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TITOLO TESI

EVALUATION OF BIOFUNGICIDES AND PLANT DEFENSE ELICITORS AGAINST BACTERIAL PATHOGENS OF AGRONOMIC IMPORTANCE

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Esame finale anno 2016

To my mother…

ABSTRACT

Plant bacterial diseases are nowadays routinely managed with scheduled treatments based on heavy metal compounds or, in the worst cases, on antibiotics; to overcome the environmental consequences linked to the use of these chemical compounds, such as pollution or selection of antibiotic resistant pathogens, an integrated control management is required. The use of bacterial antagonists, biological agents, plant defence response elicitors or resistant host plant genotypes play an important role in the frame of sustainable agriculture. In this work, the activity of plasma activated water (PAW) and different bioagents aimed to the control of the two bacterial pathogens *Xanthomonas vesicatoria* and *Pseudomonas syringae* pv. *actinidiae*, causal agents of tomato bacterial leaf spot and kiwifruit bacterial canker, respectively, were studied. All these tools were assayed for their direct efficacy and for their ability, as elicitors, to trigger the plant immune system against these two bacterial pathogens. Moreover, a study on several *Actinidia* sp. accessions was carried out to evaluate their susceptibility against bacterial canker of kiwifruit.

PAW resulted unable to direct inhibit *X. vesicatoria* growth in *in vitro* assays, however it showed the ability to trigger tomato plant immune system by reducing disease severity up to approx. 38% when tested in three experiments on two tomato genotypes conducted under greenhouse conditions. When tested in *in vitro* and *in vivo* experiments against X. *vesicatoria* and *P. syringae* pv. *actidinidiae*, the tested bioagents, based on natural extracted compounds or on different strains of *Bacillus* sp., showed a direct efficacy against both bacterial pathogens. Moreover, they were also able to elicit the plant defence response by significantly lowering the disease severity on tomato and kiwifruit leaves.

In addition, the *A. chinensis* accession NPK3 resulted the less susceptible to the bacterial canker in comparison to more than 20 accessions tested.

Keywords: kiwifruit bacterial canker, *Pseudomonas syringae* pv. *actinidiae*, tomato bacterial leaf spot, *Xanthomonas vesicatoria*, plasma activated water, *Bacillus*, bioagents, biological control, induced resistance, kiwifruit susceptibility, RNA extraction, gene expression, RT-qPCR.

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Chapter I

EFFICACY OF PLASMA ACTIVATED WATER AGAINST TOMATO BACTERIAL LEAF SPOT

1. INTRODUCTION

The bacterial plant diseases are economically significant problems in the crop production worldwide. Bacterial leaf spots disease on tomato and pepper, caused by *Xanthomonas vesicatoria,* is extensively spread in all the areas where these host species are present (Jones *et al.*, 2000; 2004; EPPO/OEPP, 2013). The lack of availability of effective pesticides and of host resistance for the management of this bacterial disease has stimulated efforts to develop alternative strategies in the framework of an integrated control management (Louws *et al.*, 2001). Usually, integrated control programs for bacterial leaf spot, are based on prophylaxis (seed testing), applications of copper compounds, antagonistic/beneficial bacteria and resistance inducers (Lucas, 1998; El-Hendawy *et al.*, 2005; Byrne *et al.*, 2005; Obradovic *et al.*, 2005).

The plasma is the fourth physical condition of the matter and it exists in many forms in nature - over 99% of the visible matter of the universe, *e. g.* the sun, lightening and the polar lights (*e. g. aurora borealis*) - and has a widespread use in science and technology. In 1927, Irving Langmuir adopted the word "plasma" to indicate the fundamental nature of a volume of ionized gas essentially free of space charge (Mott-Smith, 1971; Guy and Miles, 1974). The plasma is formed by mixtures of heavy (positive and negative ions, atoms, free radicals and excited or non-excited molecules) and light (electrons and photons) particles in permanent interaction; all of them are forming an electrically neutral medium. Usually, it is classified in thermal and non-thermal plasma (Moreau *et al.*, 2005; 2008; Nehra *et al.*, 2008; Heinlin *et al.*, 2010) based on the relative energetic levels, temperature and electronic density (Petitpas *et al.*, 2007). Thermal plasmas are characterized by an equilibrium or near equality between electrons, ions and neutrals (Nehra *et al.*, 2008); whereas, non-thermal plasma ions and uncharged molecules are more cold than those of thermal, due to the carrier gas (argon, helium, air) which is only slightly ionized (typically 1 part in 1 billion) and therefore,

the ions cool down to the room temperature very rapidly – in fractions of a second (Heinlin *et al*., 2010).

Non-thermal atmospheric plasma (NTP) may be obtained by a diversity of electrical discharges, such as corona discharge, micro hollow cathode discharge, atmospheric pressure plasma jet, gliding arc discharge, one atmospheric uniform glow discharge, plasma needle and dielectric barrier discharge (also called DBD) (Liu *et al*., 1999; Nehra *et al*., 2008). NTP has been applied in diverse technological areas: industry (Xu, 2001; Paulmier and Fulcheri, 2005; Moreau *et al*., 2005; 2008; Petitpas *et al*., 2007; Morent *et al*., 2008; Nehra *et al*., 2008), medicine (Moreau *et al*., 2008; Heinlin *et al*., 2010; Kostov *et al*., 2010; Laurita *et al*., 2015), and also in agriculture. In the last decade different researchers have highlighted the possibility of plasma applications in agronomic/industrial topics such as the inactivation of bacterial pathogens of industrial interest (*Erwinia* spp.) (Moreau *et al.,* 2005; 2007). Park *et al*. (2013) evaluated the plasma effect on germination, growth rates, and overall nutritional value of various plant species; moreover, Ma *et al.* (2015) considered it as an effective method for decontamination of fresh foods. Nevertheless, plasma effectiveness against bacterial plant diseases was not yet well studied. In this last topic, it was employed for activating seed vitality and enhancing the yield of different plant species (Carvalho *et al*., 2005; Jiang *et al*., 2014a), for water decontamination (Moreau *et al*., 2008), and, very recently, seed treatment towards plant disease control (Jiang *et al*., 2014b).

NTP was recently applied to sterile deionized water (SDW): Park *et al*. (2013) and Laurita *et al*. (2015) established that the chemical properties of PAW are, acidification (pH 2.3), increase of conductivity, induction of nitrate (NO_3^-), nitrite (NO_2^-) (*r*eactive *n*itrogen *s*pecies; RNS), and also superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH) (*r*eactive *o*xygen *s*pecies; ROS).

This study was aimed to assess the *in vitro* effect of PAW against *Xanthomonas vesicatoria*, and its ability, in *in vivo* experiments, to reduce the severity of bacterial leaf spot disease by eliciting induced resistance. Transcriptomic analyses were also performed to assay the transcription kinetics of different pathogenesis related protein (PR) genes as *pr1a*, *pr4* and *pr5*; and also genes coding for lipoxygenase (*loxf*), phenylalanine ammonia-lyase (*pal*) and ethylene-response factor (*erf1*).

2. MATERIALS AND METHODS

2.1 Plasma Activated Water (PAW)

PAW was produced following the methodology described by Laurita *et al*. (2015). NTP was generated employing DBD reactor (Figure 1) belonging to Alma-Plasma group, Faculty of Engineering, University of Bologna, Italy (leaded by prof. Vittorio Colombo). The reactor consisted of a borosilicate glass circular case (94 mm) acting both as a liquid container and dielectric barrier (thickness 2 mm). Sterile Deionized Water (SDW) was treated in a closed environment without recirculation; the volume of the reactor was filled with ambient air, which was used as plasma gas. Two circular aluminum foils acted as electrodes: the liquid-side electrode (diameter 89 mm) was connected to a nanosecond pulsed high voltage generator, while the gas-side electrode (diameter 80 mm) was grounded. The plasma source was driven by generator producing high voltage pulses with a slow rate of few kV/ns and 50 mJ of energy per pulse (FID GmbH – FPG 20-1NMK) (Laurita *et al.*, 2015).

PAW was obtained operating the source at a peak voltage of 20 kV, with a pulse repetition frequency of 1 kHz, an air gap of 1 mm and 60 mL of liquid. SDW was treated for 10 min by cold-plasma (PAW production rate: 60 mL/10 min).

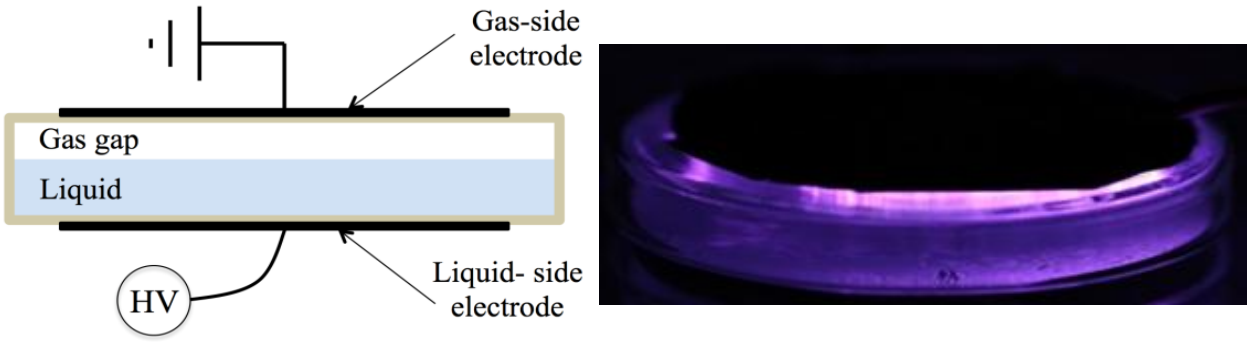

Figure 1. Plasma treatment by DBD reactor for obtaining plasma activated water (PAW). (Laurita *et al*., 2015).

2.2 Bacterial strains

The strain IPV-BO 2684 of *Xanthomonas vesicatoria* (Xv), isolated from tomato plants showing bacterial leaf spot disease in Italy (Zaccardelli, 1994) and stored in the Phytobacteriology Laboratory (University of Bologna), was grown on glucose-yeast extract calcium carbonate agar (GYCA; Dye, 1962) for 48 h at 27°C, and purified in the same medium to prepare the final bacterial suspensions.

Escherichia coli strain JM109 was used for subcloning the reverse transcriptase (RT)-PCR products of the *ß–act, loxf, pal* and *erf1* genes; it was grown at 37°C in Luria broth or on Luria agar for 24 h (Sambrook *et al*., 1989).

2.3 *In vitro* experiment

2.3.1 Diffusion method

Luria-Bertani (LB) agar medium was prepared (see appendix) and aseptically poured (*ca*. 25 mL) onto Petri plates (diameter 9 cm). From a bacterial water suspension (10^7 UFC/mL), calibrated at the spectrophotometer (OD = 0.01_{600nm}), 200 μL were inoculated and spread on LB plates. An antibiogram disc (diameter 0,9 cm) was then kept into the middle of the plates and a drop, containing the treatment (all treatments are shown in Table 1), was put on the antibiogram disc. The plates were then incubated for 24 h at 27°C. The antibacterial effect, shown as an inhibition halo, was then measured (cm) by subtracting the antibiogram disc diameter from the halo diameter. The assay was repeated 3 times, and standard deviations were calculated.

2.3.2 Dilution method

LB broth medium was autoclaved and aliquots were divided into 15 mL sterile Falcon tubes (50 mL). The different treatments that were added to Falcon tubes before bacterial inoculation are shown in Table 1. From a bacterial aqueous suspension (OD_{600nm} = 0.1; approx. 10^8 UFC/mL), 150 μL were inoculated into each tube obtaining a final population of approx. 10^6 CFU/mL. All Falcon tubes were then incubated in rotative incubator at 27°C for 24 h at 80 rpm. The bacterial population of each tube was evaluated at 0 h and 24 h by collecting 1 mL of inoculated broth. Each sample was tenfold diluted and, 10 µL from each

dilution were dropped in GYCA medium; the plates were incubated at 27°C for 48-72 h and the bacterial populations were evaluated by counting the colonies. The assay was repeated 3 times, standard deviations were calculated and data were elaborated with ANOVA test (p 0.05).

Table 1. *In vitro* treatments used for diffusion and dilution methods against *Xanthomonas vesicatoria* strain IPV-BO 2684.

Code	Details	Active Ingredient	Volume or Concentration	References
SDW-150	Sterile Distilled Water Negative control	/	150 μL	/
SDW-300	Sterile Distilled Water Negative control	/	300 μL	/
PAW-150*	Plasma Activated Water	ROS, RNS	150 μL	AlmaPlasma Group
PAW-300*	Plasma Activated Water	ROS, RNS	300 μL	AlmaPlasma Group
ASM	BTH (50 WG) Resistance inducer Negative control	Acibenzolar-S-Metil	60 μL** 100 ppm	Syngenta
H_2O_2	Hydrogen Peroxide 30%	H_2O_2	60 μL**	Merck
Sm	Streptomycin Sulphate (SP) Positive Control		60 μL**; 10,000 ppm *** 100 ppm ****	Aldrich

WG (Water granulate); SP (Soluble Powder); ROS (Reactive Oxygen Species); RNS (Reactive Nitrogen Species)
(*) PAW was applied both in diffusion and dilution methods within 1 hour from its production (**) volume used in diffusion method; (***) concentration used in diffusion method; (****) concentration used in dilution method

2.4 *In vivo* experiments

2.4.1 Pre-treatments and inoculation with the pathogen (Xv)

Under controlled conditions (Figure 2), three experiments were carried out on tomato plants disposed in randomized blocks (3 plants X 4 blocks/treatment): tomato cultivars, treatments, number of the applications, modes and times of application are shown in Table 2.

