virulence assay

The effect of plant extracts or xylem saps on PsaCFBP7286 virulence was performed *in vivo* by inoculating kiwifruit plants. Three months old micropropagated plants of *Actinidia deliciosa* cv. Hayward. The plants were grown on MS medium (Murashige and Skoog, 1962) 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}), GA3 (0.1 mg l^{-1}), adjusted to pH 5.7 with KOH. The plants were kept in a growing chamber for the whole duration of the experiments (22°C, 70% RH and a light/dark cycle of 16:8 hours). Psa was grown to log phase in LB or in filter-sterilized plant extracts or xylem saps. Before the inoculation, LD e HD cultures were pelleted by centrifugation at 9000 rpm, 18°C for 10 minutes and re-suspended in sterile 10mM $MgSO_4$ in order to adjust them to the same cell concentration in the suspension (OD_{600} 0.200 corresponding approx. to 10^8 CFU ml^{-1}). The cell concentration in the suspension was successively confirmed by sequential dilutions and plating.

Psa was grown to log phase in filter-sterilized plant extracts, xylem sap or LB. Before the inoculation, Psa cultures were pelleted by centrifugation at 9000 rpm, 18°C for 10 minutes and re-suspended in sterile 10mM $MgSO_4$ in order to adjust them to the same cell concentration in the suspension (OD_{600} 0.200 corresponding approx. to 10^8 CFU ml^{-1}). The cell concentration in the suspension was successively confirmed by sequential dilutions and plating. Fifteen plants were used for each treatment and divided into three repeats of three plants. At 1, 3, 6, 9, 24, 32, 48, 96 hours plant weight, symptoms and endophytic bacterial population was assessed. Each plant was ground 5 ml of sterile $MgSO_4$ (10mM) addition, the suspension was filtered and centrifuged for 5 minutes at 14000 rpm. Bacterial pellets were frozen in liquid N_2 and stored at -80°C for subsequent molecular quantification epiphytic populations. At the same time points, plant material was frozen in liquid N_2 and stored at -80°C for subsequent molecular quantification endophytic populations.

Transcriptional analysis

The effect of plant extracts or xylem saps on PsaCFBP728 gene expression was investigated by qPCR. A number of genes putatively responding to cell density and social behavior were selected according to the current knowledge on Psa related bacterial species. The list of selected genes is reported in table 2. The aminoacidic sequences of the reporter genes were blasted in PsaCFBP7286 genome using FASTA sequence similarity searching tool (EMBL-EBI, Cambridge, UK). Standard parameters were maintained. Only identities greater than 60% were accepted (table 2). The corresponding nucleotidic sequences in Psa genome were elaborated with Geneious software (version R8) (Kearse *et al*., 2012).

Gene expression studies were performed on a comparable amount of bacterial cells obtained from two cell densities: 10^5 and 10^8 CFU ml^{-1}. Psa cultures either grown in LB or in plant extracts or xylem saps were centrifuged (14000rpm, 4°C, 10min) and the pellet obtained were suspended with differential amount of $MgSO_4$ (10 mM) in order to obtain the same OD_{600} 0.200 corresponding approx. to 10^8 CFU ml^{-1}. The cell concentration in the suspension was successively confirmed by sequential dilutions and plating.

Total bacterial RNA was extracted form Psa culture using Total RNA Purification kit (Norgen Biotek Corp., Thorold, CA), whereas were extracted Spectrum Plant Total RNA kit (Sigma-Aldrich) was used for Psa suspension obtained from infected plants. RNA purity and quantity was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). An aliquot of 1 μg of purified RNA was reverse-transcripted using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem Life Technologies, Carlsbad USA) according to the manufacturer's recommendations. cDNA samples were used as template for qPCR which was performed with Quick SybrGreen chemistry (Applied Biosystem) in a 96 well spectrofluorometric thermal cycler StepOnePlus (Thermo Fisher Scientific Inc.). Each template was adjusted to a final concentration of 100 ηg of cDNA per reaction and run in triplicate. qPCR cycles were performed as follows: 1 cycle of 50°C 2 min, 1 cycle of 95°C 10 min, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute Melting-curve analysis was performed immediately after completion of the real-time PCR (95°C for 15 seconds, 60°C for 15 seconds). Gene expression was expressed as relative expression to reference housekeeping genes *recA*, *rpoD* that used in *Pseudomonad* (Shi-En Lu et al., 2005; Narusaka et al., 2011; Greenwald et al., 2012) The relative quantification of gene expression was evaluated using the comparative C_t method (Pfaffl, 2001). Prior of raw C_t analysis all primers efficiency was assessed using LingRegPCR software (Ruijter *et al*. 2009). The qPCR primers developed for each gene are listed in table 3. Beacon Designer V 8,0 (PREMIER Biosoft, Palo Alto CA, USA) and Primer3Plus (Untergasser *et al*., 2007; Thornton and Basu, 2010) were used to design the primers. All primers pair were checked for specificity by end point PCR (performed as described for qPCR with Psa Genomic DNA as template).

RNA-silencing of *Tr* gene

A Psa *Tr* silenced strain was preliminary tested in biofilm and motility assays. Tr resulted from a bioinformatics research of signal synthase to which PsaR1 and PsaR3 responded (Steindler et al., 2008; Patel *et al*., 2014). In previous studies Tr gene expression was related to density (Fiorentini 1) and bacterial cross-talk (Fiorentini 2). To silence *Tr* gene the plasmid pHN678, kindly provided by Dr. N. Nakashima was used as scaffold (Nakashima and Tamura, 2009). Following the author's specifications, the plasmid was digested with the appropriate enzymes (NEB, New England Biolabs, Massachusetts, USA) (table 4). *Tr* was amplified with complementary restriction site at 5' and 3' ends (primers table 2), followed by amplicons purification using the QIAquick Gel Extraction Kit (Qiagen, Redwood City, USA) according to the manufacturer's instructions. The purified amplicon was digested as described above. Linearized vector and the purified amplicon were checked for purity and digestion on a 1% agarose gel stained with GelRED (Biotium Inc., Hayward, USA). The linearized vector and the amplicon (1:6 ratio) were ligated in a volume of 15 μl with Quick Ligation Kit (NEB). An aliquot of 2 μl was used to transform electrocompetent *E.coli* DH5α cells. Transformed cells were plated on selective LB amended with chloramphenicol (25μg/ml). The presence of the plasmid inside the growing colonies was confirmed by sequencing. The plasmid pHN678-TR was extracted and purified from the selected colonies by using a QIAprep minipre kit (Qiagen). Plasmid quantity and purity was verified by NanoDrop spectrophotometer and agarose gel analysis. The purified plasmid was

used to transform by electroporation competent Psa-CFBP7286 cells as described above. The efficacy of the silencing was tested on transformed Psa-TRsile cultures grown on liquid LB amended with 0.5% IPTG for plasmid expression. From these cultures, RNA was (i) extracted, (ii) purified using total RNA extraction KIT (Sigma-Aldrich), (iii) reverse-transcripted to cDNA by using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.) (iv) and amplified with gene specific primers.

PsaR2 cloning

Sequence of PsaR2 was amplified with high fidelity Pfu DNA Polymerase (Promega) following manufacturer's protocol. Primer were designed specific digestion site at 5' and 3' (table 5) in order to be cloned in the expression plasmid pH6HTN His6HaloTagT7 Vector (Promega). Amplicons so obtained and purified plasmid were digested with proper digestion enzyme (New England Biolabs, NEB, Ipswich, MA, USAfollowing manufacturer's protocol. Digested and purified vector and amplicons (checked for purity and complete digestion on 1% agarose gel stained with GelRED (Biotium, Hayward, CA, USA)) were ligated with Quick Ligation Kit (NEB) in a ratio of 1:6 insert:vector in a total volume of 15 µl. 2 µl was used to transform electrocompetent *E.coli* strain DH5α strain cells (Green and Sambrook, Molecular Cloning: A Laboratory Manual 2012 CSH press ISBN: 978-1-936113-42-2.) using 2mm cuvette (Gene Pulser Cuvette, Bio-Rad, Hercules, CA,USA) by standard electroporator (Gene-Pulser, Bio-Rad with subsequent parameters: 25 µF, 2,5 KV, 200 Ω). Transformants were plated and selected on LB added with ampicillin (100µg/ml). Growing colonies were checked by colony PCR with the same primer set use for cloning and following the same PCR program for qPCR and using Taq DNA polymerase master mix and TAQ Dna polymerase (Qiagen, Hilden, Germany) following manufacturer indications; amplicons were purified and correct vector sequence was checked by sequencing. Positive colonies were purified using QIAprep miniprep kit, (Qiagen) yield , and purification was checked by spectrophotometer and agarose gel analysis. Purified plasmids were electroporated in electrocompetent *E. coli* strain BL21 cells and plated on LB added with ampicillin (100µg/ml). Growing colonies were check for insertion by colony PCR and sequencing as describe above..

Protein expression and purification

A fresh grown single colony of *E. coli* strain BL21 harbouring pHisHaloR2 was grown in 10 ml of LB medium at 37°C shaking at 150 rpm until absorbance reached O.D. of 0.5-0.6 at 600 nm. Then the culture was centrifuged for 10 min at 10000 x g at room temperature. Supernatant was removed and pellet was resuspended in same volume of fresh LB containing IPTG to a final concentration of 1% and incubated at 37°C for 5 hours, shaking at 150 rpm. Cells were then centrifuged at 10000 x g for 15 min at 4°C and resuspended in 1 ml of homogenization buffer (10 mM Tris-Cl pH 7.5, 1mM proteinase inhibitor PMSF) and transferred into a 50 ml falcon tube (Corning, NY, USA). To lyse cells, fresh lysozyme (10mg/ml in double distilled water) (Sigma-Aldrich) was added 1/10 V/V, then incubated at 37°C for 30 minutes in gentle shaking (70 rpm). To complete cell lysis, sample was then frozen in liquid N_2 and subsequent thawed by

incubation at 37°C for 20 minutes. In order to get rid of contaminant DNA, DNase (stock solution of 1mg/ml in water, Sigma-Aldrich) was added in a final concentration of 1/10 V/V kept on costant shaking at 150 rpm for 30 min. at room temperature then solution was centrifuged at 100000 x g at room temperature. Supernatant were kept at -20 for further investigation; pellet was resuspended in halogenation buffer Protein purification was then performed by His tag-based affinity chromatography applying cell lysate on a homogenization buffer pre- equilibrated Nickel-resin (NiNTA resin, Qiagen) following the manufacturer's protocol. In particular, His-Tag purification was performed on (i) the final lysate corresponding to soluble fraction, (ii) resuspended pellet corresponding the insoluble fraction; (iii) membrane fraction obtained as described (Smith, 2014) and resuspended in homogenization buffer.

Enzyme-Linked Immunosorbent assay **ELISA**

Tthe assay was performed at room temperature and NUNC ELISA plastic plates (Sigma-Aldrich) were implied. All the antibodies were diluted in PBS-2%fat free milk(Sigma-Aldrich). The assay was conducted as follow: 100 µL of sample were put on each well; plate was covered and incubated at room temperature for 2 hours or overnight at 4 °C. Solution from wells decanted and discarded . Wells were washed 4 times with PBS-Tween 20 0,5% and 4 times with PBS. 100 µl of PBS -5% fat-free milk was added to wells, plate was covered and incubated for 2 hours at room temperature. Plate was washed as above. 100 µL of 1000 times diluted anti-HIS epitope antibody to wells. Plate was covered incubated at room temperature for 1 hour at 37°C. Solution was decanted from wells and discarded. Wells were washed as above and 100µL of 800 times diluted HRP-conjugated Anti-mouse secondary antibody were added to each well. Plate was covered incubated at room temperature for 1 hour at 37°C. Solution was decanted from wells and discarded. Wells were washed as above. It was added added 100 µL of chemioluminisense substrate (POD, Roche) and let it to develop plate at room temperature in the dark for 30 minutes. It was added 100 µL of stop solution (Roche) to each well. Plate was read by an ELISA microplate reader (Sunrise, TECAN, Männedorf, Swiss) at 405 nm.

Statistical analysis

Each experiment was independently repeated at least twice. Where indicted, difference between data are calculated with the Student's t test. ANOVA and SNK test were applied to gene expression data analysis and to motility and biofilm data assays. Statistically significant differences were assumed for $P \leq 0.05$.

Results

Experiments with plant extracts

Growth curve of *Psa* in different plant extracts

To investigate if the presence of host plant are able to influence Psa behavior, we grew Psa in xylem and plant medium derived from specific plant host (*A. deliciosa* and *A. chinensis*) and in plant derived medium of other non-correlated plant host such as *Actinida arguta* (known quite tolerant to Psa infection (Kerry et al., 2011)), *Camellia sinensis*, *Corylus avellana* (which are natural host of *pseudomonas syringae* pathovars more phylogenetic related to Psa: *Pseudomonas syrinage pv. teae* and *pseudomonas syringae pv. avellanae* (McCann et al., 2013 and Scortichini et al., 2013) and a *Nicotiana tabacum*. Preliminary studies were conducted regarding the ability of Psa to growth in such media . Therefore, growth curves of *Psa* were defined and compared with in the one in LB, in order to evaluate an eventual lack of growth in these media due to, for example, the scarcity of nutrients or inhibitory substances released by the plant. Our results demonstrated that tested media differentially affected growth of *Psa* (fig. 1).

A. deliciosa and *A. chinensis* plant extracts growths are the only ones were slightly comparable to LB, while the trend is not overlapping during all the time points , Psa in plant extracts was able to reach the same final amount. Also into xylem sap of *A. deliciosa* and *A. chinensis* growth of *Psa* was guaranteed, but reduced compared to the other plant extracts and LB in details, the trend is similar in trend, but lower in population quantity, except for the final time point, where it reaches a comparable value. Moreover, in all the other media tested, Psa was just able to survived. In fact, compared to the initial inoculum, the growth remained constant throughout the whole time.

Motility of Psa on different plant extracts

We investigated the potential effect of host recognition on *Psa* epiphytic/endophytic fitness. In particular, we checked the potential of different plant extract (miming the phyllosphere or the plant apoplast) and specific plant host xylem sap (miming the vascular vessels, which are colonized by *Psa* during plant systemic colonization (Donati et al., 2014) to prime *Psa* motility. We tested the above described conditions both on LD and on HD Psa population. Our results demonstrated that *Psa* mostly showed a marked enhanced swarming motility at HD population only on media based on extracts of *A. deliciosa* and *A. chinensis* (fig. 2) and xylem saps. In particular, *A. deliciosa* based medium showed the maximum motility induction respect to *A. chinensis*. Subsequent, xylem saps were able to induce swarming motility approximately 4 to 5 times the control. In this case, *A. chinensis* xylem sap led to a greater induction than *A. deliciosa* one. On the other species, motility resulted comparable (*A. arguta*) or slightly inhibited (*Camellia sinensis*, *Corylus avellana*, *Nicotiana tabacum*) respect to the control LB (fig 3). The assay on *Psa* LD population showed same trend with lower value of 20% in average, so it was detectable only in plate with *A. deliciosa* and *A. chinensis* derived medium and plant saps.

Biofilm quantitation in different plant extracts

Biofilm formation is crucial for pathogenesis both for epiphytic and for vascular system colonization and mainly driven by chemical signalling (Danhorn and Fuqua, 2007). We tested the ability of plant derived medium to induce biofilm formation in HD Psa population, not in LD because of is established that biofilm is specific for HD population (Monier and Lindow, 2003) . Plant extract from *A. deliciosa* induced the highest quantity of biofilm. Xylem sap from *A. chinensis* induced more biofilm then *A. deliciosa* one and quite comparable to *A. chinensis* plant extracts. *A. arguta* extract was able to induce a little but statistical significative quantity of biofilm. The other plant extract did not induce biofilm formation.

In vivo virulence of *Psa* cultures grown in plant extracts

Motility and biofilm assays demonstrated that *A. deliciosa* and *A. chinensis* derived medium was able to prime the phenotypes tested at the higher manner. Moreover, plant extract could mimic the plant host phyllosphere and growing Psa in this medium may "pre arm" the bacteria leading to an heavier and faster pathogenesis *in vivo* in order to verify this hypothesis *Psa* was harvested from high density LB liquid colture and resuspended in *A. deliciosa* and *A. chinensis* plant derived medium and finally used for inoculating micropropagated plants of *A. deliciosa* cv. Hayward.

Different substrates affect both epiphytic fitness and endophyte colonization. Until 3 hours post inoculation, Psa showed the same behaviour despite the different cultural conditions; starting from 6 hours post inoculation epiphytic population of Psa grown in plant medium (especially the grown on *A deliciosa* medium) decreased significatively respect to control which remain stable and slightly increased during time except at 24 hours after inoculation, where Psa grown in A. chinensis medium decreased significatively then raised again following the described trend (fig. 5a). Moreover, the inoculum of *Psa* from plant extracts consisted in a severe endophytic infection that led plants to death after 10 days in average between different experimental repetitions days. At 3 hours post inoculation, endophytic population of Psa grown *in A. deliciosa* medium was just detectable, while *A. chinensis* derived Psa reached a detection level 3 hours later. Inoculum from *A. deliciosa* and *A. chinensis* plant extracts resulted extremely high just 9 hours post inoculation: 10^4 $CFUg^{-1}$ FW for *A. Chinensis* derived inoculum and 10^6 $CFUg^{-1}$ FW for *A. deliciosa* derived inoculum, while at same time point endophyte population of the control inoculum was still undetectable (fig. 5b). In the following time points, the endophytic populations deriving from plant extracts showed a very stable trend with a remarkable difference of 3 logs between *A. deliciosa* derived media and *A. chinensis* one.

Gene expression of planktonic populations in plant extracts at low and high cell density

At the phenotypic level, motility biofilm and virulence were greatly stimulated by plant extracts. Therefore, we analyzed gene expression of *Psa* populations of different density (low and high) grown in that media in order to describe at molecular level the priming effect of plant extract, moreover we also tried to understand if this behavior is or not affected by cell density. In particular, genes involved in biofilm

formation/motility/virulence and density perception were evaluated. *Psa* grown in standard LB was set as control. Summary of the results is grouped in table 6.

Density-related genes. Plant extracts were able to enhance relative gene expression even at low cell density (LD) (fig. 6a). In particular *AefR, Fur, GacA.* Ad Tr (which is significative different from control only in *A. deliciosa* derived medium. Gene expression at high cell density (HD) magnified a similar trend; in particular quite all of the tested genes are inducted with some exceptions (fig. 6b). The differences of gene expressions in the two extracts at HD are not statistically significant, with the exception of the putative *Tr*, that in *A. deliciosa* is more expressed, both at LD and HD. Furthermore, *psaR2* is highly expressed in both plant extracts. Members of the interspecific signaling, such as the homoserine-lactones acylases (*hacA, hacB, hacC*) and *psaR1 psaR3*, had little or non-significative expression. Particularly at HD, the two component system genes *gacS*/*gacA* was significantly more expressed compared to the control in both extracts (fig. 6b).

Motility-related genes. Motility related genes presented an opposite behaviour regarding cell density in presence of plant extracts. Flagella genes *fliP* and *rpoN* and pila genes *pilA/C* resulted more expressed at LD rather than HD, while *pilO* was nt significatvely different from control (fig. 7 a-b). The two types of extracts induced an overall comparable expression except for *fliP* at LD and *pilC* at HD which expressions were significatively boosted by A. deliciosa plant derived medium.

Biofilm-related genes. The presence of both plant derived medium triggered the induction of biofilm-related genes already at LD (fig. 8a), but the effect was greater at HD (fig. 8b), in contrary manner than motility genes. Only *clpP* gene behaviour differs from this trend. In fact, its relative expression in HD is not statistically different from the control population, while it is at LD. There was no statistical difference in genes induction between the medium, but for *wssB* gene which is more boosted by *A. deliciosa* plant extract at HD popultion.

Virulence-related genes. All virulence genes are activated by the extracts of both plant species. The trend between LD and HD is the same, but in HD it is highly more pronounced (fig. 9a-b). Three of the tested genes (*oprM, hopS2* and *virB4*) display same induction despite the *Psa* population density. Moreover, a number of genes resulted differently primed by *A. deliciosa* extracts as indicated (Fig. 9a-b)

Gene expression of planktonic populations in xilems saps at low and high cell density

Density-related genes. At LD population, only *gacS*/*gacA* and *rpoS* were induce by *A. deliciosa* xylem, while *Tr* is only slightly, but significantly induced in *A. chinensis* sap (fig. 10a). At HD populations, the situation followed the trend described for plant extracts, with no differential genes expression between the two xylems (fig. 10b). Interestingly, *psaR2* and *Tr* were induced by both xylem saps in a density-related manner.

Motility-related genes. As described for motility assay, the xylem sap of *A. chinensis* primed the genes more than *A. deliciosa* (especially *fliP* and *pilC* genes at LD) (fig. 11a-b).

Biofilm-related genes. Also in case of biofilm-related genes, xylem sap was able to induce an higher overall expression especially in *algD* gene. The induction is still present at LD condition (fig. 12a), but it rose in a

density related manner at HD condition, and in particular for genes linked to biofilm biosynthesis (*mdoH* and *algD*) rather than for aggregation (*wspR*) which expression remained stable (fig. 10b).

Virulence-related genes. Virulence genes analyzed showed an overall induction less robust than when primed by plant extracts. Gene expression profile described an induction related to population density (fig. 13a-b). Xylem saps, exept for few cases (*Avrpt0, HopS2* were more more induted by *A. deliciosa* xylem sap in HD population, while *virB4* was more expressed by *A. chinensis* xylem sap

Gene expression of planktonic populations in non-host plant extracts.

Gene expression of planktonic high density populations of *Psa* was also evaluated in plant extracts deriving from non-host plants (*A. arguta*, *C. sinensis*, *C. avellana* and *N. tabacum*). The extracts did not trigger biofilm nor motility in *in vitro* assays. Either gene expression analysis performed on a restricted subset of genes on *Psa* showed quite no inductions. Only *A. arguta* primed the expression of *GacS* ,*Tr* and *psaR2* genes, but in a very lower way than extracts and xylem saps of host plant species. Moreover, other plant extract not only did not enhance gene expression, but in isolated cases it depressed some genes such as *aefR*, fur (except for *A. Arguta*) (Fig 14a) regarding density related genes; *AlgD, wssB, FliP* (except for *A. Arguta*) and *PilC* regarding biofilm and motility genes. (Fig 14b) Virulence genes were differentially affected by other plant medium. *AvrPt0, LysR* and *Enolase* were inhibited by all plant medium tested; *HopR21, HopS2* and *VirB4* were differentially slightly boosted by non-related plant derived medium.(Fig 14-c)

Bioassays with Psa mutants

Psa-mR2 in bacterial in plant extracts

PsaR2 is a receptor that, from an *in silico* research, is capable of binding plant signals and it is most likely involved in interkingdom signalling and *in vivo* virulence (Patel *et al*., 2014). Therefore, we preliminarily characterized motility and biofilm formation of a Psa-mR2 mutant in plant extracts of *A. deliciosa*. The mutant resulted in a slight decrease of swarming motility and biofilm production, but still statistically different from the control (fig. 15-16).

Psa-TRsile mutant biofilm phenotyping in plant extracts

The putative transcriptional regulator named *Tr* was identified by bioinformatics research for promoters of homoserine-lactones synthases. *Tr* expression resulted to be density-dependent and from *in silico* research it would be related to biofilm regulation (Fiorentini 1). *Tr* relative expression was also greatly inducted by neighbouring bacteria (Fiorentini 2). Therefore, these results together would suggest that it may play a pivotal role in the communication systems within *Psa* communities and within the bacterial consortium. Furthermore, we observed that plant extracts enhanced its relative expression. For these reasons, *Tr* gene silencing was performed with the aim of clarifying its involvement in signal transduction. We were mainly interested in biofilm induction since the *Tr* is involved in biofilm formation (Fiorentini 1-2). Analysing biofilm production in plant extracts of *A. deliciosa*. The biofilm produced by the mutant in LB is half of that produced by the wild type (fig. 17). Approximately the same was shown by the wild type and the mutant grown in plant extracts.

Characterization of the plant signal

In order to identify the plant signal that would bind to PsaR2 signal receptor PsaR2 was cloned in an expression vector in order to obtain a recombinant protein with fused a 6-His-tag at N terminus and a Halo tag at c-terminus. 6His tag was used for purification and Halo Tag will be used for affinity column building up. PsaR2 was successively expressed in *E. coli* and it was mainly present in soluble fraction (fig. 18). From soluble fractions, PsaR2 was purified, and its presence was checked by ELISA assay (fig. 19).

Discussion

Motility and biofilm are induced by the plant host

The biofilm is a complex structure in which bacteria develop social behaviour and may, *inter alia* better defend themselves from the defence mechanisms employed by the plant and then implement a more efficient host invasion (Bogino et al., 2013). Moreover, biofilm growth mode of *P. syringae* is involved in plant pathogenicity (Renzi *et al*., 2012; Donlan and Costerton, 2002) and a direct expression of interkingdom chemical communication (Hughes and Sperandio, 2008). Motility is an essential trait for epiphytic and endophytic colonization (both apoplastic and vessels) (Broek et al., 1995)Starting from this issue, we verified the effect on the interaction *Psa*-host plant and non-related plants by testing plant extracts or xylem sap in different assays. First we evaluated the effect of several plant extracts on *Psa* growth curve in order to verify if the trend in the extracts was comparable with that in the standard LB, so as to avoid that the results were spoiled by the lack of growth in these media due to, for example, the scarcity of nutrients or inhibitory substances released by the plant. Results revealed that *A. deliciosa* and *A. chinensis* extracts and xylem saps sustained the bacterial growth. Whereas in non-host extracts, presumably the lack of recognition of the substrates by *Psa* has meant that the initial inoculum not grow but remained constant all the time. *In vitro* experiments showed also that *Psa* plant extracts of *A. deliciosa* and *A. chinensis* provided *Psa* of the ability to swarm in the totality of the petri dishes. Biofilm production was greatly induced by the presence of plant extracts and xylem sap of *A. deliciosa* and *A. chinensis*. Whereas this induction in *A. chinensis* xylem and extracts is comparable, *A. deliciosa* plant extracts significantly induced more biofilm with respect to its xylem saps. The biofilm involves surface adhesion, cell proliferation and it is probably triggered by some signalling that occur between *Psa* and the plants. Since plant extracts are essentially made of the leaves, cell juices and epiphytic tissues, it is also plausible that biofilm production is induced by plant-pathogen interaction in the epiphytic part of the pathogenesis cycle, prior endophytic invasion. Evidence of biofilm stimulation by xylem saps would also indicate that biofilm represents an important trait during endophytic colonization of tissues and vessels. Importantly, extracts from non-host plants did not promote neither swarming nor biofilm production. Thus we can suppose that *Psa* establishes a specific relationship with the host that triggers a typical group behaviours, where plant extracts would provide. This ability would also facilitate the environmental epiphytic diffusion of the pathogen.

Gene expression reveals the close relation between *Psa* and plant-hosts

Our studies of the relative gene expression have established the close intercommunication that takes place between *Psa* and the host-plant. In fact, recognition of the host triggered all types of genes analysed. These results together are in line with most of the experiments present in the literature where extracts from plants stimulated rather than inhibited QS-gene expression (Venturi and Fuqua, 2013). As regards the experiments with the plant extracts, gene expression at high cell densities was higher than at low density, with the only exception of the motility genes, whose induction was greater at low cell density. The effect of *A. deliciosa* and *A. chinensis* extracts was in fact confirmed by the expression of the genes for responsible for the biosynthesis of flagellar protein (*fliP*, *rpoN*) and pila (*pilA, pilC, pilO*). This would suggest that even when there are few cells in the environment, there is a strong stimulus to colonization and movement caused by the recognition of the plant. Therefore the biofilm, that was especially stimulated at high densities, would take over at a later time, when populations are bigger, sanctioning the colonization of the environment only when the number is large enough to lead to successful formation of the biofilm itself. A confirm of that can be partially explained by the induction of *wspR*, that is a regulator required for cells aggregation and adhesion. Interestingly, one of the main genes responsible for alginate biosynthesis *algD* and also *mdoH*, that is required for the biosynthesis of membrane-derived oligosaccharides, are highly induced, indicating that they are two important constituents of the biofilm in *Psa*. In addition, in the tested conditions, density perception would not rule only the biofilm formation, but also functional expression of QS-signal receptors *psaR1*, *psaR3*, the plant signal receptor gene *psaR2* and the homoserine-lactones acylases *hacA, hacB, hacC*. Therefore, *Psa* would display of intercommunication systems that are used not only in interspecific interactions (Fiorentini 2), but also in establishing interkingdom connections with the plant-host. However, none of the plant compounds able to stimulate AHL-mediated gene expression have yet been chemically identified. Many of these compounds have been detected in root exudates, and their chemical properties suggest that they are distinct from AHLs (Venturi and Fuqua, 2013). Also *aefR* that coordinates swarming and virulence, and the two component system genes *gacS*/*gacA* positively responded to both *A. chinesins* and *A. deliciosa* extracts, confirming that *Psa* ecological fitness relies on these regulators.

Xylem saps obtained from *A. deliciosa* and *A. chinensis* were able to stimulate both biofilm formation and motility in *in vitro* assays. Analysis of gene expression in xylems saps of *A. deliciosa* and *A. chinensis* revealed that there was a significant induction of the genes studied. Despite it resulted overall slightly lower than the one in plant extracts, gene profiles in the two types of substrates are comparable.

Moreover, reading table 6 we can evaluate the differences in gene expression of the two *Actinidia* species and in which particular growth phase of *Psa* (low density or high density). In fact, low density populations of *Psa* in the plant extracts of *A. deliciosa* caused an overall greater gene induction with respect to *A. chinensis*. The effect of the two plant extracts generally vanished in high density conditions. Regarding the xylem saps, *A. chinensis* enhanced the relative expression of biofilm and motility genes at low density. As for the plant extracts, at the high density the expressions were practically homogenous. Interestingly, *Psa* virulence genes

were generally more expressed in *A. deliciosa* than in *A. chinensis*, and in all conditions (LD/ HD; xylem saps/plant extracts).

Role of Tr in the plant-pathogen interactions

The putative transcriptional regulator named *Tr* identified from a bioinformatics research for promoters of signal synthases (Fiorentini 1), positively responded to plant extracts of *Actindia* spp. Moreover, *Tr* was overexpressed in high density cultures grown in plant extracts and xylem saps of both *A. deliciosa* and *A. chinensis*, suggesting that it could function as a pivotal regulator during the interkingdom communications. The RNA silencing of *Tr* confirmed that this regulator should act as a positive regulator of the biofilm. The deletion of the *Tr* caused a persistent reduction in the biofilm quantified and therefore we can suppose an involvement of this transcriptional regulator in the expression of this phenotype. These preliminary results will be supported by further investigations about virulence *in vivo* and qPCR analysis with the aim to elucidate the role of *Tr* in the manifestation of such phenotypes.

A "pre-armed" *Psa* is more virulent

It was supposed that plant mimics acting as agonists of AHL-QS might lead to pathogen confusion and decreased pathogenicity because they can stimulate premature expression of virulence genes (Venturi and Fuqua, 2013). However, it was not the case of *Psa*. In fact, the gene expression of virulence factors was significantly induced by plant extracts and xylems saps of *A. deliciosa* and *A. chinensis*. These observations were confirmed in *in-vivo* infections, where a "pre-armed" *Psa* grown in plant extracts resulted detrimental for the kiwifruit plantlets that died after 10 days. Populations of *Psa* treated with *A. deliciosa* extracts resulted overall more virulent than *A. chinensis* ones. However, similar is the trend either of epiphyte and endophyte populations of the two treatments. In particular, while the epiphyte populations from both plant extracts after 6 hours from the inoculation significantly decreased whereas the control inoculum survived constantly over time, the endophyte populations increased, reaching inside the plantlets levels higher than the initial population inoculated. In conclusion, this remarking result underlines the importance of gene priming occurred during the growth in the plant extracts and that the ecological fitness and virulence of a non-primed *Psa* is therefore less efficient.

Host-pathogen recognition is species-specific

When plant extracts of non-host plants (*A. arguta, C. sinensis, C. avellana* and *N. tabacum*) were tested in the bioassays, neither biofilm nor motility were induced. Even though conducted on a restricted subset of genes, also the gene expression analysis showed quite no inductions in *C. sinensis, C. avellana* and *N. tabacum* extracts, leading to the conclusion that the influence of host extracts is species-specific. Thus it was confirmed that the manifestation of biofilm and motility is regulated by specific signalling that exist between the pathogen and its host and that non-host plant misrecognition does not occur only at a pathogenic level, but also genetically and phenotypically. However, the peculiar activation of *gacS*, *psaR2* and *Tr*, in the

extracts of *A. arguta* would suggest that these receptors operate during host recognition processes and that this identification occurs at genus level, but it is not sufficient to trigger typical traits of plant-host infections.