The plants to be treated at root apparatus were uprooted at 3rd-4th leaf stage and the roots were soaked for 10 min into PAW, SDW (negative control), ASM (positive control) and then put back into the pots; moreover, ASM and SDW were also applied at the leaf surface using a sprayer as well as streptomycin sulphate (as extra positive control). The inoculation was

carried out spraying a water suspension (OD_{600nm} = 0.01; *ca*. 10^7 CFU/mL), containing the pathogen strain IPV-BO 2684, on the leaf surfaces; tomato plants were then sealed in polyethylene (PE) bags for two days to favour the water congestion and to allow the pathogen penetration. The disease assessments were carried out by counting the leaf spots (on 4 to 6 leaves/plant) 21 days after the experimental inoculation. The controlled conditions, hold until disease assessment, were 16 h of day light and 8 h of darkness, 30°C and 24°C during day and night respectively; moreover, the relative humidity was maintained up to 70-75%. Data were then elaborated with ANOVA test (Tukey HSD, p 0.05).

Figure 2. *In vivo* experiment: tomato plants cv. Moneymaker inoculated with *Xanthomonas vesicatoria* strain IPV-BO 2684 after treatments applied at root apparatus.

2.4.2 Pathogen isolation and identification

Selected symptomatic leaf samples (Figure 3) were used for Xv isolation and identification. Leaf surface was sterilized with 2% sodium hypochlorite. Necrotic lesions were aseptically collected and crushed into a mortar with 2 mL of SDW; 30 μL of the extract were dropped on GYCA medium and incubated up to 48-72 h. Xv-like colonies were subcultured and identified with molecular assays (Koenraadt *et al*., 2009; EPPO, 2013).

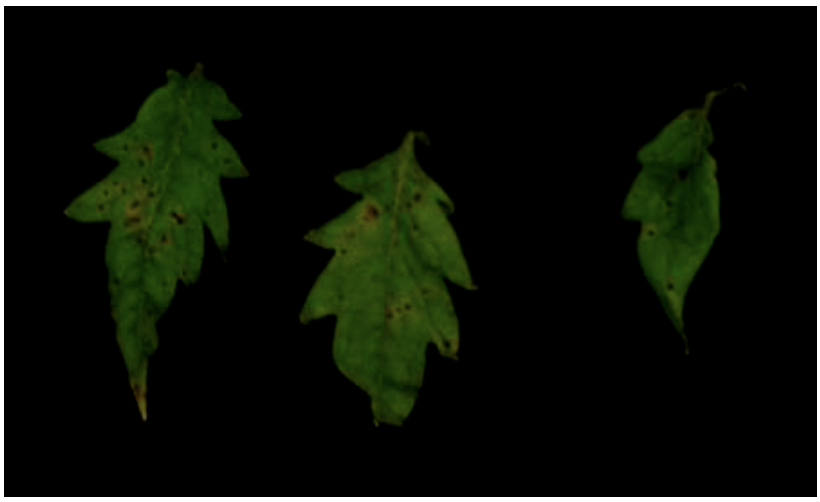

Figure 3. Bacterial leaf spot severity caused by *Xanthomonas vesicatoria* strain IPV-BO 2684 experimentally inoculated on tomato plants under greenhouse conditions.

Table 2. Experiments and treatments carried out on tomato plants under greenhouse conditions.

Code	TREATMENTS			
	Details	**Types of application**	**Volume** (mL/plant) **or Concentration** (ppm)	**Application timing** (hours or days)
Experiment 1. **Tomato cv. Moneymaker**				
SDW-R	Sterile Distilled Water	Drench	500 mL	6 d BPI
SDW-L	Sterile Distilled Water	Leaf spray	*ca.* 50 mL/plant	24 h BPI
PAW-R	Plasma Activated Water	Drench	500 mL	6 d BPI
PAW-RW	Plasma Activated Water	Drench+ irrigation	500 mL + 50 mL/plant	6 d BPI; 2 d API
ASM-R	BTH 50 WG	Drench	500 mL (a.i.75 ppm)	7 d BPI
ASM-L	BTH 50 WG	Leaf spray	*ca.* 50 mL/plant (a.i. 75 ppm)	7 d BPI
Sm	Streptomycin Sulphate (SP)	Leaf spray	*ca.* 50 mL/plant (a.i. 100 ppm)	24 h BPI
Experiment N°2. **Tomato cv. VF-10**				
SDW-R	Sterile Distilled Water;	Drench	500 mL	6 d BPI
PAW-R	Plasma Activated Water	Drench	500 mL	6 d BPI
PAW-R10	Plasma Activated Water	Drench 10 min later PAW-R	500 mL	6 d BPI
ASM-R	BTH 50 WG;	Drench	500 mL (a.i. 75 ppm)	7 d BPI
Experiment N°3. **Tomato cv. Moneymaker**				
SDW-R	Sterile Distilled Water;	Drench	500 mL	14 d BPI
PAW-R	Plasma Activated Water	Drench	500 mL	14 d BPI
PAW-RW	Plasma Activated Water	Drench+irrigation	500 mL + 50 mL	14 d; 9 d BPI
PAW-RWW	Plasma Activated Water	Drench+irrigation+irrigation	500 mL + 50 mL+50 mL	14 d; 9 d ; 4 d BPI
ASM-R	BTH 50 WG;	Drench	500 mL (a.i. 75 ppm)	7 d BPI

(R) application at root apparatus; **(RW)** 1° application at root apparatus + 2° application watering with 50 mL; **(RWW)** 1° application at root apparatus + 2° application watering with 50 mL + 3° application watering with 50 mL **(L)** Leaf application with sprayer. **(BPI)** Before pathogen inoculation; **(API)** After pathogen inoculation. (*) PAW was applied within 1 hour from its production. **(a.i.)** active ingredient.

2.5 Evaluation of defence related gene transcription

2.5.1 Treatments and sample collection

Tomato plants (cv. Moneymaker, 2 plants x 2 blocks/treatment) at 3rd-4th leaf stage maintained under controlled conditions (Figure 4), were uprooted and treated by root drenching and/or irrigation as shown in Table 3. Negative controls consisted in non-treated (NT) tomato plants and plants treated by drenching the root apparatus in sterile distilled water (SDW).

Table 3. Treatments carried out on tomato plants for evaluation of PRs gene transcription under greenhouse conditions.

Code	**TREATMENTS**		
	Details	**Types of application**	**Volume** (mL/plant) **Concentration** (ppm)
SDW-R	Sterile Distilled Water	Drench	500 mL
PAW-R	Plasma Activated Water	Drench	500 mL
PAW-RW*	Plasma Activated Water	Drench+irrigation	500 mL + 50 mL/plant
JA	Jasmonic Acid, Sigma cod. J2500	Drench	500 mL (a.i 100 mM)
ASM-R	BTH 50 WG, Syngenta	Drench	500 mL (a.i.75 ppm)
NT	Non treated	/	/

(*) Watering consisted in 50 mL/plant at 120 h from initial treatments. **(a.i.)** active ingredient.

Three youngest tomato leaves were collected from each replicate/treatment at six time-points: 0, 7, 24, 48, 120 h and 8 d. The tissues were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. The greenhouse conditions were set as 16 h light at 25°C and 8 h dark at 19°C, maintaining a RH% of approx. 85% until the last leaf collection day.

Figure 4. Tomato plants cv. Moneymaker treated at root apparatus at 3rd-4th leaf stage grown under greenhouse conditions for analysis of defence related gene transcription.

2.5.2 RNA Extraction

Total RNA extraction was carried out by Qiagen RNeasy Plant minikit (cat.: N° 74904); the RNase-Free DNase Set (Qiagen; cat. No. 79254) was used in combination with Qiagen RNeasy Plant minikit to assess DNA digestion. Procedure consisted in grounding *ca*. 100 mg of tomato leaves stored at -80°C in liquid nitrogen using pre-chilled mortar and pestle. The powdered tissue was added with 1.2 mL of extraction buffer (see appendix), and then homogenized. The extract was transferred into 2 mL Eppendorf tube and centrifuged at room temperature at 17,500 *g* for 5 min; 1 mL of supernatant was added with 100 μL of Na-lauroyl sarcosine (30%; see appendix) and mixed by inversion. The sample was then incubated at 70°C for 30 min.

The total volume of the extract, was placed into QIA shredder spin columns and centrifuged at 17,500 *g* for 2 min. The supernatant was loaded into a 1.5 mL Eppendorf tube and amended with 0.5 volume of absolute ethanol; the mixture was then homogenized. 700 μL of the mixture were transferred into RNeasy spin column, centrifuged at 9,900 *g* for 1 min; the flow-through was discarded. This step was repeated using the remaining part of the mixture.

From this step the kit RNase-Free DNase Set was applied to digest co-extracted DNA. RNeasy spin column was washed by adding 350 μL of buffer RW1, and centrifuged for 15 s at 8,000 *g*; the flow-through was discarded. A solution with 10 μL of DNase I stock solution

and 70 µL of buffer RDD was prepared and mixed by inverting the tube. DNase I incubation mixture (80 µL) was directly added to the membrane of RNeasy spin column, and incubated at room temperature for 15 min. Buffer RW1 was added (350 µl) to the column membrane and then centrifuged for 15 s at 8,000 *g*; the flow-through was discarded. The extraction continued using Qiagen RNeasy Plant minikit following the instructions of the manufacturer. The total RNA (50 µL of nuclease free distilled water) was stored at -20°C.

2.5.3 Total RNA quality evaluation and quantification

Quantity and quality (A_{260nm}/A_{280nm}) of the RNA extracts (3 µL) were evaluated using Tecan Infinite® 200 Pro NanoQuant instrument and i-control™ software (Tecan Group Ltda., Switzerland). The quality was also assayed by loading 5 µL of RNA extracts into a 1.2% agarose gel: the electrophoresis was carried out at 40 V for 100 min in TAE buffer. The gel was stained in ethidium bromide solution (0.03%) for 20 min and distained in distilled water for 5 min; visualisation was then carried out under UV light (312 nm).

2.5.4 Sequencing of reference/target genes and primer design

RT-PCR on RNA plant extracts from tomato plants treated with ASM, were performed as follows: reverse transcription was carried out in a final volume of 5 µL containing 1x Buffer (Promega, cod. M531A), 1 mM dNTPs (Cod. U1330, Promega); 50 µM random oligo-dT primer (Roche Diagnostic, cod. 11034731001), 50 U of M-MLV reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase; Promega, cod. 1705A,); 0.5 µL of RNA template were used. The thermal profile was: 37°C for 60 min and at 94°C for 5 min to inactivate M-MLV enzyme.

The PCRs were performed using 5 µL of cDNA samples, in a final volume of 25 µL which contained 1× PCR Go Taq Flexi buffer (Promega, 5X Colorless GoTaq Flexi Buffer, M890A), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.2 µM each primer, 1 U Go-Taq Flexi DNA polymerase (Promega, cod. M830A). The primers used to obtain reference/target gene sequences were from studies carried out on Moneymaker (Biondi, 2008) and Craigella tomato plants (Danve, 2010). The design of new primers, performed on NCBI sequences, was carried out using Lasergene 7.0 software (Table 4).

The thermal profiles were set up depending on the primer sequences as follows:

- Act3-fw and Act3m-rev primers: initial denaturation step (94°C for 5 min), followed by 35 cycles at 94°C for 1 min, 67°C for 30 s, 72°C for 30 s, and a final extension step for 10 min at 72°C.

- LoxF6-fw and LoxF6-rev primers: initial denaturation step (94°C for 5 min), followed by 35 cycles at 94°C for 1 min, 65°C for 30 s, 72°C for 30 s, and a final extension step for 10 min at 72°C.

- ERF1.8-fw and ERF1.8-rev primers: initial denaturation step (94°C for 5 min), followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension step for 10 min at 72°C.

- IDT130 and IDT132 primers: initial denaturation step (94°C for 5 min), followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension step for 10 min at 72°C.

The amplification products were analyzed on 1.5% (w/v) agarose gel in 1x TAE buffer, stained with 0.03% ethidium bromide and visualised under UV light (312 nm).

Table 4. Primers used to obtain tomato cv. Moneymaker sequences.