Role of PsaR2 receptor

PsaR2 is a receptor that, from an *in silico* research, is capable of binding plant signals and it is most likely involved in interkingdom signalling and *in vivo* virulence (Patel *et al*., 2014). Being a LuxR-like type receptors, it may be involved in regulation of QS-dependent phenotypes. For this reason, we used a knock-out mutants in order to elucidate its potential involvement in the manifestation of biofilm and motility phenotypes under the effect of bacterial supernatants. The PsaR2 mutant of *Psa* demonstrated that the ability of motility and biofilm is not impaired, but still lower than the wild type. Therefore we can conclude that, from our studies, the effect of this receptor on these phenotypes is fairly bland.

We were also interested in purifying PsaR2 receptor protein in order to detect the plant signals that could bind to it. To this end, the receptor was purified, extracted and covalently immobilized to a resin. We are only in preliminary stages of this work. However, we will proceed with clean-up of plant extracts over the resin. Through this operation we will be able to highlight all those molecules having binding affinity with the receptor. The subsequent analysis of the ligand components will then be done with identification techniques such as GC-MS or NMR. The completion of the experiments will have characterized the plant component potentially capable of regulating the interkingdom communication mechanisms.

In order to characterize the plant signal, plant sap from *A. deliciosa* and *A. chinensis* will be fractionated so as to differentiate chemically different components. Each fraction will be phenotyped with biofilm and motility assay. Only the active fraction will be applied on chromatographic column build up with recombinant R2 as “capture protein” with the final aim of analyzing the resulting fractions to determine the exact nature of the plant signal.

Conclusions

The premise of this work intended to establish if *Psa* is able to interact specifically to the plant host,, in particular by analysing phenotypes related to the interkingdom signalling and corroborating these views with gene expression analysis of reporter genes linked to these phenotypes. Biofilm and motility were increased by xylem saps and the plant extracts derived from *Psa* host plant species, i.e. by *A. deliciosa* and *A. chinensis*. The qPCR revealed that the plant extracts induce an increased expression compared to the xylem saps, and in particular this effect was greater on the part of *A. deliciosa*. This close plant-pathogen relation was further confirmed by the study of the interactions with non-hosts species, which have not been primed neither the phenotypes nor the expression of the gene analysed, and therefore the ecological interaction *Psa*-plant resulted to be strictly species-specific. In addition, the reporting systems of *Psa* pre-activated by the growth in plant substrates have led to a better penetration into the host and overcome of the plant defence barriers. The aggressiveness of *Psa* against *A. deliciosa* was further confirmed by this evidence. Therefore, these results demonstrated that the co-evolution of plant pathogen led to the manifestation of different phenotypes related to bacterial communities such as the motility, the biofilm formation and the trigger of virulence mechanisms, which participate together to the ecological fitness of *Psa*.

Reference

Appleby, Jeryl L., John S. Parkinson, and Robert B. Bourret. 1996. "Signal Transduction via the Multi-Step Phosphorelay: Not Necessarily a Road Less Traveled." *Cell* 86(6): 845–48.

Argenio, David a D et al. 2002. "Autolysis and Autoaggregation in." *Microbiology* 184(23): 6481–89.

Bogino, Mercedes Oliva , Fernando G. Sorroche, and Walter Giordano (2013) The Role of Bacterial Biofilms and Surface Components in Plant-Bacterial Associations *Int. J. Mol. Sci.* 14, 15838-15859

Bomchil, Natalia, Paula Watnick, and Roberto Kolter. 2003. "Identification and Characterization of a Vibrio Cholerae Gene, mbaA, Involved in Maintenance of Biofilm Architecture." *Journal of Bacteriology* 185(4): 1384–90.

Brachmann, Alexander O et al. 2013. "Pyrones as Bacterial Signaling Molecules." *Nature chemical biology* 9(9): 573–78. http://www.ncbi.nlm.nih.gov/pubmed/23851573.

Broek and Vanderleyden (1195) The role of bacterial motility, chemotaxis, and attachment in bacteria-plant interactions *Mol Plant-Microbe Interact*

Brencic, Anja, Stephen C Winans, and Plant Colonization. 2005. "Detection of and Response to Signals Involved in Host-Microbe Interactions by Plant-Associated Bacteria." 69(1): 155–94.

Cha, Ji Young et al. 2008. "Functional Analysis of the Role of Fur in the Virulence of Pseudomonas Syringae Pv. Tabaci 11528: Fur Controls Expression of Genes Involved in Quorum-Sensing." *Biochemical and Biophysical Research Communications* 366(2): 281–87.

Chancey, S T, and D W Wood. 1999. "Two-Component Transcriptional Regulation of N -Acyl-Homoserine Lactone Production in Pseudomonas Aureofaciens." 65(6): 2294–99.

Danhorn and Fuqua, 2007 Biofilm Formation by Plant-Associated Bacteria *Annu. Rev. Microbiol.* 2007. 61:401–22

Donati, Irene et al. 2014. "New Insights on the Bacterial Canker of Kiwifruit (Pseudomonas Syringae Pv. Actinidiae)." *Journal of Berry Research* 4(2): 53–67.

Donlan, Rodney M, J William Costerton, Rodney M Donlan, and J William Costerton. 2002. "Biofilms : Survival Mechanisms of Clinically Relevant Microorganisms." *Clinical Microbiology* 15(2): 167–93.

Ferluga, Sara, and Vittorio Venturi. 2009. "OryR Is a LuxR-Family Protein Involved in Interkingdom Signaling between Pathogenic Xanthomonas Otyzae Pv. Oryzae and Rice." *Journal of Bacteriology* 191(3): 890–97.

Green and Sambrook, Molecular Cloning: A Laboratory Manual 2012 CSH press ISBN: 978-1-936113-42-2

Gonzàlez, Juan F., Michael P. Myers, and Vittorio Venturi. 2013. "The Inter-Kingdom Solo OryR Regulator of Xanthomonas Oryzae Is Important for Motility." *Molecular Plant Pathology* 14(3): 211–21.

Guan, Rong et al. 2012. "Methylthioadenosine Deaminase in an Alternative Quorum Sensing Pathway in Pseudomonas Aeruginosa." *Biochemistry* 51(45): 9094–9103.

Heeb, Stephan, and Dieter Haas. 2001. "Regulatory Roles of the GacS / GacA Two-Component System in Plant-Associated and Other Gram-Negative Bacteria." *Molecular Plant-Microbe Interactions* 14(12): 1351–63.

Hughes and Sperandio, 2008 Inter-kingdom signalling: communication between bacteria and their hosts *Nat Rev Microbiol.* 6, 111-120

Kalia, Vipin Chandra. 2013. "Quorum Sensing Inhibitors: An Overview." *Biotechnology Advances* 31(2):

224–45. http://dx.doi.org/10.1016/j.biotechadv.2012.10.004.

Kawakita, Yuichiro et al. 2012. "Characterization of Each aefR and mexT Mutant in Pseudomonas Syringae Pv. Tabaci 6605." *Molecular Genetics and Genomics* 287(6): 473–84.

Kearse, Matthew et al. 2012. "Geneious Basic: An Integrated and Extendable Desktop Software Platform for the Organization and Analysis of Sequence Data." *Bioinformatics* 28(12): 1647–49.

Kerry Everett & Robert K. Taylor, Megan K. Romberg, Jonathan Rees-George, Robert A. Fullerton, Joel L. Vanneste , Mike A. Manning 2011 First report of Pseudomonas syringae pv. actinidiae causing

kiwifruit bacterial canker in New Zealand Australasian Plant Dis. Notes 6:67–71

Kinscherf, Thomas G., and David K. Willis. 1999. "Swarming by Pseudomonas Syringae B728a Requires gacS (lemA) and gacA but Not the Acyl-Homoserine Lactone Biosynthetic Gene ahlI." *Journal of Bacteriology* 181(13): 4133–36.

Li, Yi et al. 2013. "NO-Induced Biofilm Dispersion in Pseudomonas Aeruginosa Is Mediated by an MHYT Domain-Coupled Phosphodiesterase." *Journal of Bacteriology* 195(16): 3531–42.

Livak, K J, and T D Schmittgen. 2001. "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and." *Methods* 25: 402–8.

Marcelletti, Simone et al. 2011. "Pseudomonas Syringae Pv. Actinidiae Draft Genomes Comparison Reveal Strain-Specific Features Involved in Adaptation and Virulence to Actinidia Species." *PLoS ONE* 6(11).

Martin, Paul R., Alison A. Watson, Thomas F. McCaul, and John S. Mattick. 1995. "Characterization of a Five-Gene Cluster Required for the Biogenesis of Type 4 Fimbriae in Pseudomonas Aeruginosa." *Molecular Microbiology* 16(3): 497–508.

McCann, Honour C. et al. 2013. "Genomic Analysis of the Kiwifruit Pathogen Pseudomonas Syringae Pv. Actinidiae Provides Insight into the Origins of an Emergent Plant Disease." *PLoS Pathogens* 9(7).

Monier and Lindow, 2003 Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces *Proc Natl Acad Sci U S A*. Dec 23; 100(26): 15977–15982.

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*15, 473-497.

Nakashima, Nobutaka, and Tomohiro Tamura. 2009. "Conditional Gene Silencing of Multiple Genes with Antisense RNAs and Generation of a Mutator Strain of Escherichia Coli." *Nucleic Acids Research* 37(15).

Narusaka, Mari, Tomonori Shiraishi, Masaki Iwabuchi, and Yoshihiro Narusaka. 2011. "rpoD Gene Expression as an Indicator of Bacterial Pathogens in Host Plants." *Journal of General Plant Pathology* 77(2): 75–80.

O'Toole, G A, and R Kolter. 1998. "Initation of Biofilm Formation in Pseudomans Fluorescens WC365 Proceeds via Multiple, Convergent Signalling Pathways: A Genetic Analysis." *Molecular Microbiology* 28: 449–61.

Patel, Hitendra Kumar et al. 2014. "The Kiwifruit Emerging Pathogen Pseudomonas Syringae Pv. Actinidiae Does Not Produce AHLs but Possesses Three luxR Solos." *PLoS ONE* 9(1).

Penaloza-Vazquez, Alejandro, Aswathy Sreedharan, and Carol L. Bender. 2010. "Transcriptional Studies of the hrpM/opgH Gene in Pseudomonas Syringae during Biofilm Formation and in Response to Different Environmental Challenges." *Environmental Microbiology* 12(6): 1452–67.

Phan, Gilles et al. 2010. "Structural and Dynamical Insights into the Opening Mechanism of P. Aeruginosa

OprM Channel." *Structure* 18(4): 507–17.

Quiñones, Beatriz, Glenn Dulla, and Steven E Lindow. 2005. "Quorum Sensing Regulates Exopolysaccharide Production, Motility, and Virulence in Pseudomonas Syringae." *Molecular plant-microbe interactions : MPMI* 18(7): 682–93.

Renzi, Marsilio et al. 2012. "Bacterial Canker on Kiwifruit in Italy: Anatomical Changes in the Wood and in the Primary Infection Sites." *Phytopathology* 102(9): 827–40.

Ruijter, J M, S. van der Velden, and A. Ilgun. 2011. "LinRegPCR (11.0) - Analysis of Quantitative RT-PCR Data." *Heart Failure Research Center*: 1–29. papers://476b58c2-a19a-46f3-a8ef-897be4da28ce/Paper/p17039.

Sambrook, J., E.F. Fritsch, and T. Maniatis, 1989, Molecular cloning: a laboratory manual, 2nd edition ed., Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Schuster, Martin, C Phoebe Lostroh, Tomoo Ogi, and E. Peter Greenberg. 2003. "Identi Cation, Timing, and Signal Speci City of." *Society* 185(7): 2066–79.

Segura, A., E. Duque, A. Hurtado, and J. L. Ramos. 2001. "Mutations in Genes Involved in the Flagellar Export Apparatus of the Solvent-Tolerant Pseudomonas Putida DOT-T1E Strain Impair Motility and Lead to Hypersensitivity to Toluene Shocks." *Journal of Bacteriology* 183(14): 4127–33.

Sheng, Li et al. 2012. "Quorum Sensing and Alternative Sigma Factor RpoN Regulate Type VI Secretion System I (T6SSVA1) in Wsh Pathogen Vibrio Alginolyticus." *Archives of Microbiology* 194(5): 379–90.

Shepherd, Ryan W., and Steven E. Lindow. 2009. "Two Dissimilar N-Aeyl-Homoserine Lactone Acylases of Pseudomonas Syringae Influence Colony and Biofilm Morphology." *Applied and Environmental Microbiology* 75(1): 45–53.

De Souza, Alessandra A. et al. 2004. "Gene Expression Profile of the Plant Pathogen Xylella Fastidiosa during Biofilm Formation in Vitro." *FEMS Microbiology Letters* 237(2): 341–53.

Scortichini M, Marcelletti S, Ferrante P, Firrao G. (2013) A Genomic redefinition of Pseudomonas avellanae species. *PLoS One* Sep 25;8(9):e7579

Smith SM (2011) Strategies for the purification of membrane proteins. *Methods Mol Biol* ;681:485-96

Spiers, Andrew J et al. 2013. "Cellulose Expression in Pseudomonas Fluorescens SBW25 and Other Environmental Pseudomonads." *Cellulose - Medical, Pharmaceutical and Electronic Applications*: 1–26.

Spinelli F., Donati I., Vanneste I.L., Costa M., Costa G. (2011) - Real time monitoring of the interactions between Pseudomonas syringae pv. actinidiae and Actinidia species. *Acta Horticolture*, 913: 461-465

Spinelli F., Donati I., Mauri S., Preti M., Fiorentini L., Cellini A., Buriani G., Costa G. (2012) – Osservazioni
sullo sviluppo del cancro batterico. Frutticoltura, 9: 32-35.

Subramoni, Sujatha, and Vittorio Venturi. 2009. "LuxR-Family 'Solos': Bachelor Sensors/regulators of Signalling Molecules." *Microbiology* 155(5): 1377–85.

Taguchi, Fumiko et al. 2006. "Identification of Glycosylation Genes and Glycosylated Amino Acids of Flagellin in Pseudomonas Syringae Pv. Tabaci." *Cellular Microbiology* 8(6): 923–38.

Thornton, Brenda, and Chhandak Basu. 2011. "Real-Time PCR (qPCR) Primer Design Using Free Online Software." *Biochemistry and Molecular Biology Education* 39(2): 145–54.

Tian, Yang et al. 2008. “Role of RpoS in Stress Survival, Synthesis of Extracellular Autoinducer 2, and Virulence in Vibrio Alginolyticus.” *Archives of Microbiology* 190(5): 585–94.

Ude, Susanne et al. 2006. “Biofilm Formation and Cellulose Expression among Diverse Environmental Pseudomonas Isolates.” *Environmental Microbiology* 8(11): 1997–2011.

Untergasser, Andreas et al. 2007. “Primer3Plus, an Enhanced Web Interface to Primer3.” *Nucleic Acids Research* 35(SUPPL.2): 71–74.

Venturi, Vittorio, and Clay Fuqua. 2013. “Chemical Signaling between Plants and Plant-Pathogenic Bacteria.” *Annual review of phytopathology* 51: 17–37. http://www.ncbi.nlm.nih.gov/pubmed/23915131.

Zhang, Lili, Yantao Jia, Li Wang, and Rongxiang Fang. 2007. “A Proline Iminopeptidase Gene Upregulated in Planta by a LuxR Homologue Is Essential for Pathogenicity of Xanthomonas Campestris Pv. Campestris.” *Molecular Microbiology* 65(1): 121–36.

Tables and figures

Table 1. Bacterial species, strains and plasmids used in this study. The most relevant characteristics, the growing medium and temperature are also reported. Km^r, Nf^r Chl^r and Amp^r indicate resistance to kanamycin, nitrofurantoin, chloramphenicol and ampicillin respectively.