Gene	Primer Names	Primer Sequences (5' → 3')	Amplicon size (bp)	Tomato cultivar	Accession number	References
ß-actin	Act3-fw	GTTTGGATCTTGCTGGTCGTG	320	MicroTom	FJ532351	This study
	Act3m-rev	CATAATAGAGTTGTAGGTAGTC TCGTGGA				
loxf	LoxF6-fw	TTTCCGGCCATCGACTCACTT	893	Merveille des Marchés	NM_001247330	This study
	LoxF6-rev	GCTTCTCTATCAAACCTCCACT CA				
erf1	ERF1.8-fw	TATCAACTTCCCACTTCTACTG	788	MicroTom	AY192367	This study
	ERF1.8-rev	ATGATTTGCTATTTTCTGTCC				
pr1a	Pr1a-fw	CACTCTTGTGAGGCCCAAATTT CACC	427	Moneymaker	/*	Biondi, 2008
	Pr1a-rev	TACTTTAATAAGGACGTTCTCC AACC				
pr4	Pr4-fw	TGTCATCAACATGATGATGGC GGTGGC	349	Moneymaker	/*	Biondi, 2008
	Pr4-rev	ATAGCCCAATCCATTAGTGTCC AATCG				
pr5	Pr5-fw	GACTTACACTTATGCTGCCACT TTCGAG	560	Moneymaker	/*	Biondi, 2008
	Pr5-rev	GGTAGCTATACGCATCAGGAC ATCTTTG				
pal	IDT130	GAATTCACTGACTATTTG	652	Craigella	/*	Danve, 2010
	IDT132	GATAGGTTGATGACATTA				

(*) Accession number not available.

2.5.5 Reference and target genes cloning

The amplified RT-PCR band was extracted and purified from gel using Kit Wizard SV Gel and PCR Clean-Up System (Promega cod. A9281) following the manufacturer's protocols. The purified amplicon (3 µL) was recombined in pGEM-T plasmid pGEM-T Easy Vector System (Promega, cod. A1360), following the manufacturer's protocols. The reaction was incubated at 4°C overnight.

Transformation was performed using the recombinant plasmid pGEM-T and strain JM109 strain of *E. coli* through electroporation and thermoporation (Sambrook and Russell, 2001) as follows:

- Electroporation. Electrocompetent cells were prepared according to Sambrook and Russell (2001) protocol. The electroporation was achieved in an electroporation cuvette (0.1 cm diameter), using 20 ng of recombinant plasmid and the following profile: in the Gene Pulser II (Bio-Rad) electroporator the resistance was set up at 400 Ω (ohm), the capacitance at 125 μF and the voltage was set at 18 kV.

- Thermoporation. Ligase reaction (3 μL, containing the recombinant plasmid) was added to 100 μL of JM 109 competent cells, prepared following Sambrook and Russell (2001) protocol. The reaction was incubated on ice for 30 min. Successively, the samples were kept in a thermoblock for 90 s at 42°C and transferred to ice for 2 min.

For both methods, the transformed bacterial cells were grown in 900 μL of SOC broth (see appendix). The samples were kept in a shaker at 37°C for 2 h, and centrifuged for 5 min at 14,000 rpm; then, 400 μL of the supernatant were removed and the pellet was resuspended in the remaining supernatant (600 μL). Petri plates containing LB medium amended with ampicillin (100 μg/mL), IPTG (isopropy-β-D-thiogalactopyranoside, 0.5 mM) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 80 μg/mL) (see appendix) were inoculated by spreading 200 μL and 400 μL of bacterial resuspension. The plates were incubated at 37°C overnight.

The transformation and recombination were verified by colony morphology: blue colonies indicated transformation, but not plasmid recombination, whereas, white colonies indicated that the insert was inserted into the plasmid.

The transformed white colonies were sub-cultured at 37°C for *ca.* 16 h, collected with a toothpick and suspended in 50 μL of SDW. The bacterial suspension was lysated in a thermoblock at 100°C for 5 min and then immediately put in ice for 5 min and finally centrifuged at 13,000 rpm for 1 min. PCR assays were performed using 5 μL of the supernatant with chemical and thermal profiles depending on markers (internal primers) characteristics (Paragraph 2.5.4, Table 4).

The recombinant plasmid was also purified with Wizard Plus SV Minipreps DNA purification System Kit (Promega, cat. A1330). Ten mL of 24 h bacterial suspension were centrifuged at 10,000 *g* for 5 min. The pellet was resuspended in 250 μL of Cell Resuspension Solution. A volume of 250 μL of Cell Lysis Solution was added and homogenized by inverting the tube

four-times and an incubation for 5 min at room temperature followed. Alkaline Protease Solution (10 µL) was added and mixed by inverting the tube four times; the solution was incubated for 5 min at room temperature. Then, 350 µL of Neutralization Solution were added and mixed by inverting the tube; the sample was centrifuged at room temperature at maximum speed (14,000 *g*) for 10 min. The supernatant (850 µL) was transferred to a Spin Column, supplied by the kit, and it was centrifuged at 14,000 rpm for 1 min at room temperature; the flow-through was eliminated. The membrane column was then washed, by adding 750 µL of Column Wash Solution, and then centrifuged for 2 min at 14,000 rpm (room temperature). The membrane was transferred in sterile Eppendorf tube and successively added with 100 µL of nuclease free water. The tube was centrifuged for 1 min at 14,000 rpm. The column membrane was removed and the eluate, containing the recombinant plasmid, was stored at -20°C.

The extracted recombinant plasmid (200 ng) was digested with 6 U of *Eco*RI 37°C for 3 h. Restriction products were separated in 0.8% (w/v) agarose gel, stained and visualised as described above.

2.5.6 Primer design and quantitative RT-PCR (RT-qPCR)

From tomato cv. Moneymaker plant extracts, RT-PCR product sequences of the reference gene *ß-actin*, as internal control (Danve, 2010; Murshed *et al.*, 2013), and of the target genes *pr1a*, *pr4*, *pr5*, *pal*, *loxf* and *erf1* were employed for primer design using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). All the primers used for RT-qPCR are listed in Table 5.

The RT-qPCR was performed using an ABI7000 v 1.2.3 sequence detection system. Each reaction was carried out with one-step method, in a final volume of 25 µL containing 1 U M-MLV (Promega, cod. 1705), 12.5 µL GoTaq qPCR Master Mix (Promega, No. A6001, SYBR Green/ROX chemistry), 0.25 µL CXR reference dye, primers at 400 nM (Invitrogen, cod. A967) and 1 µL of RNA template (100 ng/µL). The primers specificity was evaluated on RNA extracts by dissociation curves analysis, in order to exclude non-specific amplifications or primer dimers presence (Figure 5).

Table 5. RT-qPCR primer sequences designed on the gene sequences obtained from tomato cv. Moneymaker.

Gene	Primer Name	Primer Sequence (5' - 3')	Dissociation curve (°C)	References
ß-actin	ACTFRt.1	AGCTCCTCCATTGAAAAGAACTATG	76.0	This study
	ACTRRt.1	GGTAATAACTTGTCCATCAGGCAA		
pr1a	Pr1FRt21	TGTTGGTGGAAAAATGTGTGGA	77.0	Biondi, 2008
	Pr1RRt21	GAGTTGCGCCAGACTACTTGAGT		
pr4	Pr4FRt21	TATGAACGTTAGGGCAACGTATCA	75.0	Biondi, 2008
	Pr4RRt21	CAGTTTATGTTTTGCGGATTGTACA		
pr5	Pr5FRt21	CCAGTTTAGCAACCTAGATTTCTGG	75.0	Biondi, 2008
	Pr5RRt21	TTAAATCCATCGACTAAAGAAATGTCC		
pal	PALFRt.2	TCAGCACTTTGGACATGGTTAGTC	76.0	This study
	PALRRt.2	AGAACTTCAATTCCTTGCAAATCC		
loxf	LOXFRt.1	AGCAAGCTAGACCCTGAGGTATATG	77.0	This study
	LOXRRt.1	TTTTGTAATTGCTGATTCAGGAGGT		
erf1	ERFFRt.2	AACTCAATGGCTAGGGCTTGTTT	76.0	This study
	ERFRRt.2	TTTGCTATTTTCTGTCCACTTCAAAG		

The thermal profile was set up as follows: 30 min at 48°C (reverse transcription), 95°C for 10 min (*Taq* activation), followed by 40 cycles of 95°C for 10 s (denaturation), and 60°C for 1 min (annealing/extension). Dissociation curves were performed at 95°C for 15 s, 60°C for 20 s and 95°C for 15 s.

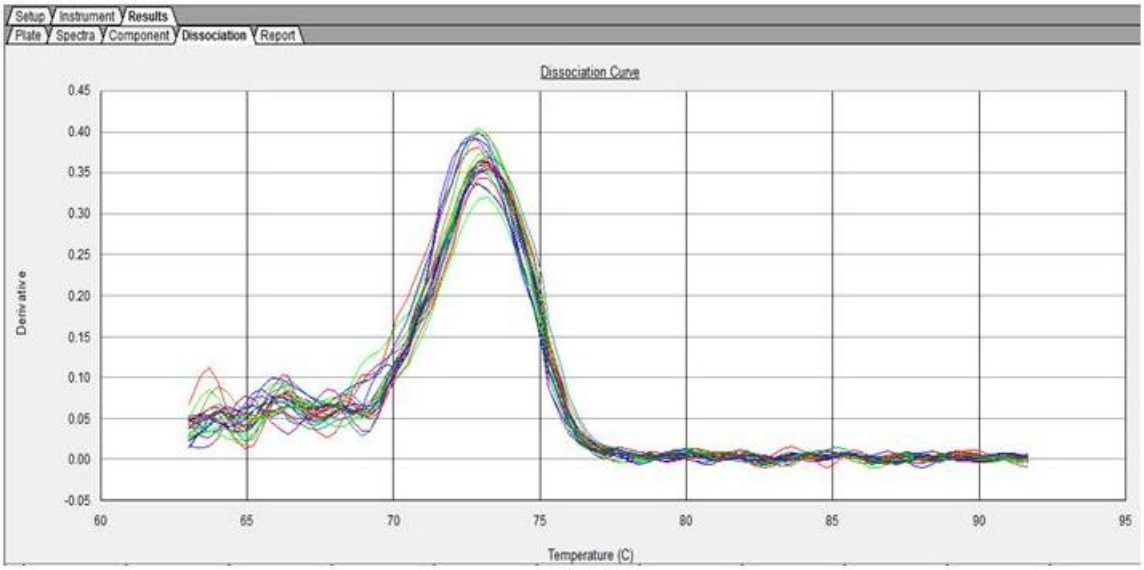

Figure 5. Melting curve analysis of the obtained amplicons indicating the primer specificity for *ß-actin* (internal control), *pr1a*, *pr4*, *pr5*, *pal*, *loxf* and *erf1* genes.

2.5.7 Experimental design and statistical analysis

Data were analyzed using SDS1.2 software (Applied Biosystems). The efficiency of each primer pair was determined using RNA ten-fold dilution series (calibration curves) to determine the fold changes. Exponential amplification was plotted on a logarithmic scale, and Rn was set to 0.32 for each RT-qPCR plate to obtain the cycle threshold (Ct) values. Ct values for all the gene studied were normalized to the reference gene *β-actin* (internal control) to evaluate possible variation between different RNA samples.

The experiment was replicated three times per time point, moreover the analysis of target gene expression, related to the reference gene (*ß-actin*), was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All gene expression levels were normalized to the appropriate non-treated (NT) control at each time point (0, 7, 24, 48, 120 h and 8 d). Standard errors (SE) were calculated to evaluate statistically significant differences among samples.

3. RESULTS AND DISCUSSION

The results highlighted that PAW, as well as negative controls (SDW and ASM), was not able to inhibit the Xv growth in *in vitro* experiments using the diffusion method (Table 6). Meanwhile, the positive controls (Sm and H_2O_2) produced a mean inhibition halo of 3.0 cm and 3.8 cm, respectively (Figure 6).

Table 6. Results on *in vitro* PAW efficacy tested against *Xanthomonas vesicatoria* on LB-agar.

Treatments	*Xanthomonas vesicatoria* IPV-BO 2684
SDW-150	-
PAW-150	-
PAW-300	-
ASM	-
H_2O_2	+ (3.8 cm)
Sm	+ (3.0 cm)

(-) non inhibited growth; (+) inhibited growth

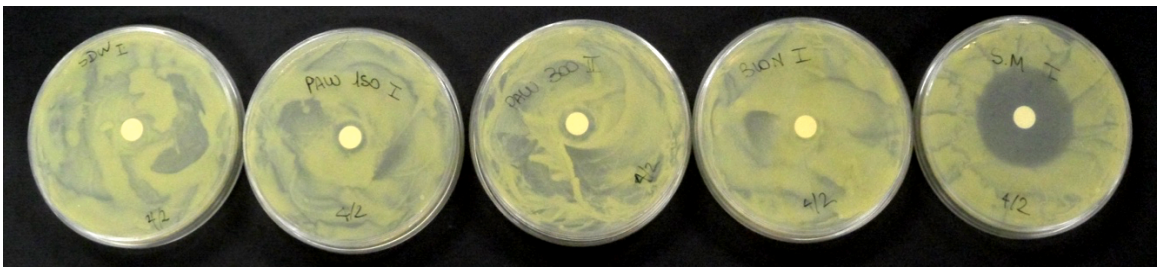

Figure 6. PAW efficacy evaluated by diffusion method on Luria-Bertani agar medium, inoculated by *Xanthomonas vesicatoria* strain IPV-BO 2684 (200 μL; 10^9 UFC/mL).