Strain or plasmid	**Relevant characteristics**	**Reference/Source**
Pseudomonas syringae pv. *actinidiae* (*Psa*)		
Psa wild type strain CFBP7286	Cultivated in LB at 27°C	Spinelli et al 2011
Psa-mR2 (psaR2::pKNOCK)	Cultivated in LB at 27°C Km^r (100 μg/ml)	Patel et al., 2014
Psa-Trsile (Psa harbouring pSileTR)	Cultivated in LB at 27°C; gene silencing is induced with IPTG 0.5%; Chl^r (25 μg/ml)	This work.
Escherichia coli (*E. coli*) strain DH5α	Cultivated in LB at 37°C; electrocompetent cells are used for cloning and protein expression	Sigma-Aldrich
pSile TR	Derived from pHN678, expressing the N terminal part and putative Shine-Delgarno sequence of *Tr* gene	Nakashima and Tamura 2009
pHisHalo R2	Derived from pH6HTN His6HaloTag® T7 Vector. Expression of R" protien in E. coli strains. Amp^r (100 μg/ml)	Promega

Table 2. Genes selected from several bacterial species and their relative function for the study of the signalling phenotypes in *Psa* by qPCR. Gene identity is a percentage value that refers to the homology of each bacterial gene to the relative gene in *Psa*. Reference for each gene is also indicated.

	Gene	Identity	Organism	Strain	Function/s	Reference
Biofilm	*algD*	99,3	*Pseudomonas syringae* pv. *tomato*	DC3000	*algD* is involved in biofilm formation both *in vitro* and *in planta*. First gene to be transcribed during the biosynthesis of alginate, a virulence factor in *Ps. syringae*.	Penaloza-Vazquez et al., 2010
	clpP	87,6	*Pseudomonas fluorescens*	SBW25	Codes for a protein required for biofilm formation of *P.fluorescens*.	O'Toole and Kolter, 1998
	mdoH	99,5	*Pseudomonas syringae* pv. *tomato*	DC3000	Membrane derived oligosaccharides, a family of glucans present in periplasmic space of Gram negative bacteria. Required for biofilm formation.	Penaloza-Vazquez et al., 2010
	wspR	85,2	*Pseudomonas fluorescens*	SBW25	Is involved in colony morphology and cellular aggregation; regulator of genes that encode a putative fimbrial adhesin required for biofilm formation	D'Argenio et al., 2002 Ude et al., 2006
	wssB	68,3	*Pseudomonas fluorescens*	SBW25	Cellulose synthase catalytic subunit. Cellulose is used by *P. fluorescens* SBW25 in the colonization of plant surfaces.	Spiers et al., 2013

	Gene	Identity	Organism	Strain	Function/s	Reference
Motility	*fliP*	81,9	*Pseudomonas putida*	W619	Flagellar biosynthetic protein FliP	Segura et al., 2001
	pilA	92,4	*Pseudomonas syringae* pv. *tabaci*		involved in the attachment of the bacteria and/or biofilm formation	A. de Souza et al., 2004
	pilC	90,4	*Pseudomonas syringae* pv. *tomato*	DC3000	type IV pilus biogenesis protein PilC	A. de Souza et al., 2004
	pilO	74,9	*Pseudomonas aeruginosa*	PAO1	type 4 fimbrial biogenesis protein PilO	Martin et al., 1995
	rpoN	60,0	*Vibrio alginolyticus*		Involved in polar flagellar formation.	Sheng et al., 2012

	Gene	Identity	Organism	Strain	Function/s	Reference
Density-related genes	*aefR*	91,6	*Pseudomonas syringae* pv. *syringae*	B728a	Potential regulator of mexEF/oprN in *Ps. tabaci*; Synthesis of 3OC6-HSL. Gene product: "AHL and epiphytic fitness regulator"	Quinones et al., 2005; Kawakita et al., 2012
	fur	100,0	*Pseudomonas syringae* pv. *tabaci*		Ferric uptake regulator. Role in the control of genes involved in QS.	Cha et al., 2007
	gacA	99,1	*Pseudomonas syringae* pv. *syringae*	B728a	The GacS/GacA system interacts positively with the PhzI/PhzR quorum sensing system by regulating the synthesis of N-acyl-homoserine lactone	Chancey et al.,1999
	gacS	100,0	*Pseudomonas syringae* pv. *syringae*	B728a	Membrane-bound sensor kinase protein that recognizes specific environmental stimuli and activates GacA	Appleby et al ., 1996 Heeb and Haas, 2001 Pernestig et al., 2001
	hacA	88,4	*Pseudomonas syringae* pv. *syringae*	B728a	AHL acylase; Peptidase S45, penicillin amidase	Shepherd and Lindow, 2009
	hacB	94,3	*Pseudomonas syringae* pv. *syringae*	B728a	AHL acylase; Penicillin amidase. Can inactivate 3OC6HSL	Shepherd and Lindow, 2009
	hacC	97,0	*Pseudomonas syringae* pv. *syringae*	B728a	Aspartate transaminase: it is an AHL acylase that degrades AHL signal	Kalia, 2013
	mexE	93,7	*Pseudomonas syringae* pv. *phaseolicola*		Related with resistance to antimicrobial agents component of multidrug efflux system; multidrug efflux RND transporter, membrane fusion protein MexE.	Kawakita et al., 2012
	oprM	73,1	*Pseudomonas aeruginosa*	PAO1	Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM precursor	Phan et al., 2010
	psaR1	-	*Pseudomonas syringae* pv. *actindiae*		Highly similar to LuxRs which bind AHLs and are part of the canonical LuxI/R AHL QS systemslo that bind to AHLs	Patel et al., 2014
	psaR2	-	*Pseudomonas syringae* pv. *actindiae*		Binds and responds to yet unknown plant signal molecules.	Patel et al., 2014
	psaR3	-	*Pseudomonas syringae* pv. *actindiae*		Highly similar to LuxRs which bind AHLs and are part of the canonical LuxI/R AHL QS systemslo that bind to AHLs	Patel et al., 2014
	rpoS	69,4	*Vibrio alginolyticus*		Part of the regulatory networks of virulence and LuxS quorum sensing system; RpoS is cell density related; RpoS positively regulates AHL levels.	Tian et al., 2008
	Tr	-	*Pseudomonas syringae* pv. *actindiae*		Transcriptional regulator derived from promoter blast analysis. Potentially involved in signaling transduction and biofilm regulation.	This work

	Gene	Identity	Organism	Strain	Function/s	Reference
Virulence	*avrpt01*	-	*Pseudomonas syringae* pv. *actindiae*		Unknown	This work
	enolase	-	*Pseudomonas syringae* pv. *actindiae*		Pathogenesis related genes	McCann et al., 2013
	hopD1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	McCann et al., 2013
	hopQ1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopR1	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopS2	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	Marcelletti et al., 2011
	hopZ5	-	*Pseudomonas syringae* pv. *actindiae*		T3SS and pathogenesis related genes	McCann et al., 2013
	lysR	93,5	*Pseudomonas syringae* pv. *tabaci*		LysR-type transcriptional regulators (LTTRs) regulate a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility.	Kawakita et al., 2012
	pamTada	74,7	*Pseudomonas aeruginosa*		Potential target for QS in most Gram-negative bacteria.	Guan et al., 2012
	virB4	-	*Pseudomonas syringae* pv. *actindiae*		Secretion system	Marcelletti et al., 2011

Table 3. Primers used for PCR and qPCR. The genes are grouped in 4 classes according to their function. The source of primer sequences is also reported.

	Gene	Forward primer	Reverse primer	Source
Biofilm				
	algD	GACCTGGAACTGGACTACATC	TGCTGCGAACCACGATAG	This work
	clpP	CTTATATTCAGCAGAACTCT	GCGAATAGATGTCATAGG	This work
	mdoH	ACGGTAACCTTGAACTTGC	CACCATCGTTCTGCTGTT	This work
	wspR	ACGACTATCTGGTCAAACTG	ATAGGCTTCATCACGCTG	This work
	wssB	CGCTGGTGATGATGATGGT	CTGACGCTCAACGCTGTG	This work
Motility				
	fliP	TCAAGACGGCGTTTCAGA	CGGCGAGAGCATCATCAT	This work
	pilA	GCCATTCCTTCCTATCAA	GTAAGACCATTGCTCCAG	This work
	pilC	CGCTGGACATCGCATTCT	GCACCTTCGGCAATGATG	This work
	pilO	CCTACAGAAAGCAGATGGA	GTGATGTCTTCAAGCAGTC	This work
	rpoN	GCACCGACTCCTGATTGA	GAATCCACAGAAGCCGAATAC	This work
Density-related				
	aefR	AACTGCTGGAATTGCTCTG	TGTATCGTGGCACCTACC	This work
	fur	TGAAAATAGCGAACTACGAAAAGC	TGTAAACATCCTCGGCACTC	This work
	gacA	GATGACCATGACCTTGTTC	TCTTCAGCGATTCCTCAC	This work
	gacS	AGAACCTGGAAACCATCG	ATCTCGTGGCTCATGTTG	This work
	hacA	AGTTCACTGAAGCCTTTGC	CCAGTTGTAGCGCCTGAA	This work
	hacB	ACGGCATCAACCAGTATC	ATGCTGACCGTGTCCTCT	This work
	hacC	GACCTTCTTCGCCTCCAG	TTCCTTCGATTCGGTGATGA	This work
	mexE	TGTACGCACGGCTGAAACTG	TCCTTGTCCATCACCAGCAC	This work
	oprM	CGCTGGACATCGCATTCT	GCACCTTCGGCAATGATG	This work
	psaR1	ATACCTGGTCAGTAGTCTCA	GCAGCACTTCAAGTTCAC	This work
	psaR2	ACTGTTTGACCAGAAGATG	CTGAACGGTTGAGTTGAT	This work
	psaR3	GGTTCGCTCATTATCTGAT	GCAATGCTTGAGGATAGG	This work
	rpoS	CGTCGCTCAAACAACACAAAT	GAGACAGCAGAGGGGAAAAC	This work
	Tr	ACATTTACCATGTCACCCGCC	TGATGGCTTCTGCGTCGTTC	This work
Virulence				
	avrpt01	GGAGCGAATCTTGCCATT	GGAGCGATATGCGTGAAG	This work
	enolase	CATCGCCAACCTCAATGG	CCTGGATGTCGATGTTGTTAT	This work
	hopD1	CAGTAGACAGCAGTAGCC	CGGGTTATCGGAAACAAG	This work
	hopQ1	GGCATTCCACTTCGTATAG	CAACGCACTCTTCTGAAC	This work
	hopR1	GACATAACTGCCGATGCT	TCCAGATAGGCTCGATCA	This work
	hopS2	CCTTAAACGGCTGGCAGAG	CGAAGTGATGCTTGAGGTGAA	This work
	hopZ5	TCAGGCTACAATACTTACGCATCA	CAGGAATAGAACGGAACTCAGGAT	This work
	lysR	TGCGGAAGTTGAAGCGGATTACG	ACCGAAATGTTGCTGCCTCCC	This work
	pamTada	ACACATGACCCAGATCAG	CAGCTTGAGGTTGGATTC	This work
	virB4	TTTGAAGACACCACTGTTTC	CTGCGTCACCTACTACTC	This work
Reference				
	rpoD	CCGAGATCAAGGACATCAAC	GAGATCACCAGACGCAAGTT	Narusaka et *al.*, 2011
	recA	CGCACTTGATCCTGAATACG	CATGTCGGTGATTTCCAGTG	This work

Table 4. List of primer set used for *Tr* cloning in pHN678 to perform *Tr* silencing. Restriction sites, putative promoter -10 box nd Shine Dalgarno sequence are indicated.

Primer_Forward	CG	GGATCC *Bam*HI	GGCTTTCCATATTTGTTCGAG -10 box
Primer Reverse	CG	GAATTC *Eco*RI	TTGAGCCGTGGACCTATCC Shine Dalgarno sequence

Table 5. List of primer set used for PsaR2 cloning in pH6HTN His6HaloTagT7 vector restrictions sites are indicated

PsaR2			
Primer_Forward	AATTCC	GAGCTC *Sac*I	ATGCATATCAGGTTGTCGG -10 box
Primer_Reverse	CTAG	TCTAGA *Xba*I	TCAGTGGTCG AGTAAACGGT Shine Dalgarno sequence

Table 6. Summary of the effects of the plant extracts and xylem saps of *A. deliciosa* and *A. chinensis* on *Psa* gene induction at low and high cell densities. (+) indicates which treatment between *A. deliciosa* and *A. chinensis* resulted in a statistically different increase of the relative gene expression. (=) indicates no statistical difference.

		LOW DENSITY				HIGH DENSITY			
		Plant extracts		Xylem saps		Plant extracts		Xylem saps	
		A.deliciosa	*A.chinensis*	*A.deliciosa*	*A.chinensis*	*A.deliciosa*	*A.chinensis*	*A.deliciosa*	*A.chinensis*
Biofilm	*algD*	+			+	=			+
	clpP		+		+	+		=	
	mdoH	=		=		=		=	
	wssB	=		=		+			+
	wspR	=			+	=		=	
Motility	*fliP*	+			+	=			+
	rpoN	=			+	=		=	
	pilA	=		=		=		=	
	pilC	=			+	+		=	
	pilO	=		=		=		=	
Density-related genes	*aefR*	+		=		=		=	
	fur	+		=		=		=	
	gacA	+		+		=		=	
	gacS	=		+		=		=	
	hacA	=		=		=		=	
	hacB		+	=		=		=	
	hacC	=		=		=		=	
	mexE	=		=		=		=	
	psaR1	=		=		=		=	
	psaR2	=		=		=		+	
	psaR3	=		=		=		=	
	Tr	+		+		+		=	
	rpoS	=		=		=		=	
Virulence	*avrpt01*	+			+	=		+	
	hopD1	=		=		=		=	
	hopQ1	=		=		=		=	
	hopR1	+		=		=		+	
	hopS2	+		=		+		=	
	hopZ5	=		=		+		=	
	lysR	+		+		=		=	
	paMTAda	=		=		=		=	
	virB4	+		+		+		+	
	enolase	+		=		=		=	
	oprM	=		=		=		=	

Figure 1. Growth curves of PsaCFBP7286 in different plant extracts, from zero up to 96 hours from the moment of inoculation. a) LB. b) *Actinidia deliciosa/chinensis* extract.. c) *Actinidia deliciosa/chinensis* xylem sap. d) *Actinidia arguta* extract. e) *Camellia sinensis* extracts. f) *Corylus avellana* extracts. g) *Nicotiana tabacum* extracts. Standard error bars are showed.

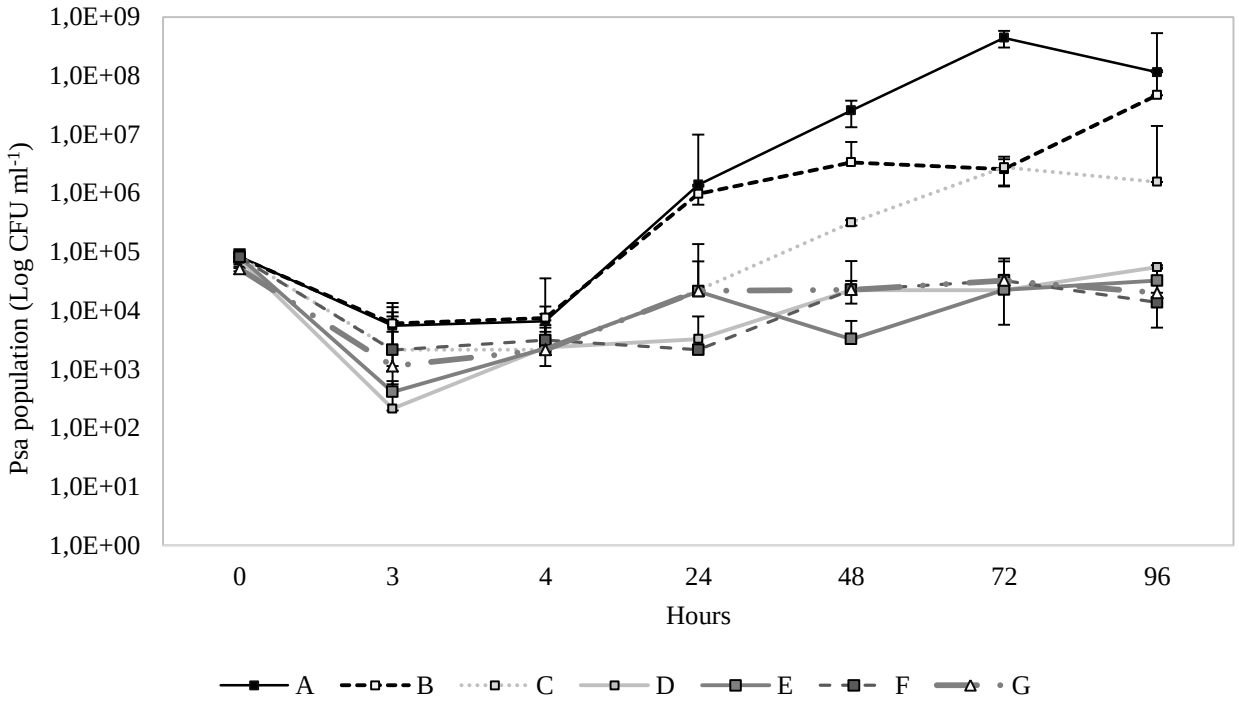


Figure 2. Effect of the application of 1 ml of different plant extracts on the swarming motility of *Pseudomonas syringae* pv. *actinidiae* wild type strain CFBP7286. Values represent the number of colonies that displayed swarming, in percentage. The control consisted of motility on plates with no addition of plant extracts. a) LB. b) *A. deliciosa* xylem sap. c) *A. chinensis* xylem sap. d) *A. deliciosa* extracts. e) *A. chinensis* extracts. f) *Actinidia arguta* extracts. g) *Camellia sinensis* extracts. h) *Corylus avellana* extracts. i) *Nicotiana tabacum* extracts. Statistically significant differences were indicated as letters on bars with $P < 0.05$.