In vitro assays, carried out using the dilution method, confirmed the results obtained with the diffusion method: PAW did not show effect against the Xv strain. At 0 h PAW 150, PAW 300 treatments as well as SDW and ASM did not show any inhibition of the bacterial growth: for those treatments Xv population was evaluated at *ca*. 10^6 UFC/mL (Figure 7), whereas for positive controls, represented by hydrogen peroxide and streptomycin sulphate at the same time point (0 h), the bacterial population was inhibited and resulted to be 0 and approx. 10^5 CFU/mL, respectively. After 24 h, the PAW treatments and the negative controls (SDW and ASM) did not inhibit the bacterial growth, the population reached *ca.* 10^8 CFU/mL, while in the positive controls Xv population was completely killed (Figure 7).

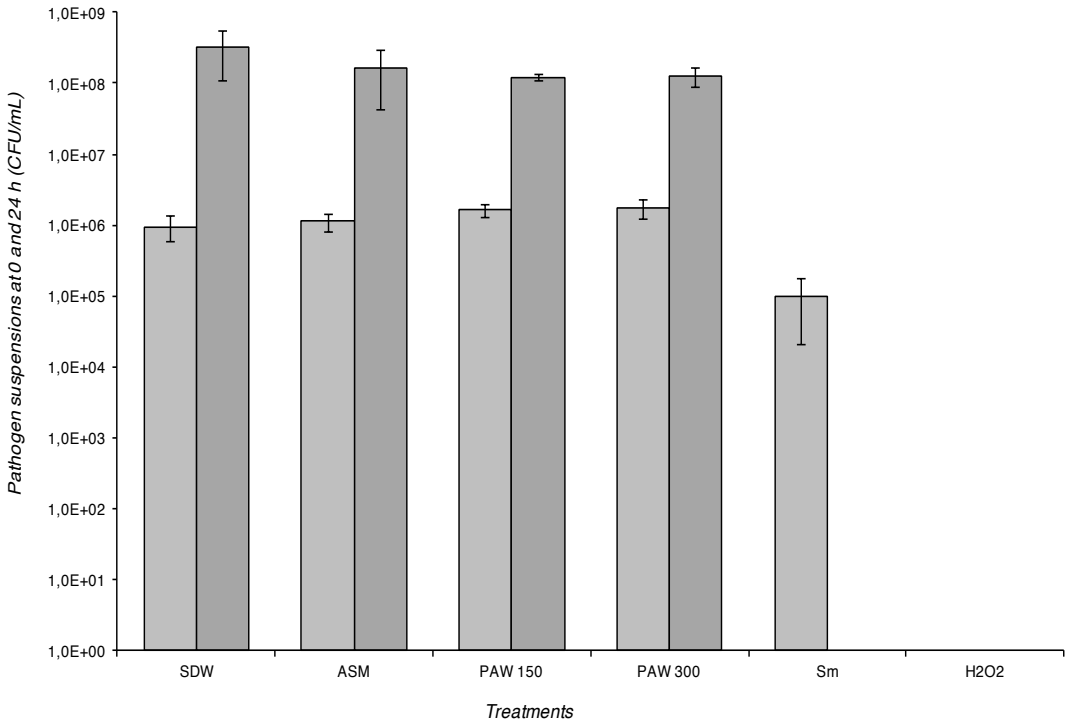

Figure 7. Results of PAW *in vitro* efficacy using dilution method against *Xanthomonas vesicatoria* strain IPV-BO 2684: at 0 h (light grey histograms), at 24 h (dark grey histograms). Each column represents the mean of 3 replicates and standard deviations (±SD).

In a recent work (Laurita *et al*., 2015), in which the efficacy of PAW against *Candida albicans* and *Staphylococcus aureus* was studied using the dilution method, it was pointed out that the loss of PAW efficacy was related to the post discharge time. After 16 minutes, a minimal concentration of H_2O_2 (100 μM) was present and that of NO_2^- was not detectable by the

instruments, therefore PAW did not show any efficacy against the two pathogens. In this study, the *in vitro* experiments were carried out after 30 up to 50 minutes from the discharge: this explained the lack of PAW efficacy against Xv. In addition, the method used for these *in vitro* experiments was based on guidelines suggested by Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS, 2006) that differ from the methods proposed and used in the other studies in which PAW was employed (Julák *et al*., 2012; Laurita *et al.* 2015; Traylor *et al*., 2011). The latter studies took into account not just the time occurring between the PAW production and its use in *in vitro* experiments, but also the effect of exposure time of the bacterial suspension to higher volumes of PAW. Traylor *et al.* (2011) demonstrated that PAW, produced 7 days before its application using dilution method, was effective inhibiting *E. coli* growth depending on its exposure time to the treatment.

The presence of minimal concentration of H_2O_2 and NO_2^-, and low pH (Laurita *et al*., 2015; Scholtz *et al*., 2015) suggested a different way for PAW employment in controlling plant pathogens *i.e*. through a defence mechanism mediated by the plant host. The typical characteristic of an elicitor for plant resistance induction is in fact, its low concentration (Agrawal *et al*., 1999; Shibuya and Minami, 2001). Moreover, the ROS (O_2, OH, O_2H) is known to be one of the upstream responses of the plant during a pathogen attack (Peng and Kuć, 1992; Doke, 1983; Grant *et al*., 2000; Miller *et al.,* 2009).

The three *in vivo* experiments that were conducted on tomato plants were aimed, indeed, to evaluate the indirect effect of PAW (treated at the roots) against Xv, through the host induced resistance.

In the first experiment, the disease severity of Moneymaker tomato plants treated with PAW-R and PAW-RW resulted slight lower significantly (approx. 6.5 and 6 spots/leaf, respectively) than that of tomato control plants represented by SDW-R and SDW-L (11 and 9, respectively); the relative protection (related to SDW-R) provided by both PAW root treatments was approx. 30%. The efficacy of both PAW treatments was almost equivalent and no statistical difference between them was observed. On the other hand, the plants treated both at leaves and roots with the resistance inducer (ASM, positive control), showed a disease severity significantly reduced, *ca.* 2 and 3 spots/leaf, respectively (Figure 8). Moreover, the relative protection provided by both positive controls was calculated as approx. 74% confirming the already well known efficacy of this resistance inducer against

bacterial diseases (Oostendorp *et al.*, 2001; Biondi *et al.*, 2006; 2009; Cellini *et al.*, 2014), and in particular, against tomato bacterial diseases (Thaler *et al.*, 1999; Louws *et al.*, 2001; Graves and Alexander, 2002; Obradovic *et al.,* 2005; Herman *et al.*, 2007). Between ASM-L and ASM-R, two of the three positive controls used in the experiment, there was not statistical difference, even though ASM-R (ASM treated by drenching the roots) apparently reduced the disease severity when compared with the plants treated with ASM-L (ASM treated at the leaves). Plants treated with streptomycin sulphate (positive control) showed the significant lowest disease severity since the mean number of leaf spots was 0.4 (Figure 8).

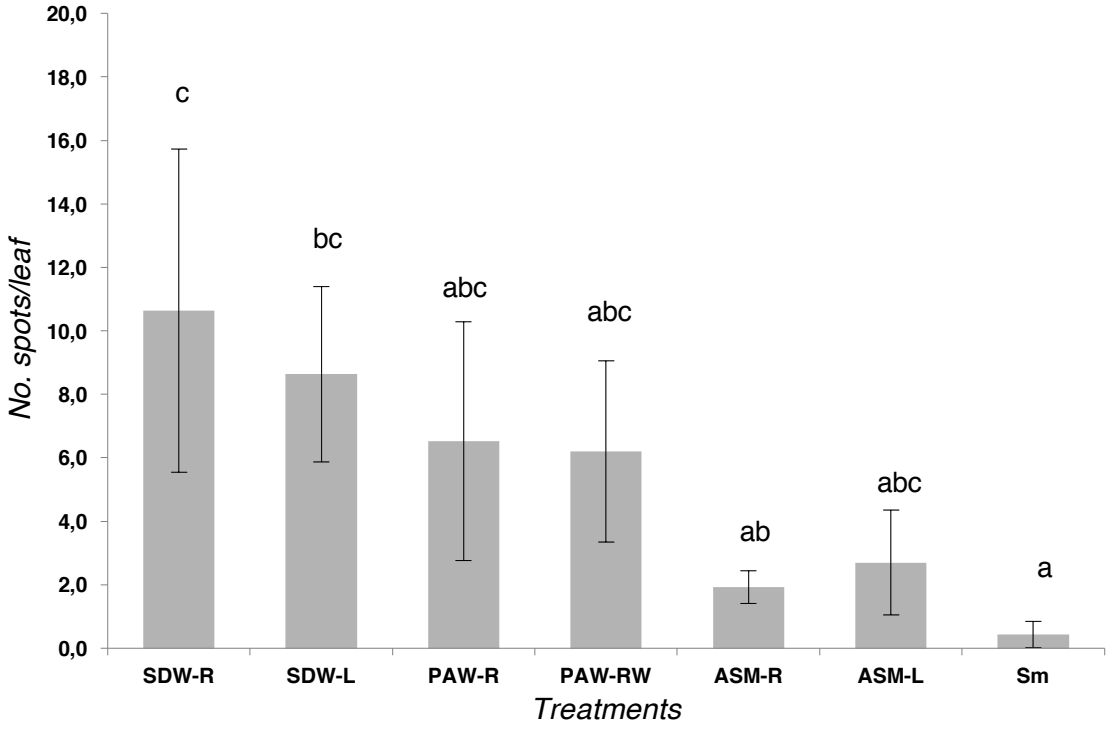

Figure 8. Results of the *in vivo* efficacy of plasma activated water (PAW), compared to the negative and positive controls, against bacterial leaf spot caused by *Xanthomonas vesicatoria* (strain IPV-BO 2684) on tomato plants cv. Moneymaker grown under greenhouse conditions. Different letters indicate significant differences (Tukey's HSD, p 0.05).

The results of the second experiment, carried out on tomato plants VF10, confirmed those obtained on Moneymaker plants. The bacterial leaf spot severity caused by the strain IPV-BO 2684 was significantly reduced on plants treated at the roots with PAW: the number of spots per leaf was *ca.* 10.5 and 11 in the plants treated with PAW-R and PAW-R10, respectively. The relative protection (related to negative control SDW-R) was confirmed and improved to approx. 35% (Figure 9). Moreover, no significant differences were evaluated in disease severity between the PAW treatments. The plants treated with SDW-R showed significantly higher disease severity (approx. 17 spots/leaf).

In the tomato plants treated with ASM by drenching the roots, the leaf spot disease severity caused by Xv was significantly reduced (approx. 3 spots per leaf). The treatments PAW-R and PAW-R10 fell into the same category (Tukey's HSD, p 0.05) of the positive control treatment represented by ASM-R (Figure 9).

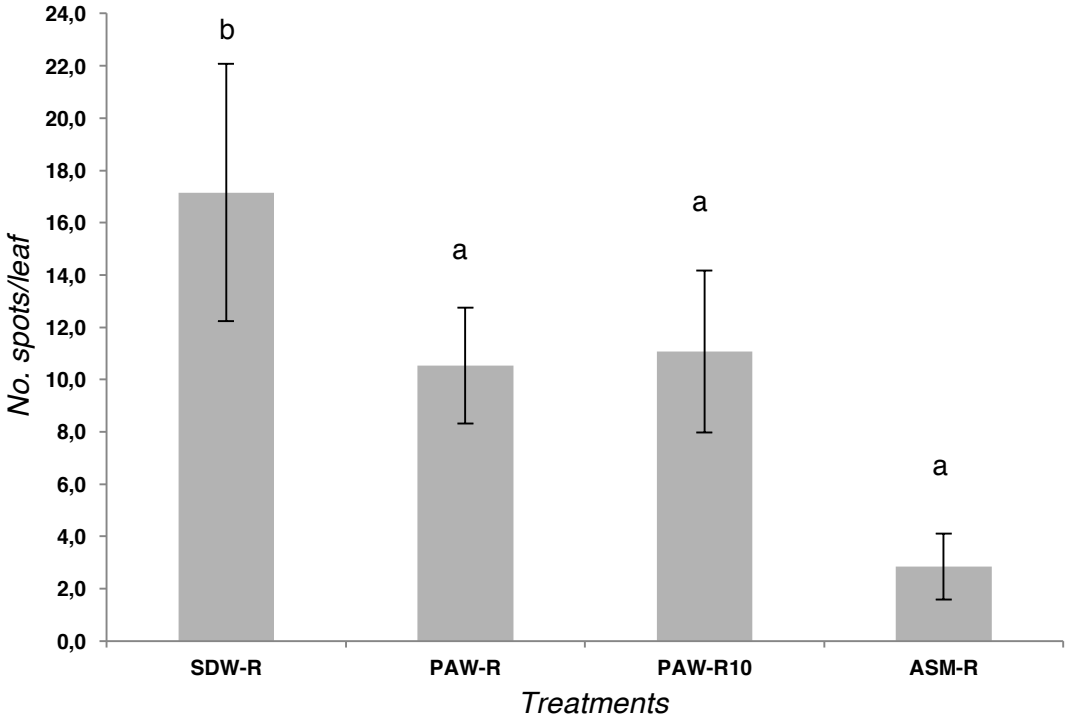

Figure 9. Results of the *in vivo* efficacy of plasma activated water (PAW), compared to negative and positive controls, against bacterial leaf spot caused by *Xanthomonas vesicatoria* (strain IPV-BO 2684) on tomato plants cv. VF-10 grown under greenhouse conditions. PAW-R only drenching (14 days BPI), PAW-RW (root drench + irrigation) (14 d and 9 d BPI). Different letters indicate significant differences (Tukey's HSD, p 0.05).