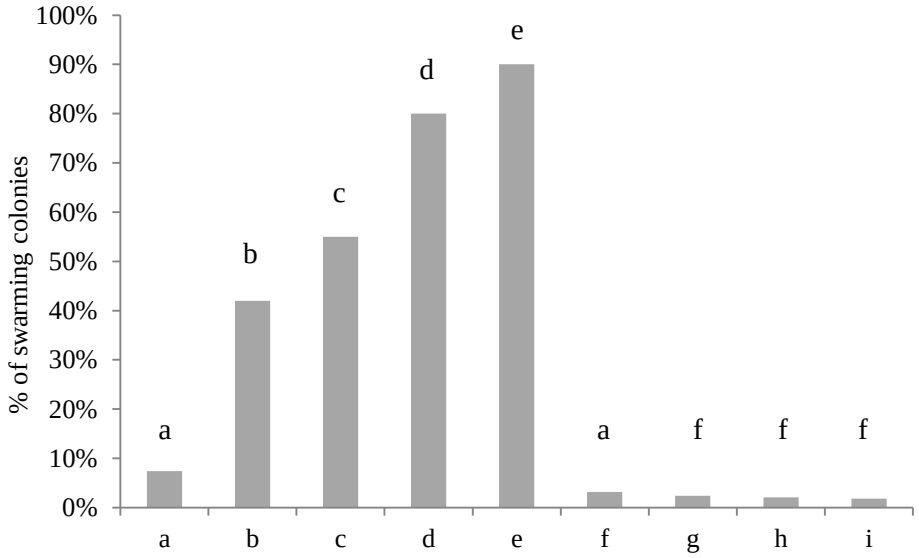


Figure 3. Pictures of PsaCFBP7286 motility on different plant extracts. Photographs were taken binocular Nikon SMZ25 fluorescence microscope (zoom magnification 1x) .a) LB. b) *A. chinensis* extracts. c) *A. deliciosa* extracts. d) *Actinidia chinensis* xylem sap. e) *Actinidia deliciosa* xylem sap. f) *A. arguta* extracts. g) *C. sinensis* extracts. h) *C. avellana* extracts

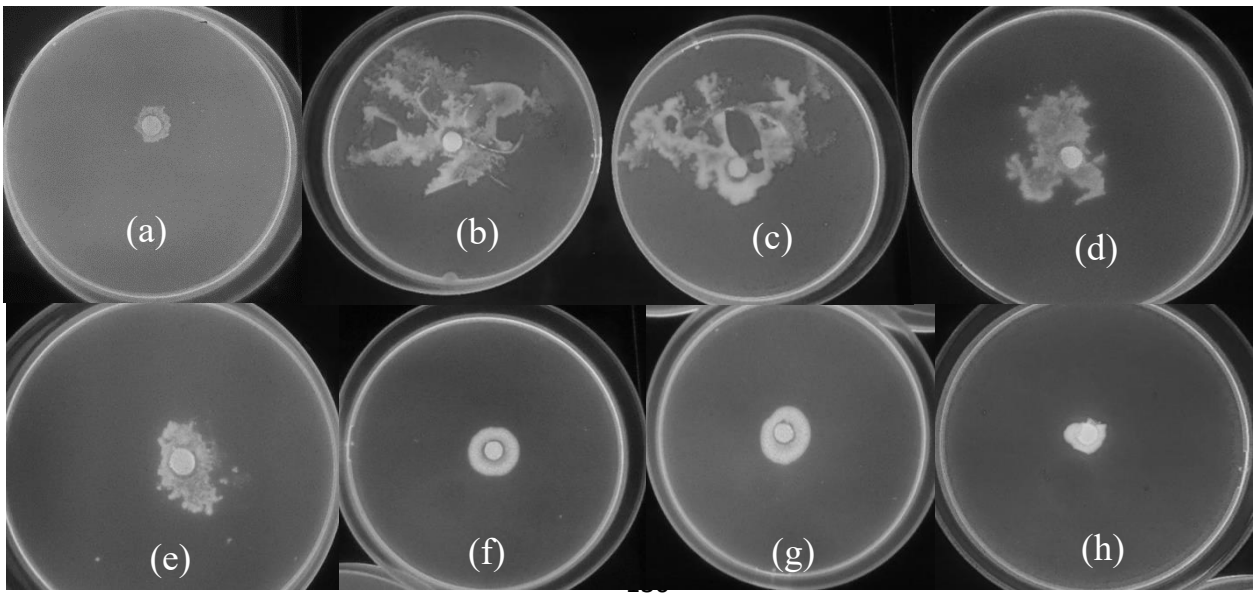


Figure 4. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 grown in different plant extracts. Biofilm was quantified after staining with crystal violet (0.5% w/v). The controls is represented by biofilm formation in LB. a) LB. b) *Actinidia deliciosa* xylem sap. c) *Actinidia chinensis* xylem sap. d) *A. deliciosa* extract. e) *A. chinensis* extract. f) *Actinidia arguta* extract. g) *Camellia sinensis* extract. h) *Corylus avellana* extracts. i) *Nicotiana tabacum* extract. Standard error bars are showed. Statistically significant differences were indicated as letters on bars with $P < 0.05$.

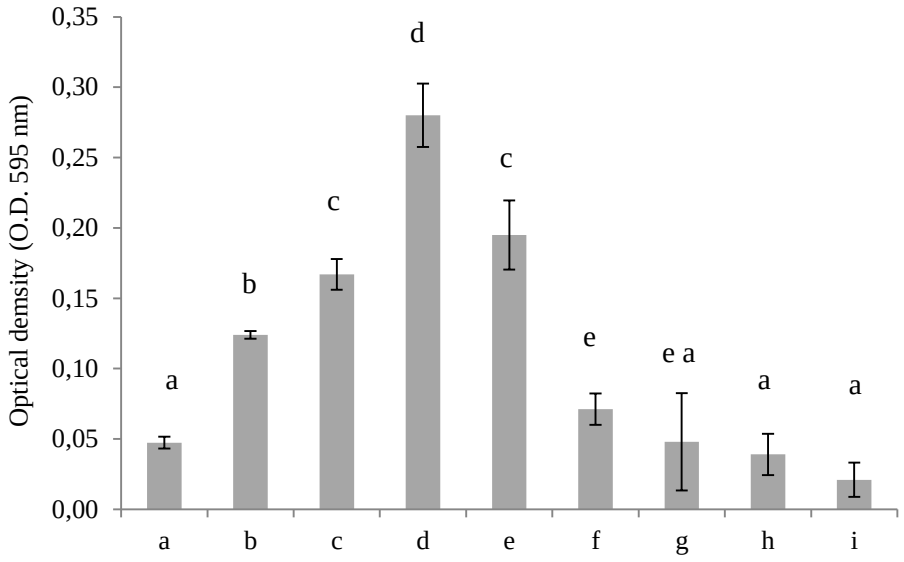
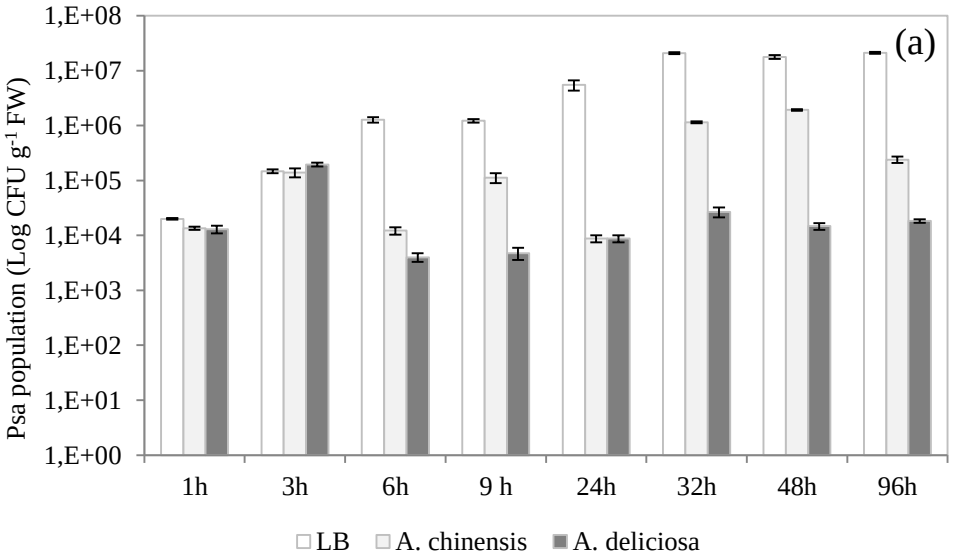


Figure 5. Epiphyte (a) and endophyte (b) populations of *Pseudomonas syringae* pv. *actinidiae* CFBP7286 analysed with qPCR of groups of plantlets inoculated with a high density inoculum grown in in LB and *A. chinensis* and *A. deliciosa* planta extracts. Y axis: CFU per gram of the fresh weight of group of plantlets of three replicates. The standard errors are showed. Data marked with an asterisk (*) were significantly different according to the Student's *t* test, with $P < 0.05$.



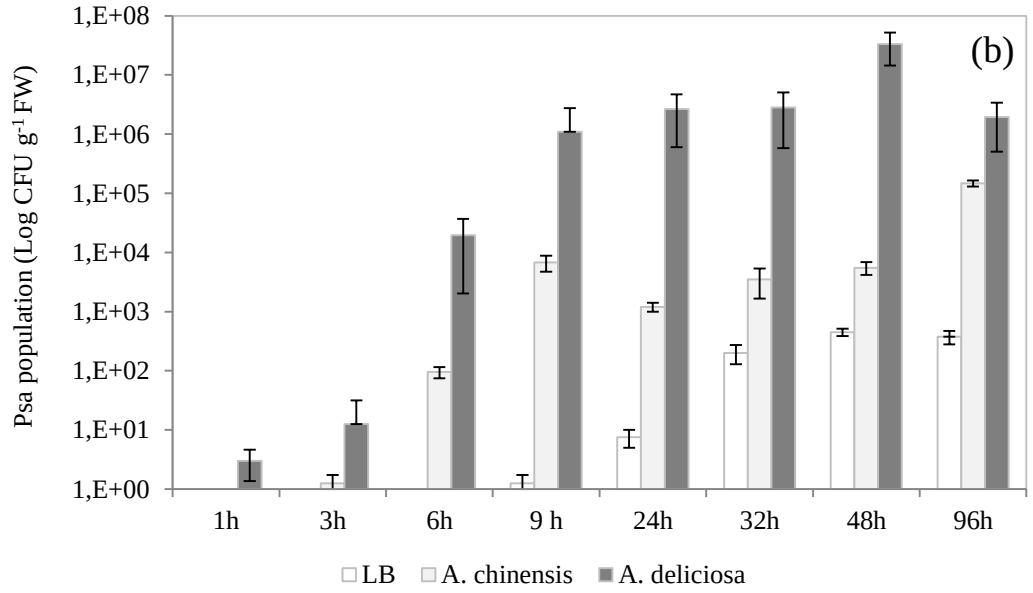
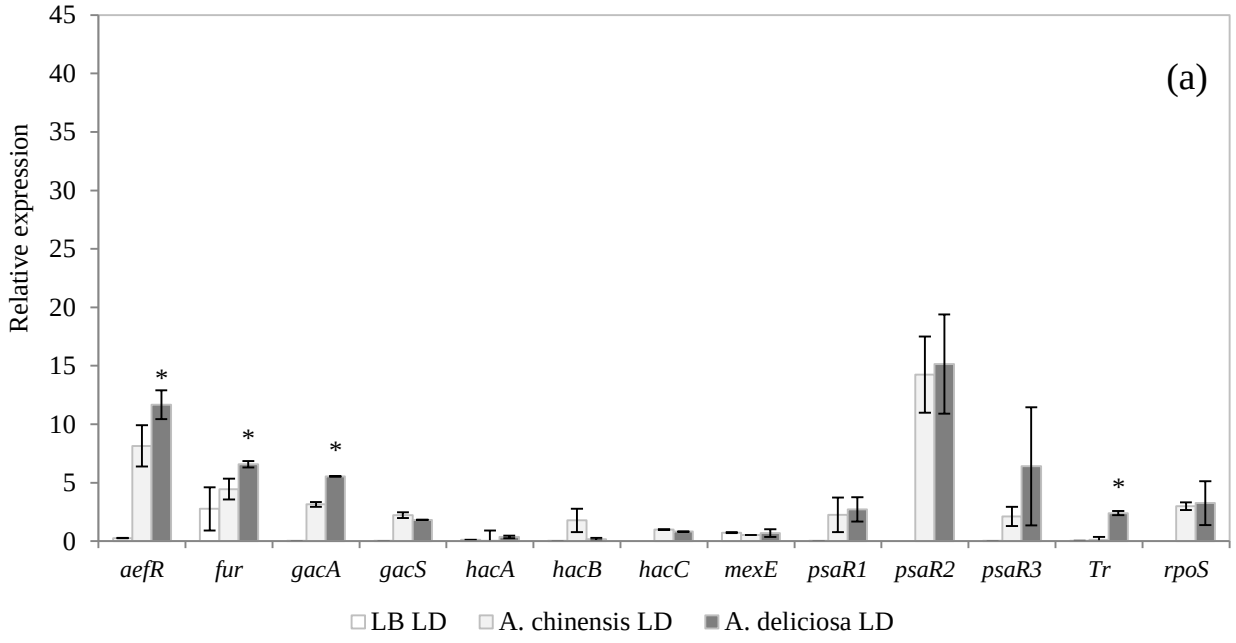


Figure 6. Expression of genes related to density of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* plant extracts. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significative difference between *A. deliciosa* and *A. chinensis* plant extracts induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*)



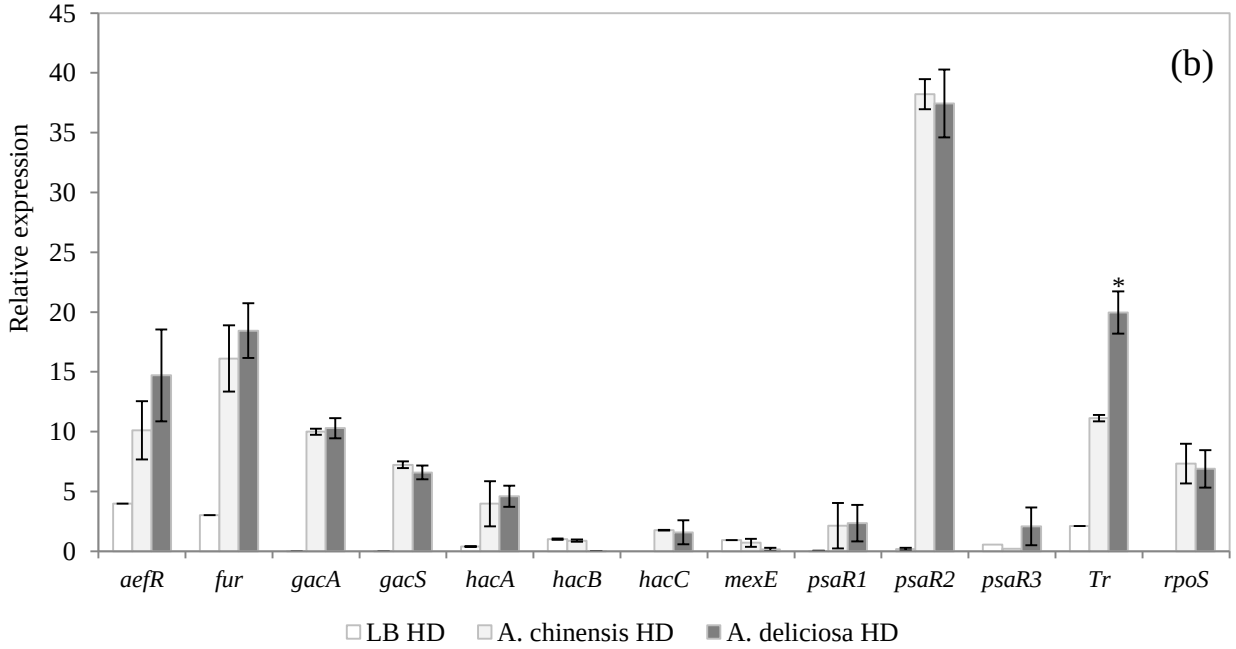


Figure 7. Expression of genes related to motility of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* plant extracts. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significative difference between *A. deliciosa* and *A. chinensis* plant extracts induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*).

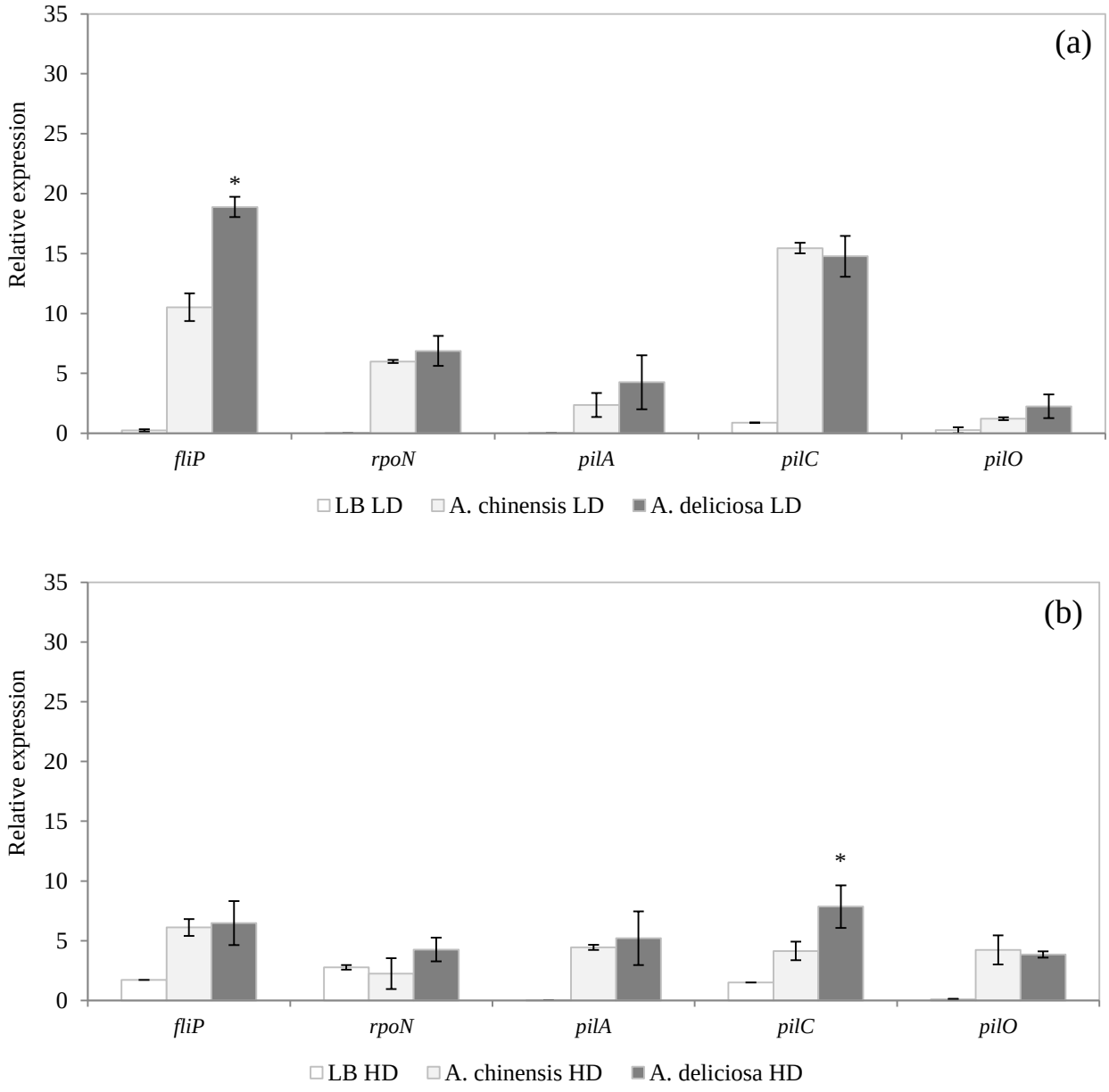


Figure 8. Expression of genes related to biofilm of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* plant extracts. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significative difference between *A. deliciosa* and *A. chinensis* plant extracts induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*).

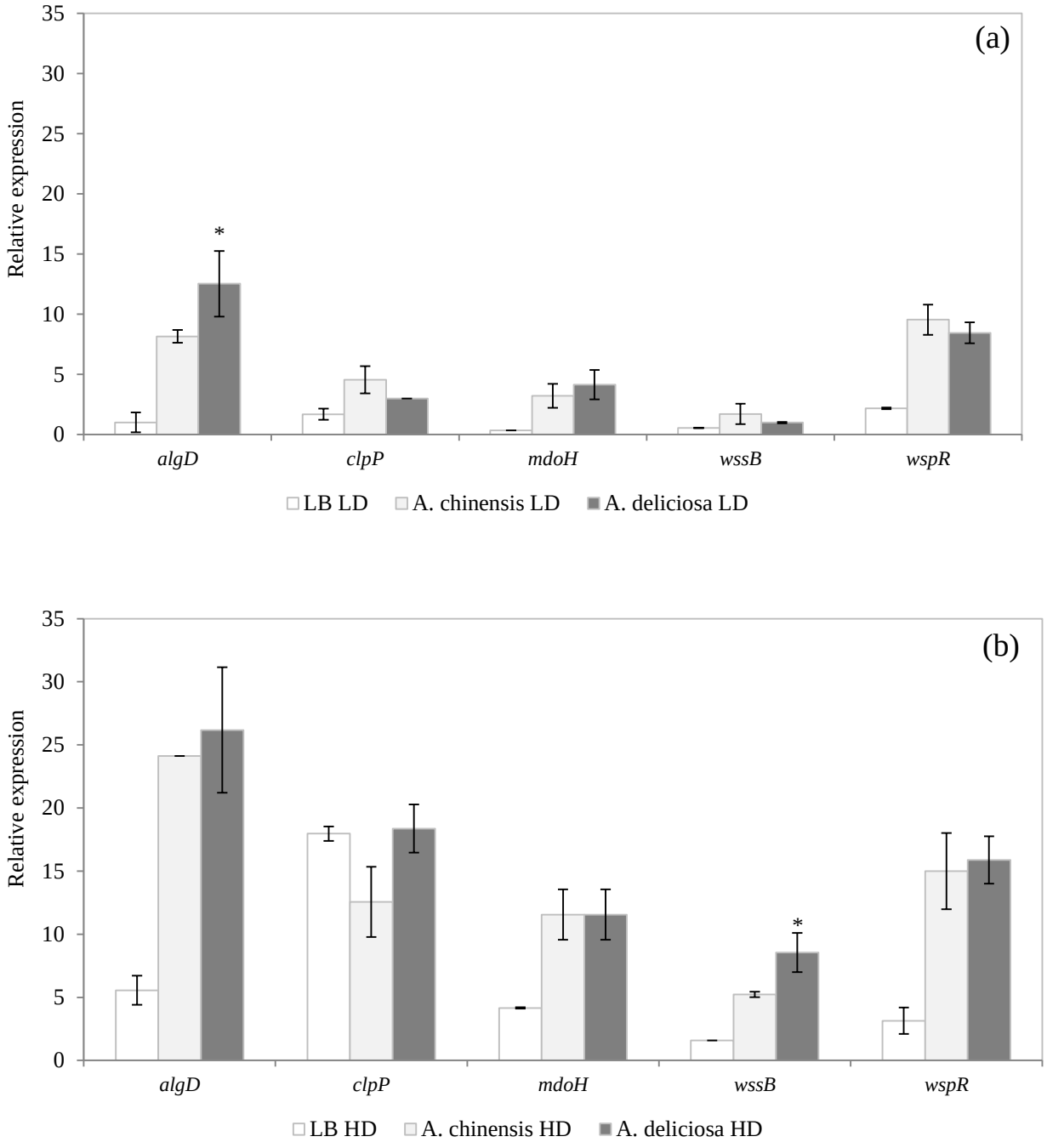


Figure 9. Expression of genes related to virulence of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* plant extracts. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significative difference between *A. deliciosa* and *A. chinensis* plant extracts induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*).

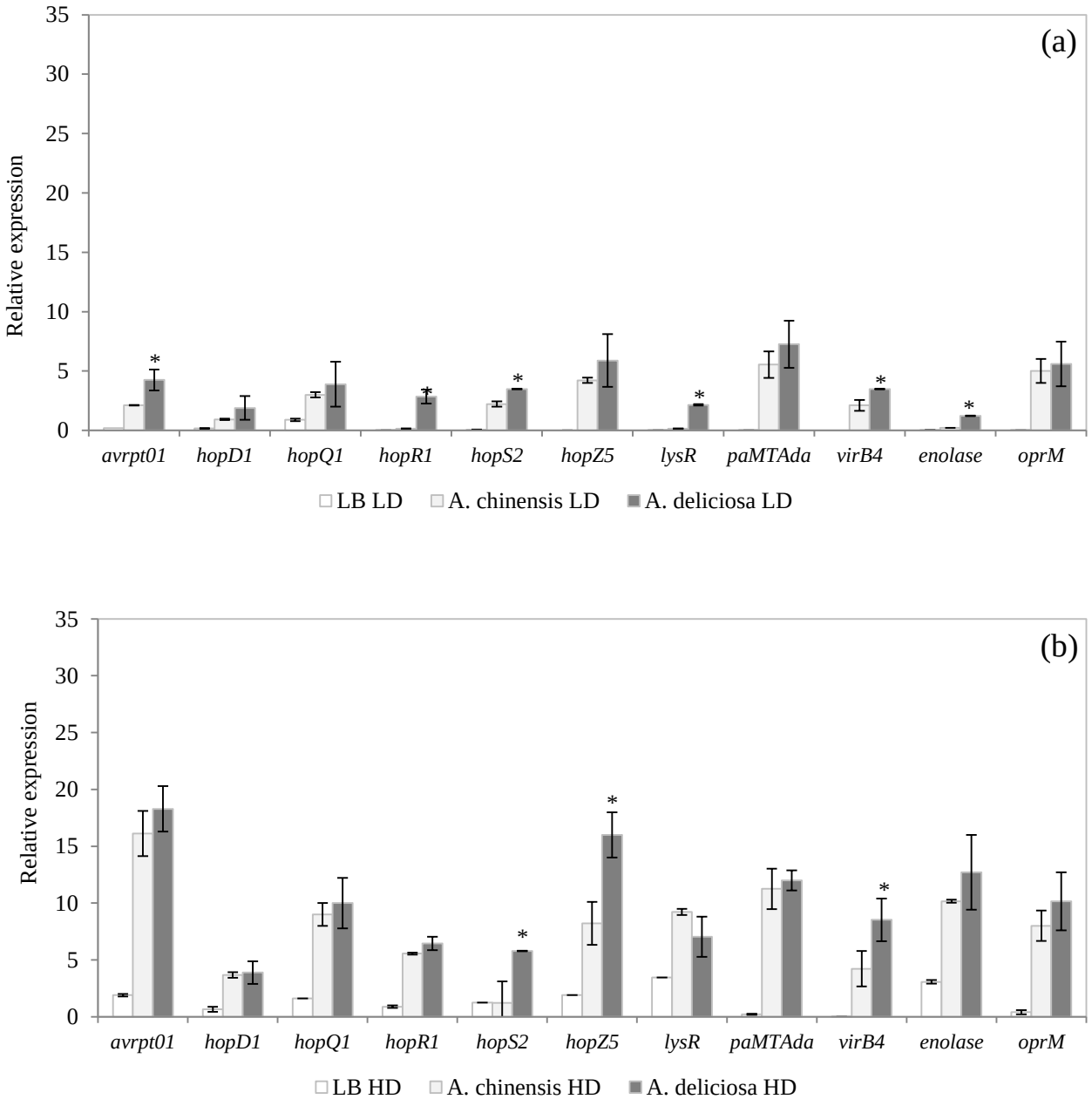
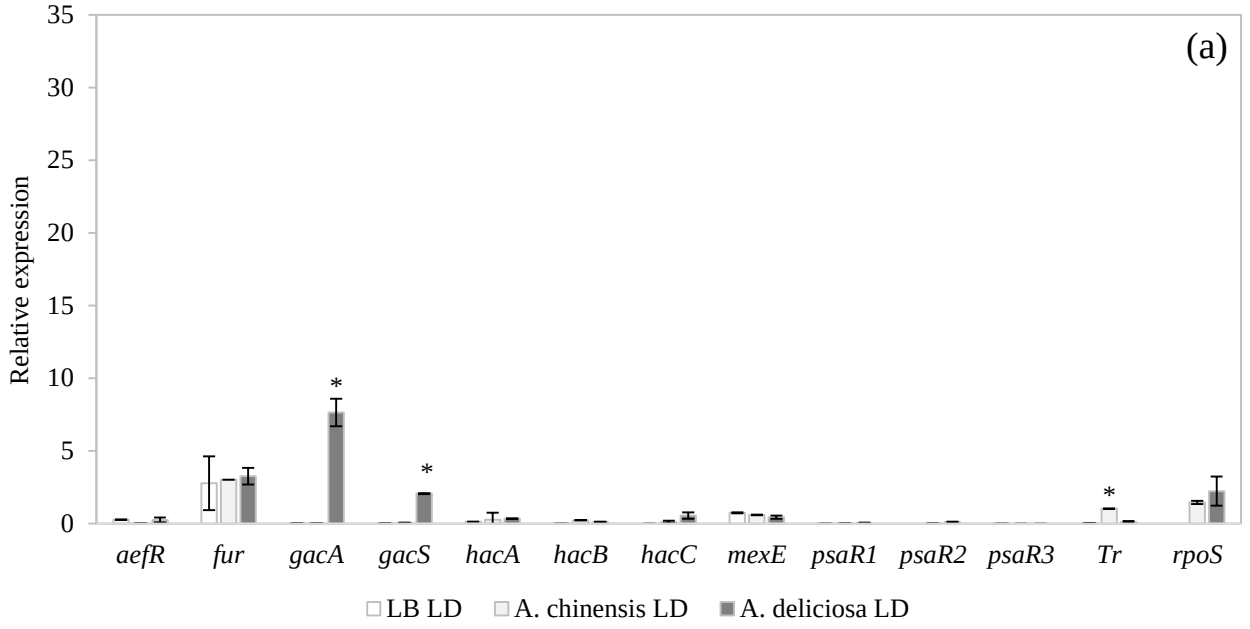


Figure 10. Expression of genes related to density of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* xylem saps. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significativity difference between *A. deliciosa* and *A. chinensis* xylem saps induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*)