The third experiment on tomato, again on cv. Moneymaker, was conducted to evaluate the effectiveness in disease protection of PAW at different combinations, types and times of application. The plants treated 14 days BPI (PAW-R) showed a disease severity (mean of approx. 15 spots/leaf) which resulted significantly similar to that of positive control plants (SDW-R, mean of approx. 12 spots/leaf). On the contrary, the disease severity of the tomato plants treated with PAW-RW and PAW-RWW (*ca.* 8 and 10 spots/leaf, respectively) resulted between the SDW-R and the negative control (ASM-R, approx. 4 spots/leaf) in a significant manner (Figure 10). These results confirm those obtained in the first experiment in which the relative protection by PAW-R treatment, was calculated as approx. 30% (Figure 8): in this experiment, the relative protection of PAW-RW, apparently the best of the PAW treatments, was *ca*. 38%. PAW-RWW (the most similar to PAW-RI, Table 2) did not show an adequate relative protection (approx. 20%), as also evidenced in the first experiment.

The positive constant that was observed in all the experiments carried out *in planta* using PAW, was the absence of phytotoxicity and the lack of reduction in the vegetative growth, as was instead rarely observed in plants treated with ASM-R.

The PAW treatments therefore show to induce a stable plant-mediated defence response. The ROS and/or RNS, components of PAW, might be linked to signalling pathways that establish a correlation between the disease reduction and the activation of pathogenesis-related protein and defence enzymes on tomato. These results induced to analyse more deeply the effect of PAW treatments on tomato plants by carrying out transcriptomic analyses of genes related to the tomato plant induced resistance.

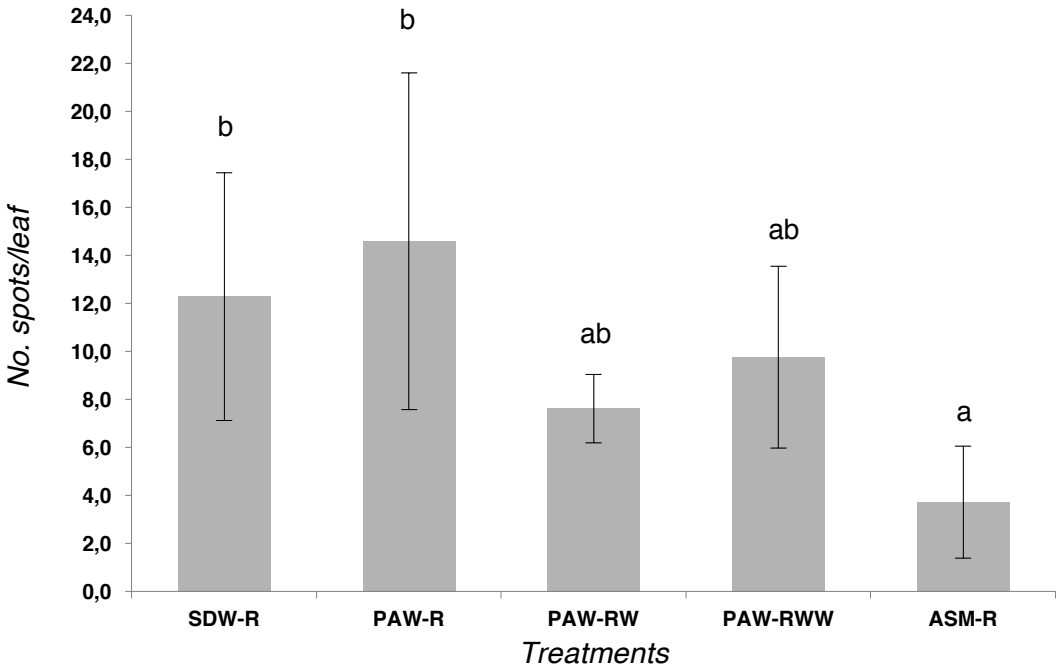

Figure 10. Results on application frequency of plasma activated water (PAW) against bacterial leaf spot caused by *Xanthomonas vesicatoria* (strain IPV-BO 2684) on tomato plants cv. Moneymaker grown under greenhouse conditions. PAW-1 only drenching (14 days BPI), PAW-RW (drench+irrigation) (14 d and 9 d BPI), PAW-RWW drenching+watering repeated twice (14 d, 9 d and 4 d bpi). ASM-R and SDW-R represented the positive and negative controls. Different letters indicate significant differences (Tukey's HSD, p 0.05).

In Figure 11 are shown the RNA extracts used in the RT-qPCR: the integrity of purified RNA is critical to all gene expression analysis techniques to obtain meaningful gene expression data (Fleige and Pfaffl, 2006; Pfaffl, 2005). The quality and quantity of RNA extracts are also reported in Appendix.

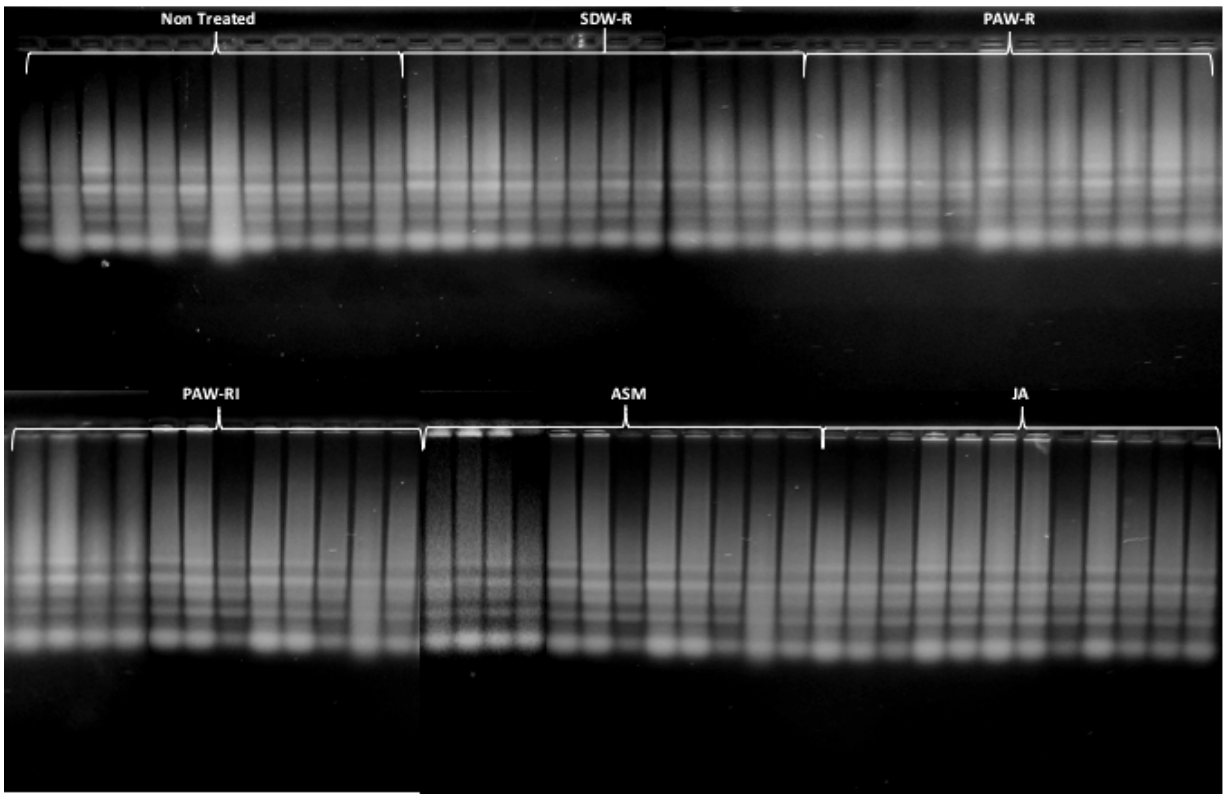

Figure 11. Total RNA extracted quality run in 1.2% agarose gel.

First, the *ß-actin* was confirmed as a reliable reference gene, because its abundance in PAW treatments, in the negative (NT and SDW-R) and positive (ASM and JA) control plants was equivalent. The data here presented show the change in transcript abundance of the studied genes in all the different treatments studied. The use of the negative control treatment (SDW-R), whose type of application was similar to that of the other treatments (PAW, ASM and JA), allowed to measure the real intensity of the plant defence response. The plant stress, in fact, caused by the type of treatment application (*e.g.* tomato plant explants and subsequent root drenching) could have influenced some gene induction. Indeed, the SDW-R control was included in the gene expression studies to ensure that results accurately reflected the host response to the treatment alone (Smart *et al.*, 2003).

ASM application (positive control for SAR), as expected, triggered the expression of PRs in the considered time intervals. Referring to PR-1a (acid PR-1), and PR-4, gene expression kinetics showed a general significant increase in tomato plants 120 h after ASM root application up to 8 days (Figures 12 and 13). The PR-5 resulted significantly inducted (approx. 90-fold) only after 8 days from ASM application (Figure 14). In the plants treated with JA (positive control for ISR) the PR-1a, PR-4 and PR-5 induction was not observed up to the 8th day (Ciardi *et al*., 2000; 2001). The only exception is represented by the tomato plants tested 7 hours after treatment; in those cases, the expression of PR-1a, PR-4 and PR-5 were approx. 35-, 8- and 10-fold. The decrease of PR-1a observed from 7 h and up to 48 from ASM application is partially contradictory in the initial stages of PR-1a induction according to what was reported by Biondi (2008) and Herman *et al*. (2007); however, the upregulation of the transcript abundance after 5 days is closely related to the present results. The early expression of PR-1a in those studies should be attributed to the ASM mobilization in the plant, that was described within 2 h from the application in tomato leaves (Scarponi *et al*., 2001). After 120 h and 8 d the PR-1a induction was demonstrated, confirming the ability of ASM as an effective elicitor in inducing host resistance; effectiveness that was also confirmed by the *in vivo* experiments against bacterial leaf spot and reported in previous works (Louws *et al*., 2001; Graves and Alexander, 2002).

A different expression pattern was observed for LOXF, PAL and ERF1 (Figures 15, 16 and 17). The plants treated with ASM, showed a relevant induction of these genes at 120 h after treatment, but this induction constantly decreased up to the 8th day: the LOXF, PAL and ERF1 expression, in fact, was close to the baseline; nonetheless, PAL induction at the last point time (8 d after treatment) of PAW treated plants, remained still significantly higher compared to the one of the control plants (SDW-R, Figure 16).

Concerning the Moneymaker plants treated with JA, the PR-1a, PR-4 and PR-5 expression was *ca.* 35-, 6- and 10-fold, respectively, after 7 hours from the treatment, but it was close to 0-fold at each time point up to the 8th day. In particular, the behavior of PR-1a kinetic expression in plants treated with JA is fully in agreement with known JA characteristics since it can suppress the induction of *m*RNA encoding the PR-1a (Thaler *et al*., 1999; Ciardi *et al*., 2000). The jasmonate pathway is reported as ISR inducer that stimulates other PRs (*i.e.* PR-4) and enzymes (*i.e.* LOXF) (Smart *et al*., 2003; Choudhary *et al*., 2007).

Moreover, in JA treated tomato plants, LOXF and ERF1 were upregulated until the 7th hour (approx. 4- and 8-fold, respectively). However, this induction decreased rapidly at each point time until the last assessment. Similar behaviour was shown by PAL: its expression, in fact, reached its maximum after 24 h (*ca.* 8-fold), but its decrease started from 48 h (5-fold) to reach the baseline after 8 days.

The tomato plants treated with SDW-R (negative control) showed an induction of PR-1a at 7 and 24 h (*ca.* 11- and 4-fold, respectively) due to the type of treatment application. The tomato uprooting (wounded roots), as already written above, and the 10 min drenching triggered also a plant response (Tournier *et al.,* 2003). At time 0, 48 and 120 h the plant response was close to the baseline; unexpectedly, at the 8th day from the SDW-R treatment, the expression of PR-1a was evaluated as approx. 12-fold. The increase of PR-4 transcripts however, was only observed in the plants tested 8 days after the treatment; in this case the overexpression of PR-4 was comparable to that of PR-1a. Similar behaviour on negative control plants (SDW-R) was observed for PR-5: its induction was close to the baseline (from *ca.* 0- to 1-fold) up to 120 h and a slight expression increase (approx. 3-fold) was evaluated in the last readings (Figures 12, 13 and 14).