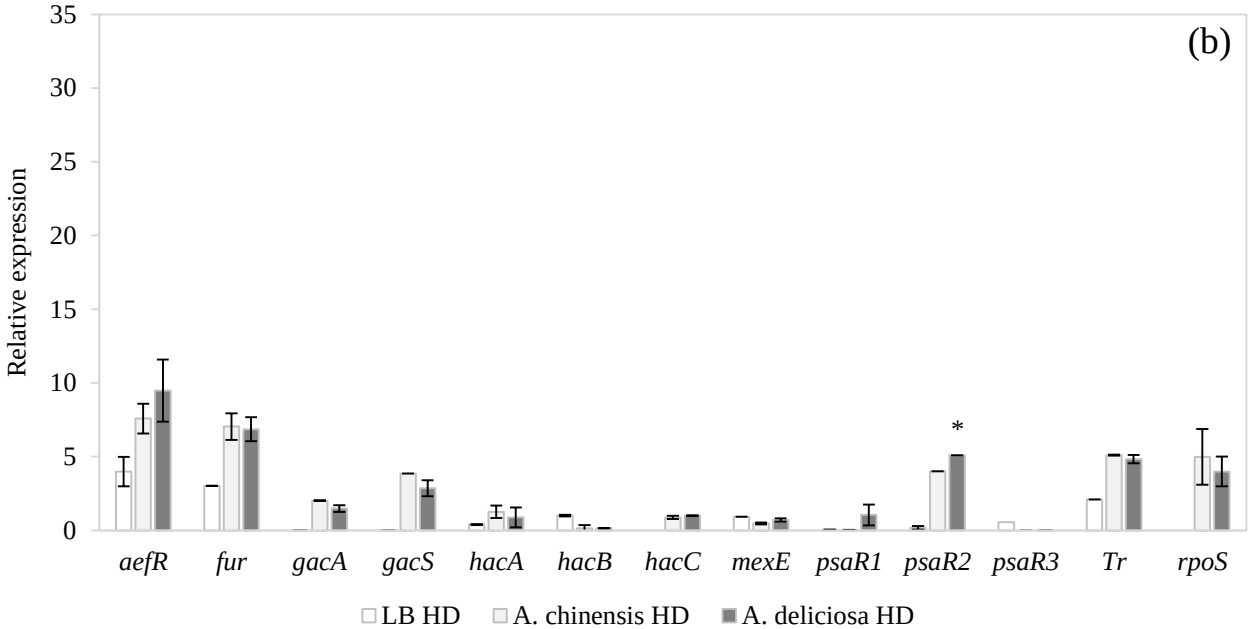
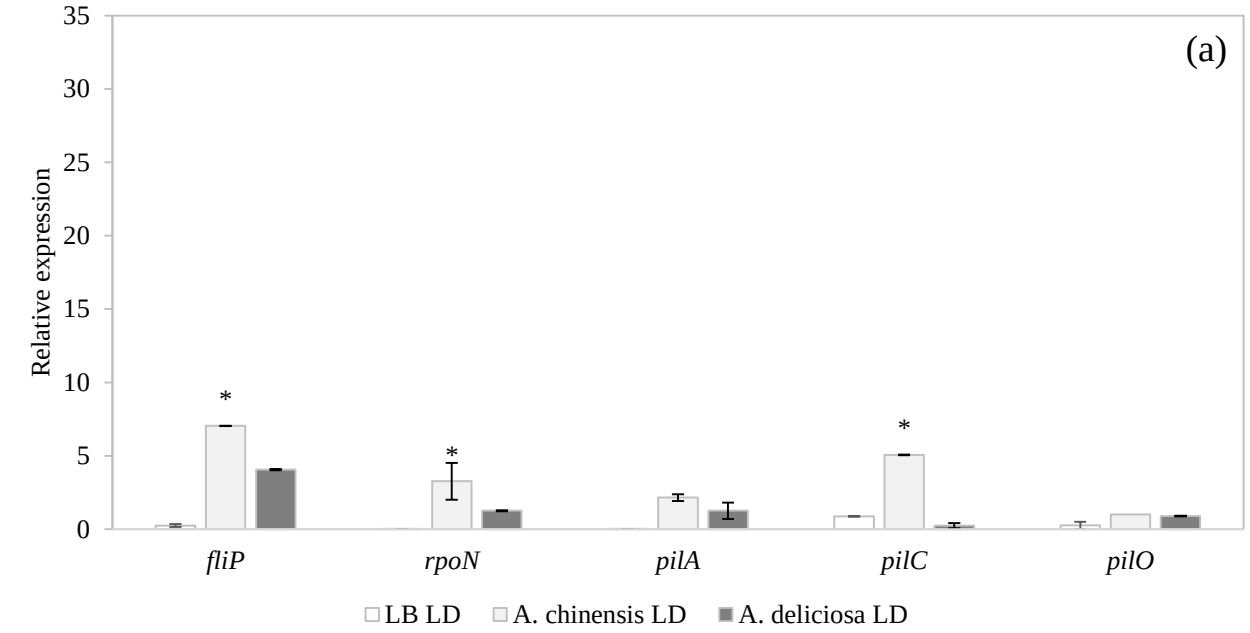


Figure 11. Expression of genes related to motility of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* xylem saps. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significative difference between *A. deliciosa* and *A. chinensis* xylem saps induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*)



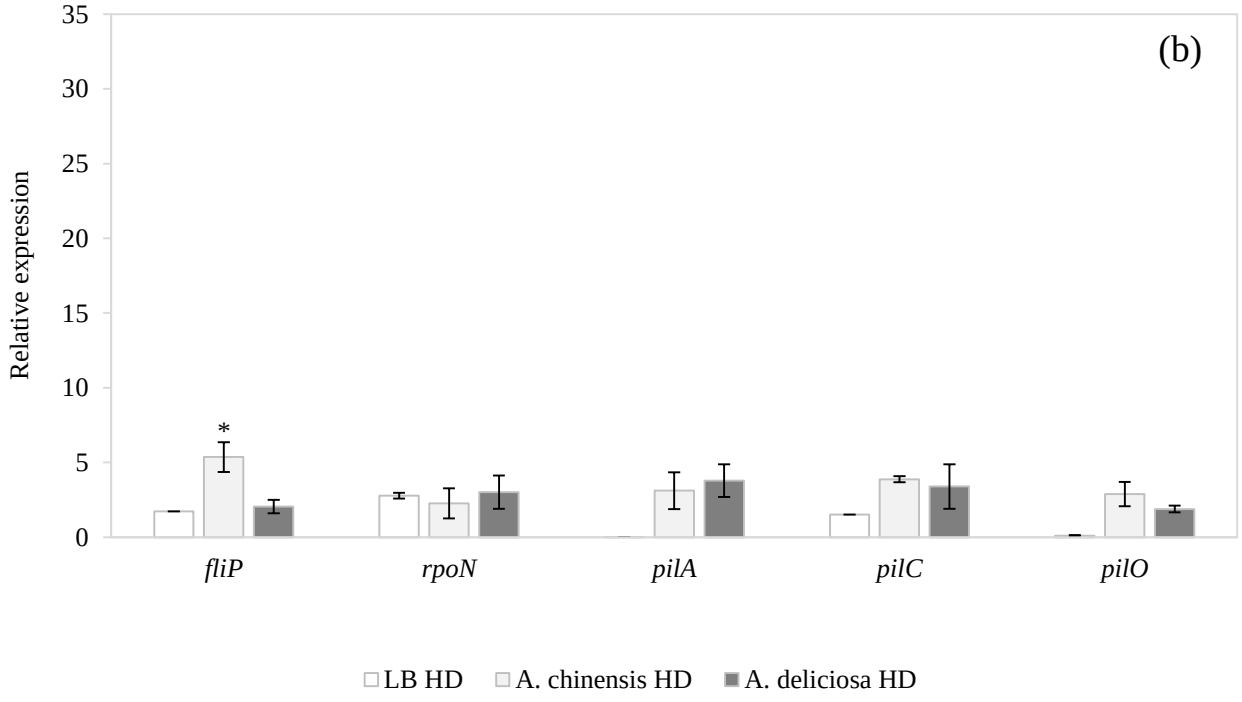


Figure 12. Expression of genes related to biofilm of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* xylem saps. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significative difference between *A. deliciosa* and *A. chinensis* xylem saps induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*).

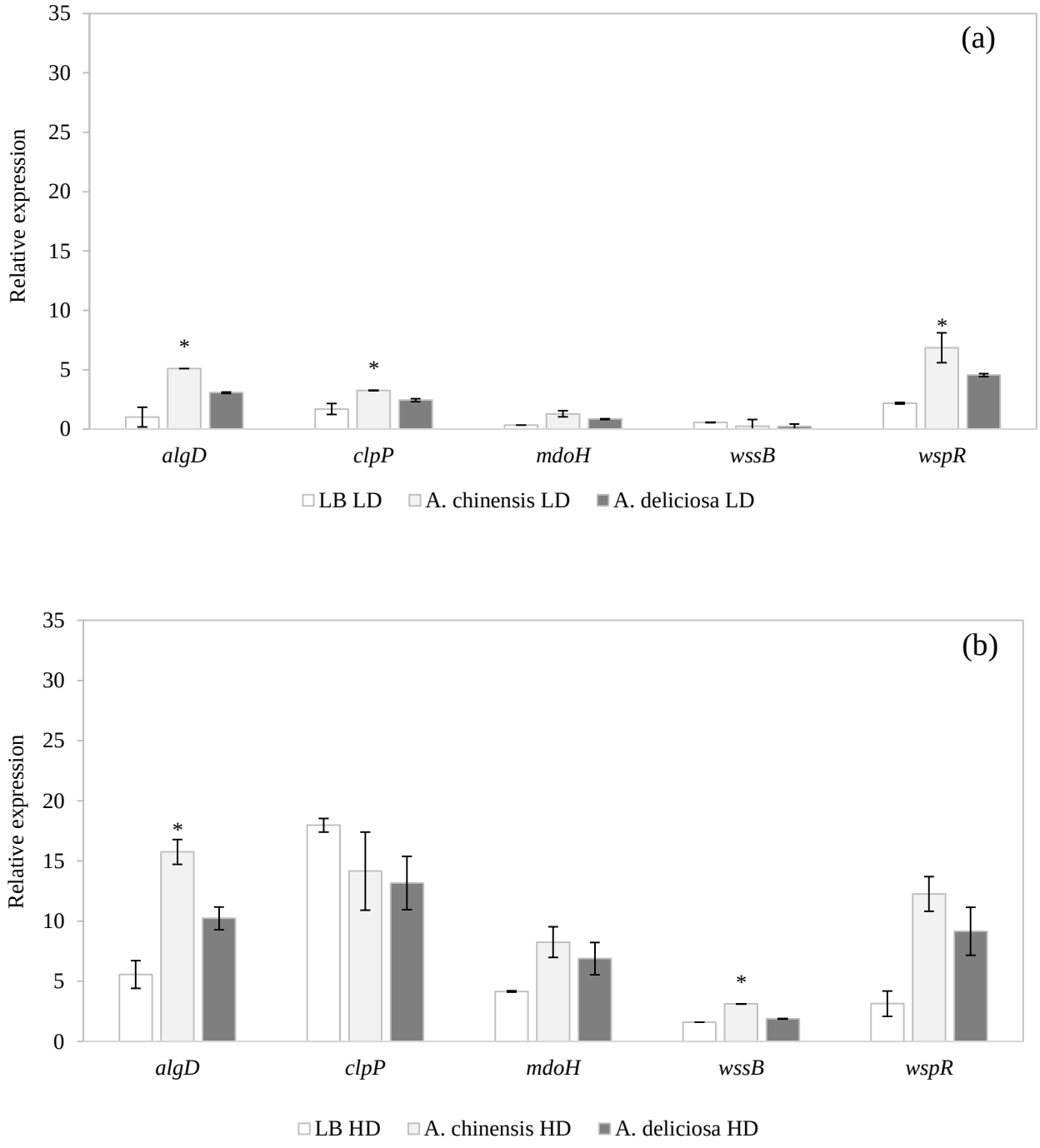
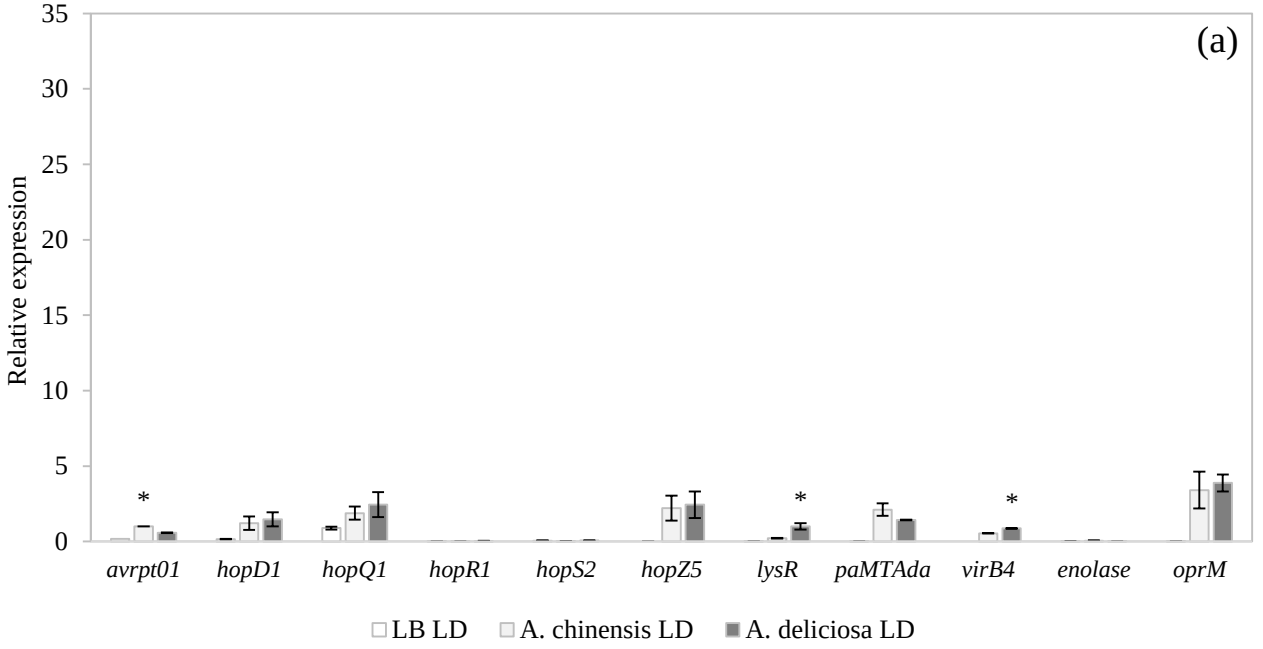


Figure 13. Expression of genes related to virulence of *Psa* liquid cultures grown at (a) low (10^5 CFU ml^{-1}) and (b) high (10^8 CFU ml^{-1}) cell density in LB and in *A. deliciosa* and *A. chinensis* xylem saps. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significative difference between *A. deliciosa* and *A. chinensis* xylem saps induction expression was calculated using Student's *t* test with $P < 0.05$ and indicated (*).



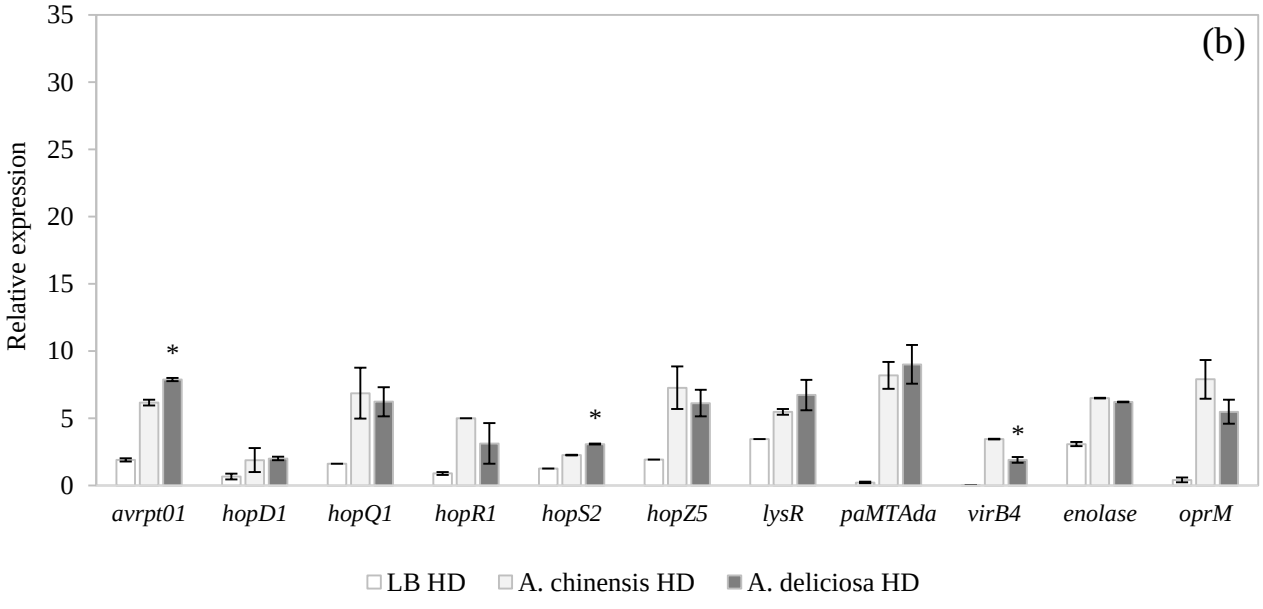


Figure 15. Motility of Psa-mR2 mutant on plant extracts of *A. deliciosa* compared to *Pseudomonas syringae* pv. *actinidiae* CFBP7286. The graph represents the total plates showing swarming motility (in percentage). The control consisted of motility on plates with no addition of plant extracts. Standard error bars are showed. Data marked with an asterisk (*) were significantly different according to the Student's *t* test ($P < 0.05$).