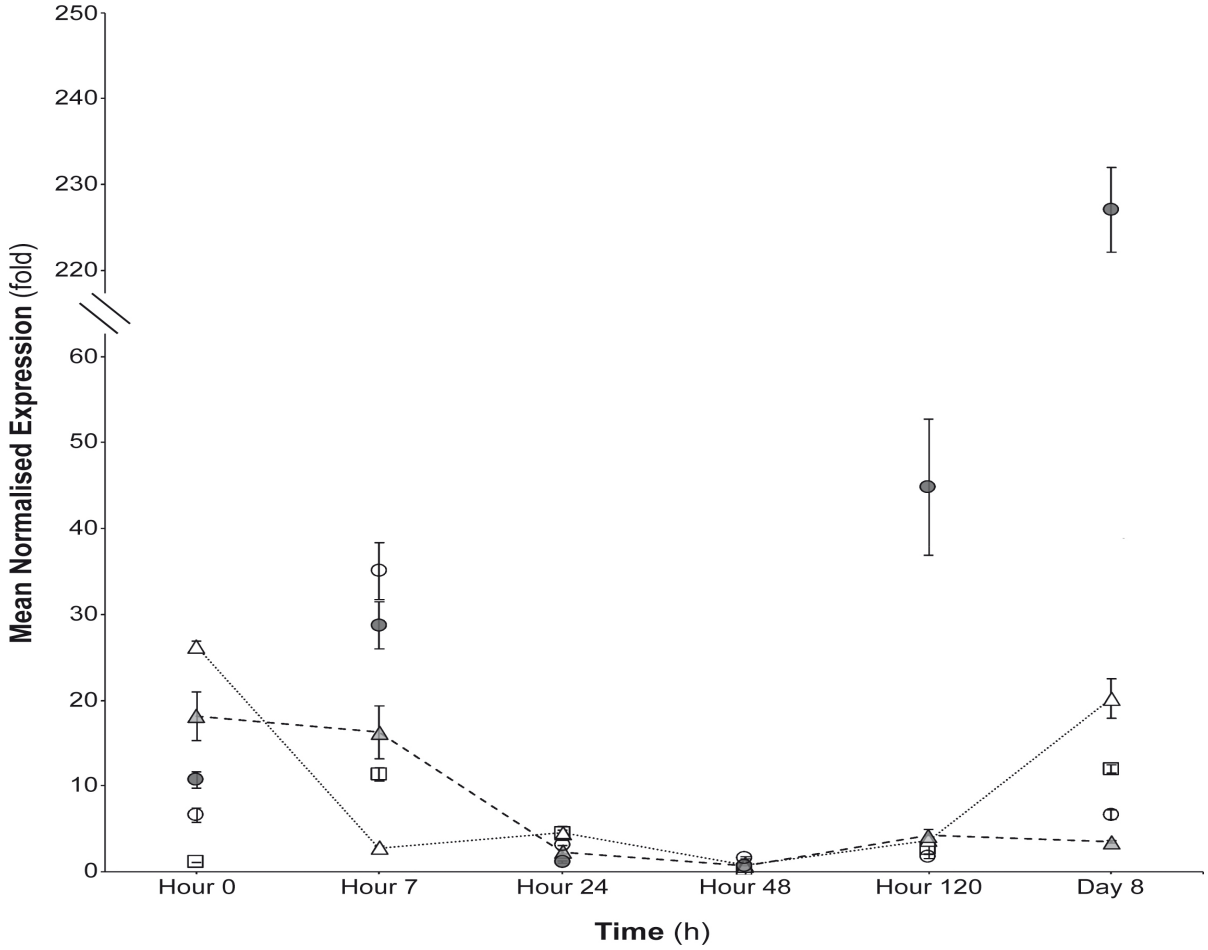

Figure 12. PR-1a expression kinetics in tomato leaves after root application of: PAW-R (empty triangles), PAW-RI (grey filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the PR-1a induction at each time point and the standard error (±SE).

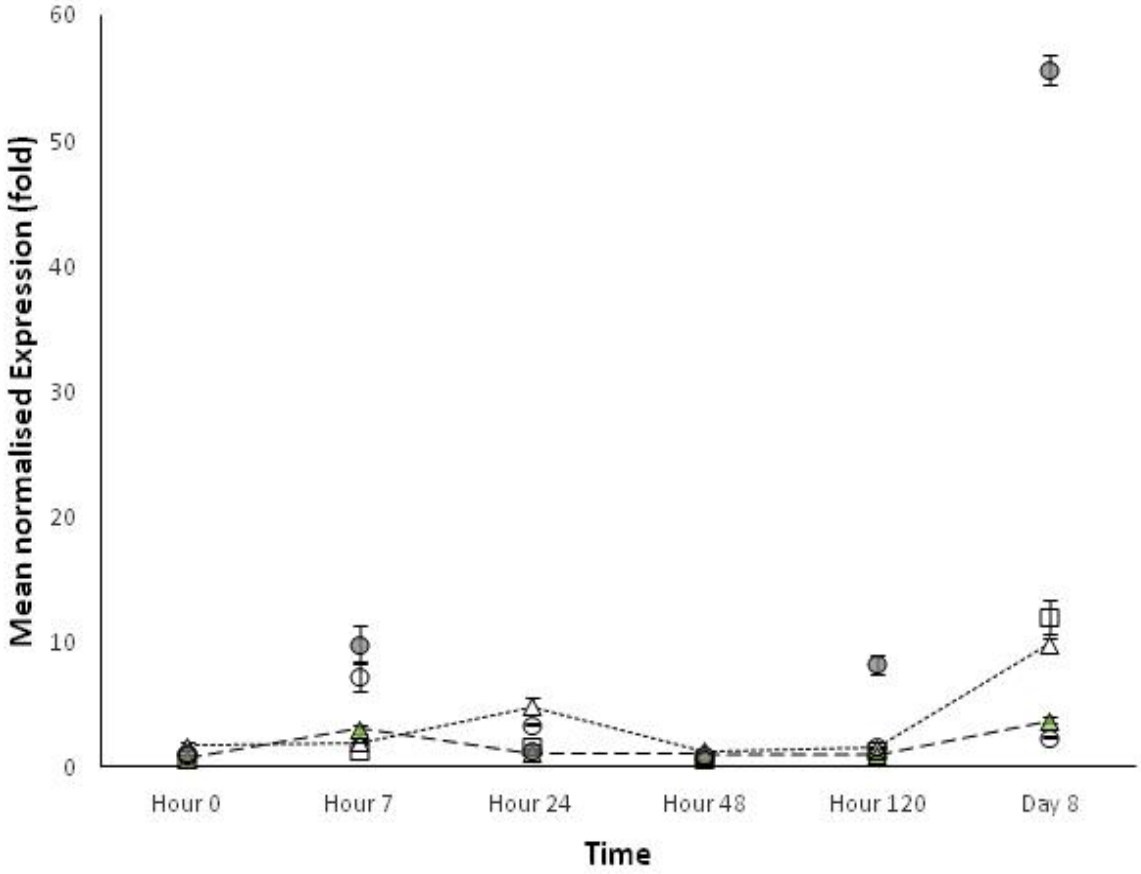

Figure 13. PR-4 expression kinetics in tomato leaves after root application of: PAW-R (empty triangles), PAW-RI (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the PR-4 induction at each time point and the standard error (±SE).

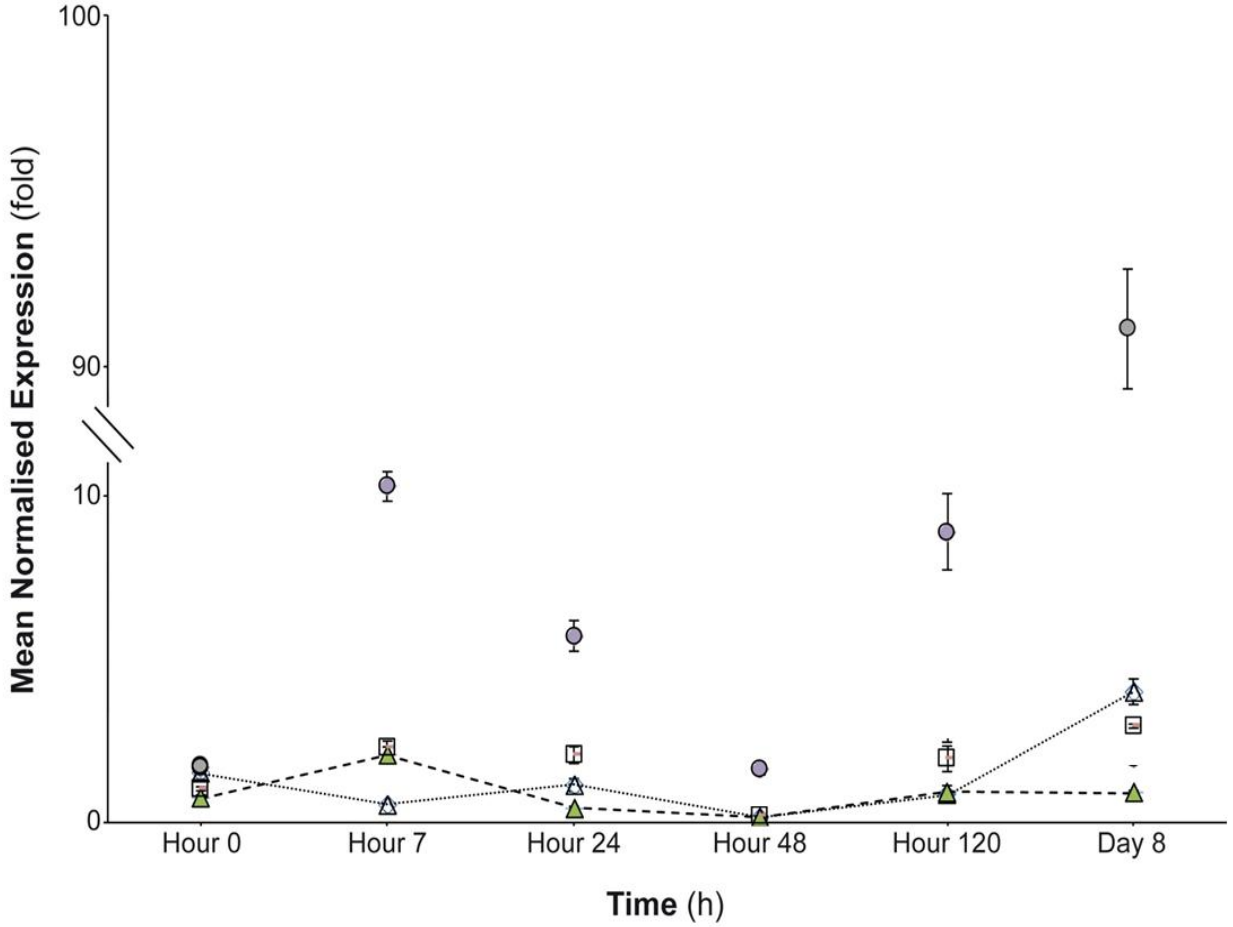

Figure 14. PR-5 expression kinetics in tomato leaves after root application of: PAW-R (empty triangles), PAW-RI (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the PR-5 induction at each time point and the standard error (±SE).

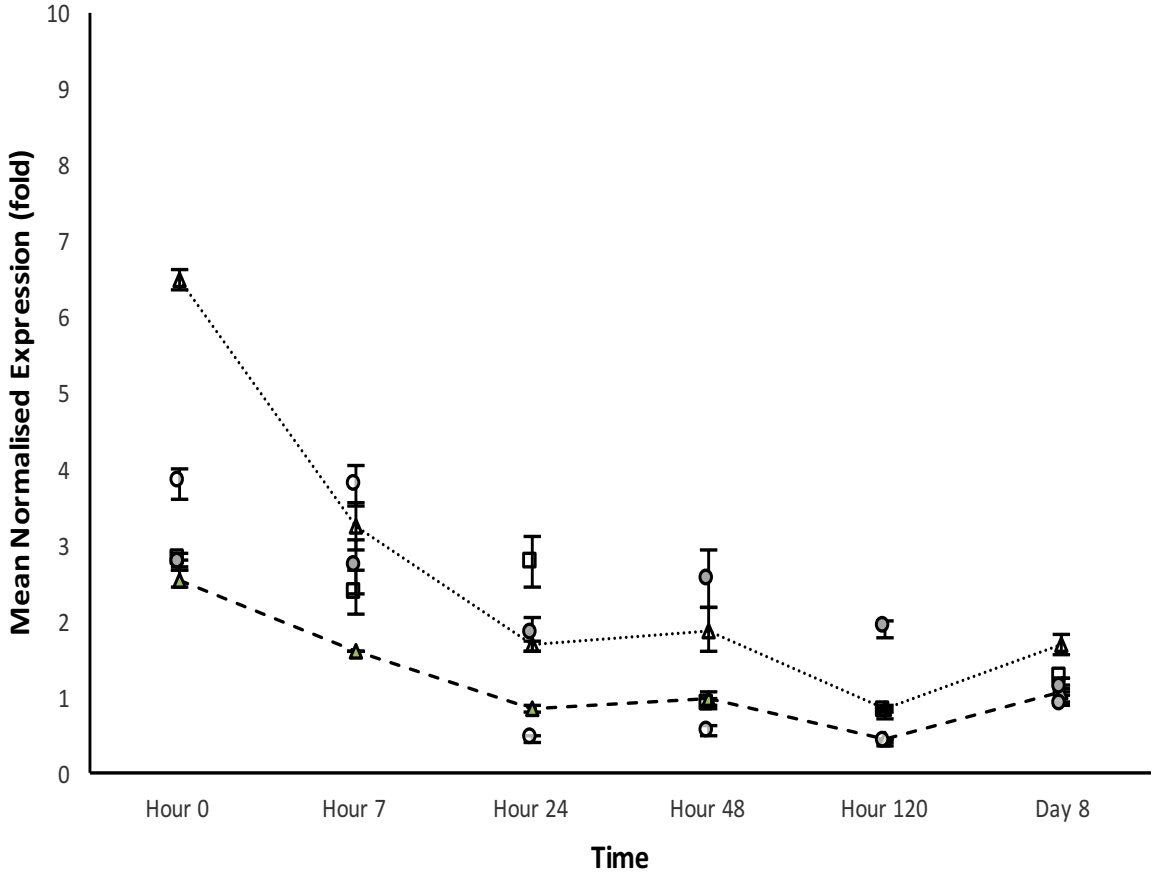

Figure 15. LOXF expression kinetics in tomato leaves after root application of: PAW-R (empty triangles), PAW-RI (filled triangles), SDW-R (negative control, empty squares), ASM (positive control, filled circles), JA (positive control empty circles). The graph shows the LoxF induction at each time point and the standard error (±SE).

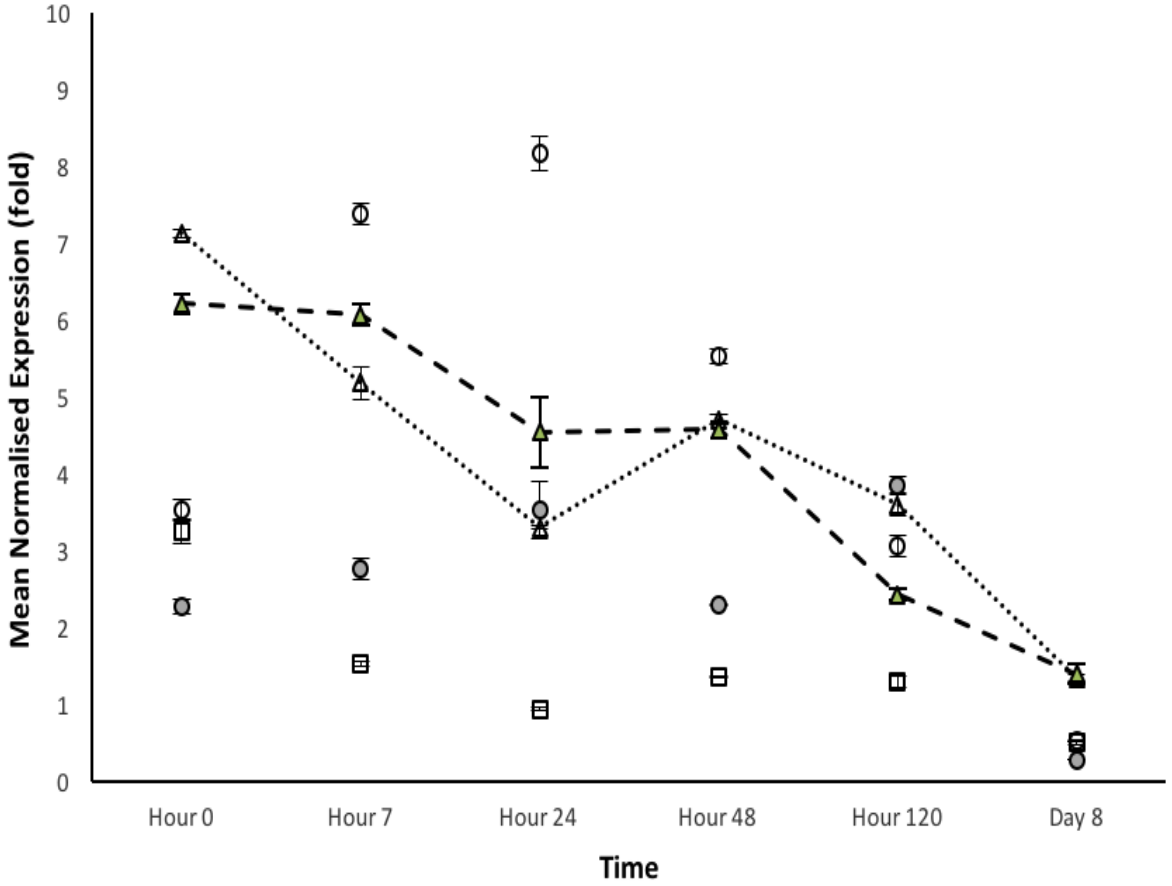

Figure 16. PAL expression kinetics in tomato leaves after root application of: PAW-R (empty triangles), PAW-RI (filled triangles), SDW-R (negative control, empty squares), ASM (positive control, filled circles), JA (positive control empty circles). The graph shows the PAL induction at each time point and the standard error (±SE).

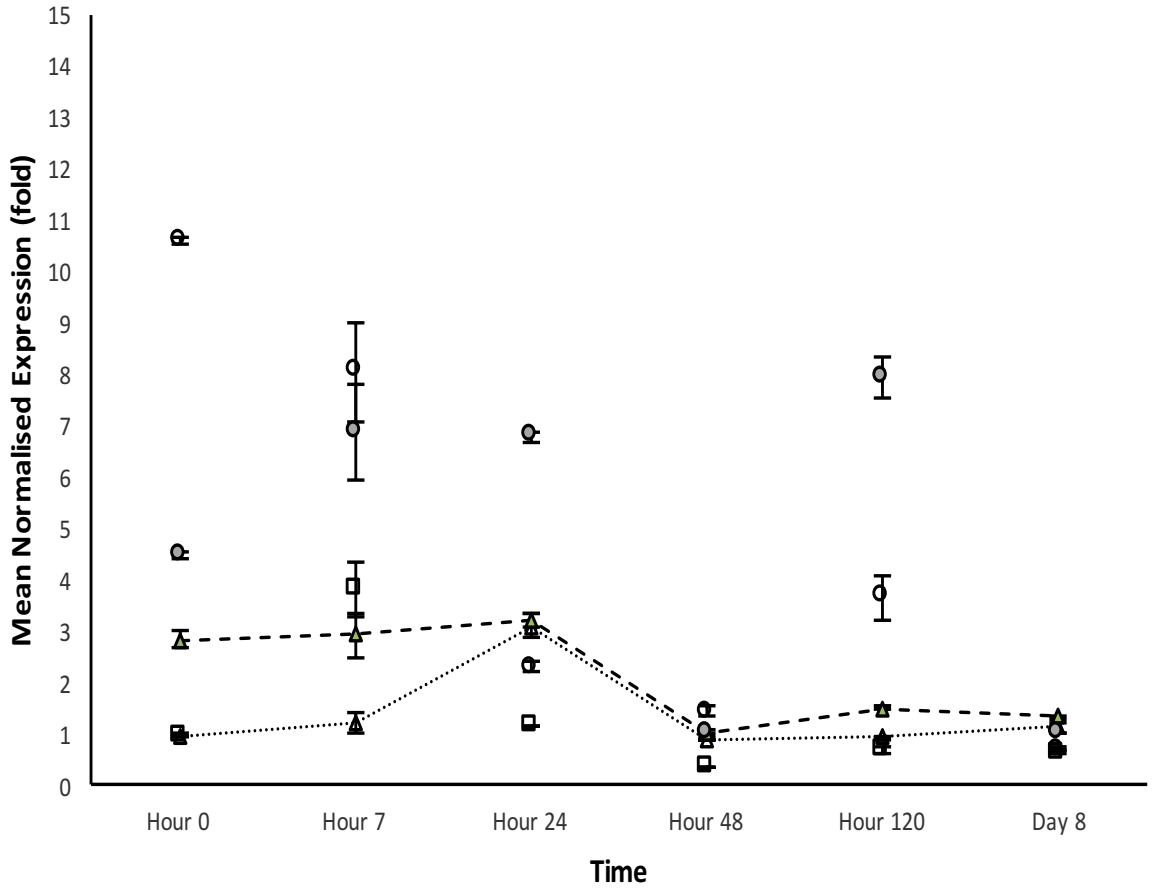

Figure 17. ERF1 expression kinetics in tomato leaves after root application of: PAW-R (empty triangles), PAW-RI (filled triangles), SDW-R (negative control, empty squares), ASM (positive control, filled circles), JA (positive control empty circles). The graph shows the ERF1 induction at each time point and the standard error (±SE).

Tomato plants treated with SDW-R did not show a relevant induction of PAL and LOXF in the considered time intervals with the only exception of PAL (at 0 h) and LOXF (from 0 h to 24 h) whose expression was calculated as approx. 3-fold. The amount of ERF1 RNA transcripts was *ca.* 4-fold at 7th hour from the root treatment; in the rest of time points the expression was close to the baseline (Figures 15, 16 and 17). These results were expected because of the wounds produced by the treatment method, as for PR-1a.

The cv. Moneymaker plants treated with PAW-R and PAW-RI triggered the expression of PR-1a since the beginning: at time 0 h, in fact, the amount of *m*RNA was approx. 26- and 19-fold, respectively, significantly higher than that of the negative control plants (SDW-R). The transcript abundance level was also significant when comparing, at the same time, to ASM (*ca.* 10.6-fold) and JA (*ca.* 6.5-fold). The induction of PR-1a from 7 h to 48 h after both PAW treatments was not different to those plants drenched in SDW; at 120 h the expression of PR-1a increased to *ca.* 4-fold. Finally, at 8 d, PAW-R treated plants highlighted a significant increase of PR-1a induction (*ca.* 20-fold), with respect to that of negative control (SDW-R, *ca.* 12-fold), but also significantly lower than that of ASM-R treated plants (approx. 227-fold). These data could be explained by the different components of PAW (*i.e.* H_2O_2). Hydrogen peroxide, in fact, is a diffusible molecule and it is involved in the regulation of several defence- and pathogenesis-related genes, in particular *pr1a* (Kuhn *et al.,* 1989; Desikan *et al*., 1998). The accumulation of H_2O_2 can lead to salycilic acid (SA) synthesis, which in turn, can stabilize H_2O_2 by inhibiting catalase activity (Leon *et al*., 1995). Elevated levels of SA along with H_2O_2 can activate the local PR-1a expression and can serve as a systemic signal to activate the SAR response that is also expressed in leaf tissues (Devadas *et al*., 2002). Unexpectedly, PAW-RI treated plants showed an induction decrease at 8 d (approx. 3.5-fold) (Figure 12). Referring to PR-4 and PR-5 gene expression kinetics in PAW-R and PAW-RI treated plants, no significant increase was detected up to 8 d after treatments (Figures 13 and 14).

More interesting results were obtained from the PAL and LOXF expression analyses of plants treated with both PAW applications: at 0 h the PAL and LOXF induction was significantly higher than that of the positive controls (ASM-R and JA, Figures 15 and 16), and this can be justified by the presence of hydrogen peroxide in the water activated with plasma (Laurita *et al*., 2015). The expression levels of these two genes was significantly higher than that observed in plants treated with SDW-R (negative control), however it

constantly decreased up to 8 d after treatment remaining, in some cases significantly lower, and in other cases similar/higher to the induction level observed in the positive controls (ASM-R and JA). PAL and LOXF are known to be involved in the defence responses against several pests/pathogens (Peng *et al.,* 2005; Mariutto *et al*., 2011) and abiotic stresses (Kuzniak and Urbanek, 2000; Dat *et al*., 2000; Miller *et al*., 2009); in particular, PAL, whose expression levels early occurred in this study on PAW treatments (from 0 h to 48 h), is known as a key factor in tomato plant defence as a regulatory enzyme in the phenylpropanoid pathway, leading to the production of phytoalexins and phenolic substances (Pellegrini *et al.,* 1994; Polle *et al.,* 1994; Guo and Wang, 2009). The early increase of PAL expression may be also explained considering that ROS – contained in PAW - are involved in signal transduction pathways triggered during stress responses (Willekens *et al.,* 1995; Alvarez *et al*., 1998; Kuzniak and Urbanek, 2000; Dat *et al*., 2000; Miller *et al*., 2008; Miller *et al*., 2009; Bhattacharjee, 2012; Suzuki *et al*., 2013) within some minutes to hours/days (Soares *et al*., 2009; Mittler *et al*., 2011). In addition, PAL induction, involved in the pre-infection resistance, could be considered one of the first barriers used by plants to contrast the bacterial penetration; moreover, the study by Kavitha and Umesha (2008) demonstrated that PAL expression is upregulated in cultivars resistant to bacterial leaf spot in tomato.