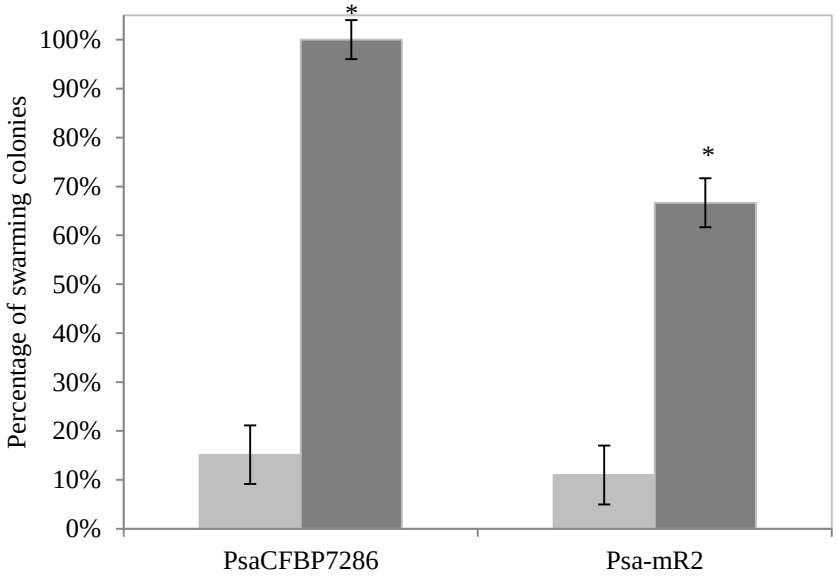


Figure 16. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 and Psa-mR2 in plant extracts of *A. deliciosa*. Biofilm was quantified after staining with crystal violet (0.5% w/v). The controls are represented by biofilm formation in LB. Standard error bars are showed. Data marked with an asterisk (*) were significantly different according to the Student's *t* test ($P < 0.05$).

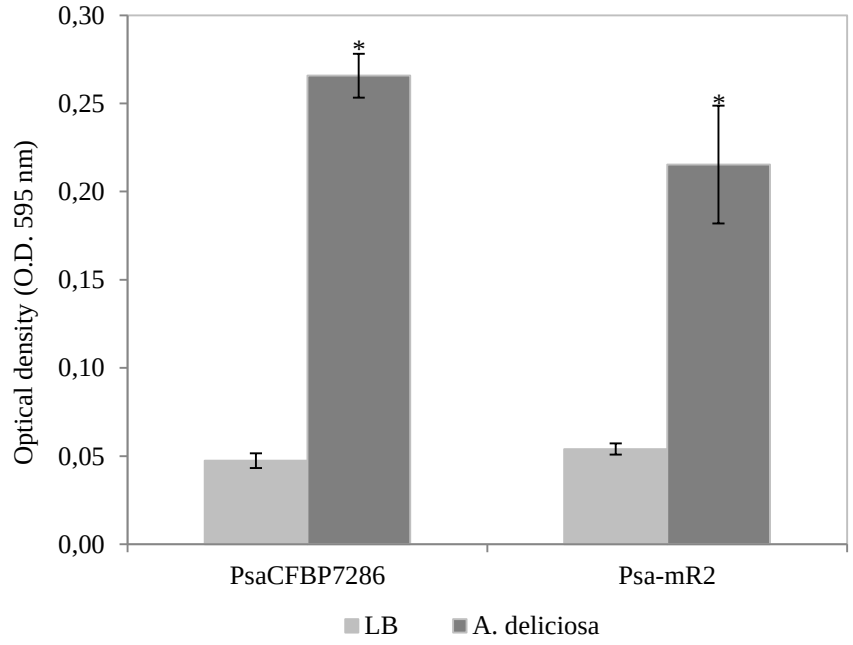
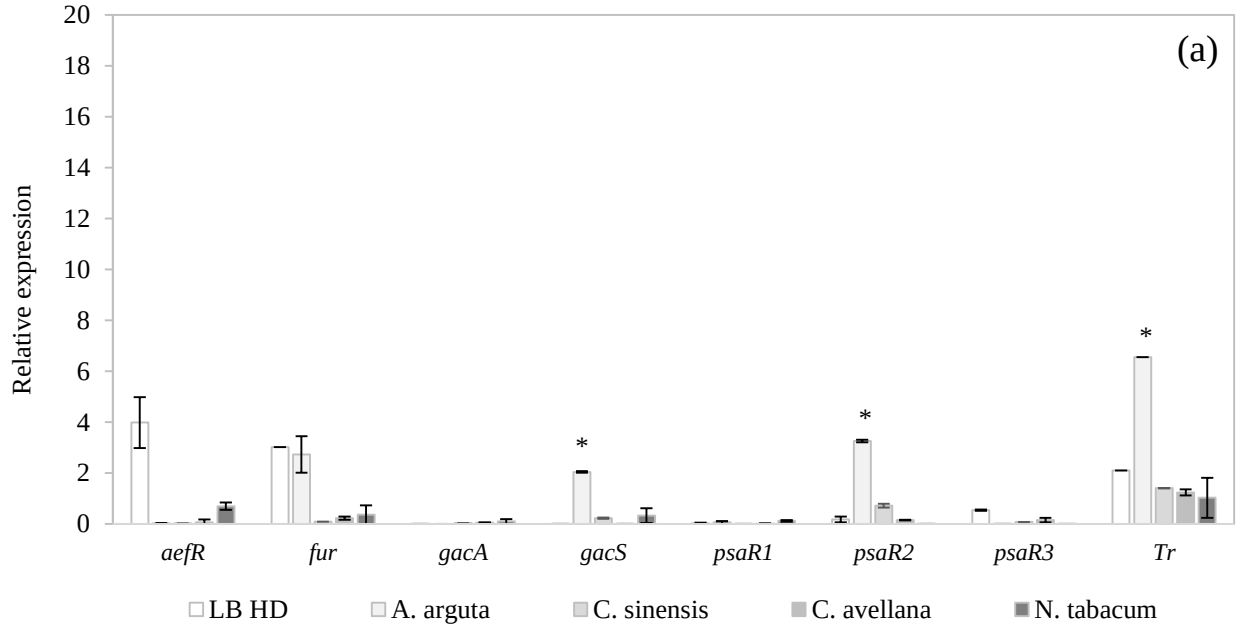
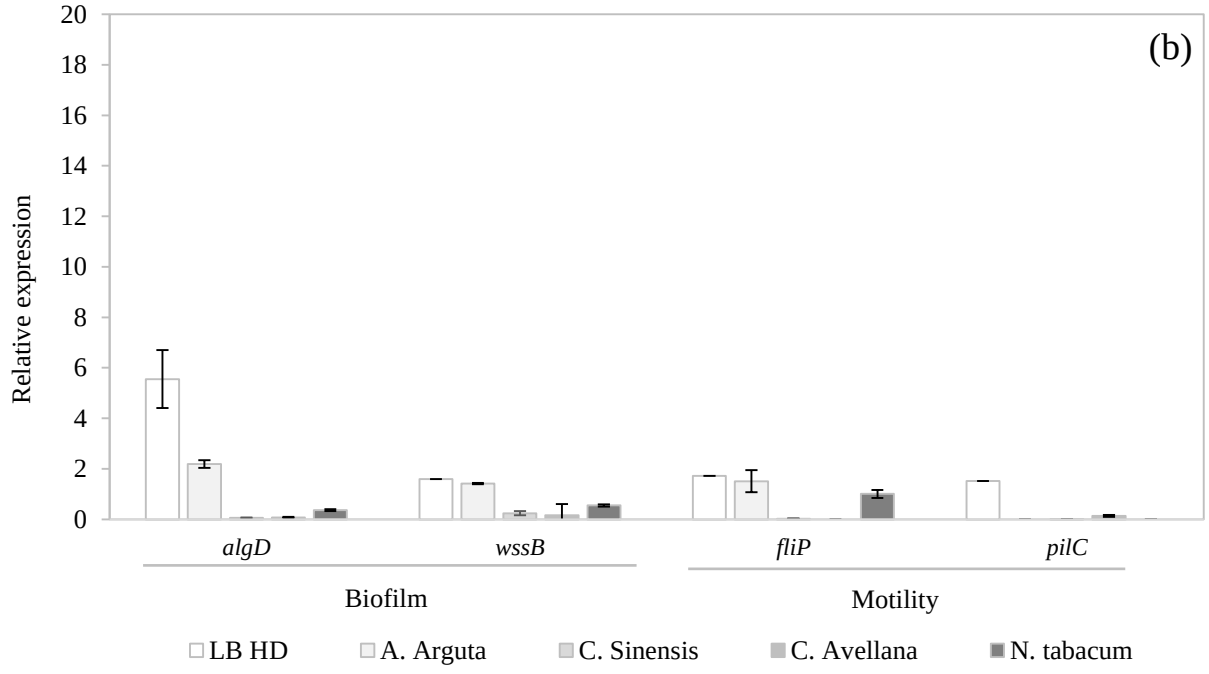


Figure 14. Expression of genes related to (a) density, (b) motility, biofilm and (c) virulence of *Psa* liquid cultures grown at high cell density (10^8 CFU ml^{-1}) in LB and in *A. arguta*, *C. sinensis*, *C. avellana* and *N. tabacum* plant extracts. Y axis: relative expression compared to *rpoD* and *recA* genes. Standard error bars are showed. Significance was calculated using Student's *t* test ($P < 0.05$). (*) indicates the difference from the control.





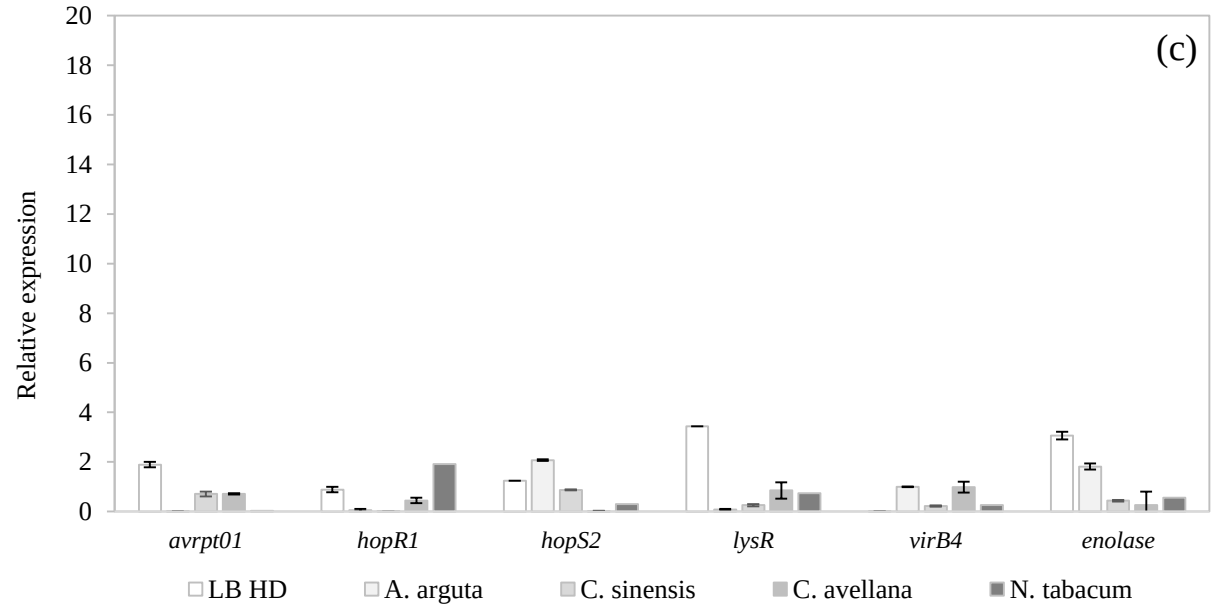


Figure 17. Biofilm production in *Pseudomonas syringae* pv. *actinidiae* CFBP7286 and Psa-TRsile in plant extracts of *A. deliciosa* in presence of IPTG (0.5%). Biofilm was quantified after staining with crystal violet (0.5% w/v). The controls are represented by biofilm formation in LB. Standard error bars are showed. Data were significantly different according to the Student's *t* test ($P < 0.05$).

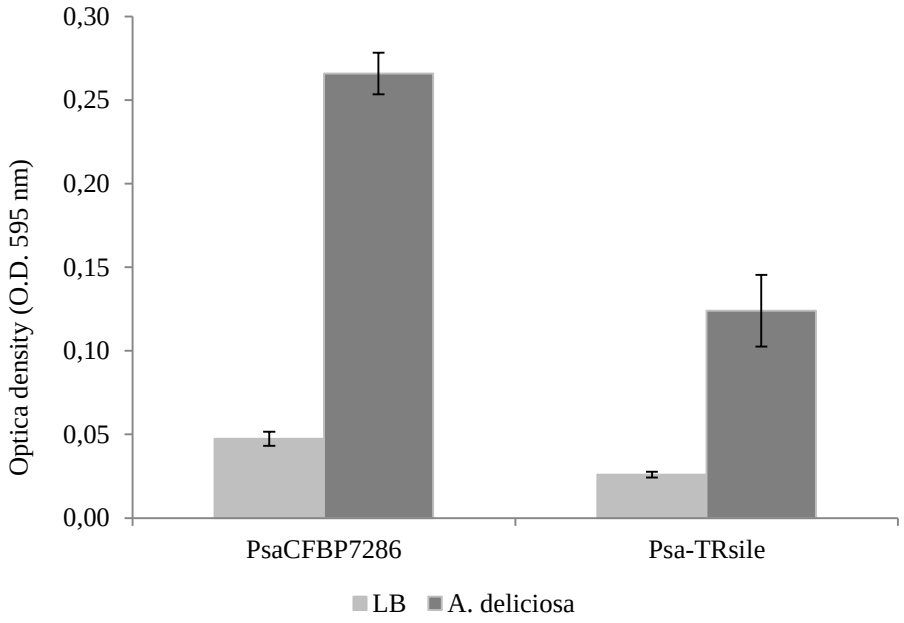


Figure 18. PsaR2 was expression in *E. coli*. ELISA assay of S= Soluble fraction; M = membrane fraction; I = insoluble fraction differentially extracted from total lysate of PsaR2 *E. coli* producing strain. Fractions statistically different from negative controls wild type total extract (WT) or PBS are indicated (*).

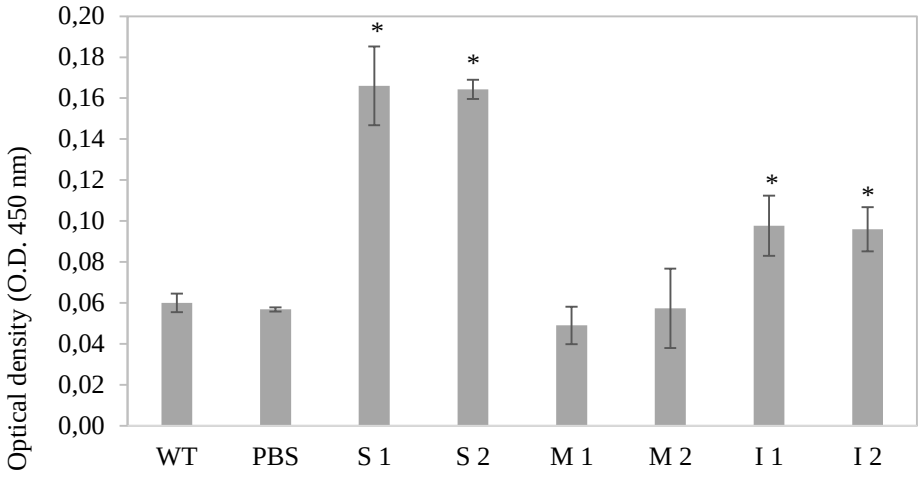


Figure 19. PsaR2 purification ELISA assay of fractions of Nickel purification of soluble fractions obtained from *E. coli* lysates.