ERF1 tomato expression levels at 24 h after both PAW-R and PAW-RI treatments resulted significantly higher (approx. 3.1- and 3.2-fold, respectively) than those of plants treated with SDW-R and JA (*ca.* 1.1-fold, and 2.3-fold, respectively), and significantly lower than those of plants treated using ASM (approx. 6.8-fold) (Figure 17). At time points 0, 48, 120 h and 8 d the relative transcript abundance of PAW treated plants resulted upregulated with respect of that of negative control (SDW-R). The only exception was represented by *m*RNA abundance in plants at 7 h after water treatment that is in agreement with the known role of *erf1* gene shown to be upregulated by wounds (Tournier *et al.,* 2003). The plant response, in fact, that was recorded at 7 h after SDW application at the root apparatus (*ca.* 4-fold) - by the plant explants and the subsequent drenching - was indeed explained by this mechanism. The ERF1 expression plants treated with PAW was significantly lower than those treated with ASM and JA.

In conclusion, PAW was not able to directly inhibit the growth of Xv strain IPV-BO 2684 when tested in *in vitro* experiments using diffusion and dilution methods (according to NCSL standard methods). However, when PAW was tested in *in vivo* experiments, by treating tomato plants at the roots to study its ability to induce plant defences against Xv, it reduced the disease severity (bacterial leaf spot of tomato) giving a relative protection that ranged between approx. 30 and 38%.

The subsequent transcriptomics study to verify the activation of 6 selected genes, selected among those that are specific markers of salicylic acid and jasmonic acid/ethylene pathways, it demonstrated an early upregulation of PAL and LOXF expression that was observed from the beginning of the experiment. This expression decreased up to the 8th day from the root treatment, but it remained significantly higher than that of the negative control. Moreover, the PR-1a (Ciardi *et al.*, 2000; 2001), PR-4 and PR-5 expression data resulted highly variable and therefore very likely that are not involved in the reduction of bacterial leaf spot severity.

The defence response represented by the expression increase of *pal* gene is involved in the phenylpropanoid pathway, and therefore in the production of lignin and some phytoalexins (Dixon *et al.*, 1995); in these experiments this response was related to the reduction of disease severity caused by the bacterial pathogen Xv. The slight effect of relative protection in combination to the PAL and LOXF expression kinetics study, suggested that the key factor for the disease severity inhibition might be the time of PAW application before pathogen inoculation. Further experiments will be also needed to assay the efficacy of PAW against Xv when treated at leaves. This will allow to evaluate the expression of selected defence genes involved in the local acquired resistance (LAR) and this, if confirmed, can open new friendly application ways to protect tomato plants from this bacterial disease.

Chapter II

EFFICACY OF THE BIOSTIMULANT V 3-2435 AGAINST DIFFERENT BACTERIAL PATHOGENS

1. INTRODUCTION

1.1 Bacterial leaf spot of tomato and pepper

Bacterial leaf spot of tomato (BLST), caused by different species of *Xanthomonas* was firstly observed in 1914 in South Africa (Doidge, 1921) and it has then worldwide spread in all the countries where pepper and tomato are cultivated (Quezado-Duval *et al.*, 2003; Jones *et al.*, 2004; Moretti *et al.*, 2009; EPPO, 2013); indeed, it is a very important disease in the seed market (Giovanardi *et al.*, 2015). The symptoms occur on leaves, stems, and fruits; leaf lesions (spots) may be dark green (water-soaked) and become later dark brown. Long-distance dissemination of those xanthomonads is ensured by contaminated seed trading (Carmo *et al.*, 2001).

1.2 Bacterial canker of kiwifruit

Pseudomonas syringae pv. *actinidiae* (Psa) is the causal agent of kiwifruit bacterial canker; it was isolated for the first time in 1984 in Japan (Serizawa *et al.*, 1989; Takikawa *et al.*, 1989). Almost two decades after the first disease appearance, it became pandemic and it was threatening the sustainability of the kiwi industry in all the major kiwi-producing countries such as China, Italy, New Zealand, Chile and Greece (FAO, 2016; Ferguson, 2015). Psa symptoms on leaves can be recognized by dark brown spots surrounded by yellow haloes; cankers with reddish exudates on twigs, leaders and trunks, and the collapse of fruits can also be observed (Serizawa *et al.*, 1989; Balestra *et al.*, 2009; Abelleira *et al.*, 2011; EPPO, 2014).

1.3 Bacterial disease control

The European Union (EU), United States of America (USA) and different other countries have undertaken regulatory changes in pesticide registration requirements expecting a half reduction of the existing active ingredients in the early 1990s; thus, compounds are becoming more selective, with a lower intrinsic toxicity and a reduced impact on the environment (Montesinos, 2007). Upon the implementation of the new regulations several pesticides have been excluded and some plant diseases of economic relevance are therefore not well managed. Indeed, the efficacy requirements of the different experimental molecules is needed for both authorisation and registration of the pesticides, but also, to guarantee their effectiveness against pathogens.

Plant protection against bacterial pathogens was mainly based on copper compounds, among them the Bordeaux mixture is the most known and used. The mechanisms of copper ions-mediated antimicrobial activity consist of directly toxicity on bacterial growth (Yuan *et al*., 2010; Liu *et al*., 2015). Moreover, some studies showed that copper might be involved also in regulating the plants defence response (Yeh *et al*., 2003; Sudo *et al*., 2008). Detrimental effects of the copper as environmental contaminants and selection of resistant plant-pathogenic bacterial strains have been reported in several crops (Loper *et al*., 1991; Sundin *et al*., 1993; Graves and Alexander, 2002).

These unfavourable effects could be reduced in a framework of integrated pest management, in which the plant protection is achieved by the combination of different preventive techniques: from prophylaxis through the use of biocontrol agents, resistance inducers and chemicals, to the use of less susceptible cultivars (Lyon and Newton, 1999; Oostendorp *et al*., 2001; Gerhardson, 2002; Bergamaschi *et al*., 2006; Gao *et al*., 2014; Perez *et al*., 2015). Nowadays in the pesticide markets, it is possible to find biological and chemical elicitors that have the ability to induce complex biochemical reactions as local acquired resistance (LAR), systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Terry and Joyce, 2004; Reglinski *et al*., 2011; Rademacher, 2004; Vallad and Goodman, 1998; Thakur and Sohal, 2013). The mode of action of the resistance inducers involves the strengthening of pre-infection, and the expression of post-infection defences, and it is the best

characterized signal pathway for systemically induced resistance, that is represented by the SAR response (Hammerschmidt, 1999; Oostendorp *et al*., 2001).

The exogenous applications of chemical resistance inducers, whose major characteristic is the lack of a direct pathogen inhibition, can elicit, at very low concentration a defence response similar to the one elicited by plant pathogens (Kessman *et al*., 1994). In particular, Acibenzolar-S-metyl (ASM) the active principle of the well known resistance inducers as BION® or Actigard®, belonging to benzothiadiazoles (BTHs) class (Kunz *et al*., 1997), satisfies these requirements, conferring a plant-mediated resistance to a broad spectrum of bacterial pathogens.

Control methods of BLST include field rotation, use of pathogen-free transplants, seed sanitation practices, and application of copper compounds and SAR elicitors. SAR has proven to be effective in tomato against fungal (Benhamou *et al*., 1998), oomycete (Cohen *et al*., 1994), and bacterial diseases (Inbar *et al*., 1998), and has been associated with a cascade of defense reactions including the accumulation of pathogenesis- related (PR) proteins and ultrastructural changes (Benhamou *et al*., 1998; Cohen *et al*., 1994). Moreover, the development of synthetic compounds that elicit plant defense reactions has good potential for a new approach to field management of bacterial diseases (Louws *et al*., 2001).

The current chemical control of Psa is based on bactericidal compounds such as antibiotics and copper formulations; both chemicals, as written above, present issues of phytotoxicity, bacterial resistance and residue in fruits (Cooksey, 1994; Nakajima *et al*., 2002; Han *et al*., 2003; Lee *et al*., 2005; Vanneste *et al*., 2011; Cameron and Sarojini, 2013). A recent study highlighted the ability of the elicitor ASM in reducing the disease severity of experimentally inoculated plants (Cellini *et al*., 2014); other studies, carried out on biocontrol agents such as *Bacillus* spp. or bacteriophages, indicate these as promising sustainable tools to control the Psa (Biondi *et al*., 2012; Di Lallo *et al*., 2014; Donati *et al*., 2014; Frampton *et al*., 2014).

In this work several aims were addressed: i) efficacy evaluation of the biostimulant V 3-2435 in *in vitro* experiments against *Xanthomonas vesicatoria* (Xv) and *Pseudomonas syringae* pv. *actinidiae* (Psa); ii) ability determination of V 3-2435 in reducing the symptoms severity provoked by Xv and and Psa in tomato and kiwifruit

plants; iii) evaluation of tomato resistance induction by V 3-2435 using transcriptomic analyses of 6 genes related to the plant immune system.

2. Materials and Methods

2.1 Experimental biostimulant

Biostimulant V 3-2435 (Valagro®) was provided by Valagro S.p.a. (Piazzano di Atessa, CH, Italy). The compound is under patent and its chemical properties cannot be disseminated.

2.2 Bacterial strains

Bacterial pathogenic strains were obtained from the collection of the Phytobacteriology Laboratory, University of Bologna (Italy) and were used in *in vitro* and *in vivo* experiments, to evaluate the efficacy of the biostimulant V 3-2435. Xv strain IPV-BO 2684 and Psa strain IPV-BO 9312 were routinely grown at 27°C for 48 h on GYCA and NSA (see appendix), respectively.

2.3 *In vitro* experiments

The *in vitro* experiments, using diffusion and dilution methods, were conducted following the procedures described in the Chapter I (Materials and Methods section). All the treatments were shown in Table 1. Sterile deionized water (SDW) and ASM were used as negative controls, streptomycin sulphate was used as positive control. The experiment was repeated 3 times and the data were processed with ANOVA test (p 0.05).

Table 1. Treatments and concentrations used in *in vitro* assays against *Xanthomonas vesicatoria* strain IPV-BO 2684 and *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312.

Treatments	Details	Diffusion method (% v/v, ppm)	Dilution method (% v/v, ppm)
SDW	Sterile deionized water	\	\
V 0.25%$_1$	Biostimulant V 3-2435	2.5 % v/v	0.25 %
V 0.5%	Biostimulant V 3-2435	5.0 % v/v	0.50 %
V 1%	Biostimulant V 3-2435	10.0 % v/v	1.00 %
ASM	BTH 50 WG	75 ppm	75 ppm
Sm	Streptomycin sulphate	10,000 ppm	100 ppm

2.4 *In vivo* experiments

For each pathosystem, experiments were carried out to assay the biostimulant effect directly against bacterial species and its indirect effect through the response mediated by the host.

2.4.1 *In vivo* experiments on tomato plants (direct efficacy against Xv)

Twelve tomato plants per treatment (cv. Perfect peel, 3 plants X 4 replicates) were distributed in randomized blocks; at the 3rd – 4th leaf stage the plants were treated at the leaves using a sprayer. Treatments, concentrations and times of application are shown in Table 2. After treatment application the plants were experimentally inoculated by spraying a water suspension (OD = 0.01_{600nm}; *ca*. 10^7 CFU/mL) containing the Xv strain IPV-BO 2684. The plants were then sealed in polyethylene (PE) bags for 2 days (humid chamber, Figure 1). The greenhouse conditions were set as 16 h light at 30°C and 8 h dark at 24°C until the disease symptom evaluation. Streptomycin sulphate and SDW treated at leaves were used as positive and negative controls, respectively (Table 2).

2.4.2 *In vivo* experiment on tomato plants (plant resistance induction against Xv)

Twelve tomato plants per treatment (cvs. Perfect peel and Moneymaker, 3 plants X 4 replicates) were distributed in randomized blocks; at the 3rd – 4th leaf stage the plants were uprooted and treated by drenching the roots for 10 min into the treatment solution; the plants were then put back into their pots. Treatments, concentrations and times of application are shown in Table 2. After treatments application the plants were experimentally inoculated by spraying a water suspension (OD = 0.01_{600nm}; *ca*. 10^7 CFU/mL) containing the strain Xv IPV-BO 2684. The plants were then sealed in polyethylene (PE) bags for 2 days (humid chamber). The greenhouse conditions were set as 16 h light at 30°C and 8 h dark at 24°C until the disease symptom evaluation. ASM and SDW treated at root apparatus were used as positive and negative controls, respectively (Table 2).

Figure 1. Tomato plants cv. Perfect peel inoculated with *Xanthomonas vesicatoria* strain IPV-BO 2684 under greenhouse conditions: plants sealed in PE bags to promote the water congestion and the penetration of the pathogen.

Table 2. Treatments, concentrations, modes and times of applications before inoculation (BPI) with *Xanthomonas vesicatoria* strain IPV-BO 2684.

	TREATMENTS			
Code	**Details**	**Application type**	**Volume** (mL/plant) **Concentration** (i.a. ppm;% v/v)	**Application timing** (hours or days)
***Experiment on* coverage applications on tomato plant cv. Perfect peel**				
SDW-L	Sterile Distilled Water; Negative Control	Leaf spray	/	24 h BPI
V 0.25%	V 3-2435	Leaf spray	0.25% v/v	24 h BPI
V 0.5%	V 3-2435	Leaf spray	0.5% v/v	24 h BPI
V 1%	V 3-2435	Leaf spray	1% v/v	24 h BPI
Sm	Streptomycin; Positive control	Leaf spray	100 ppm	24 h BPI
***Experiment on* elicitation of defence responses on tomato plant cvs. Perfect peel and Moneymaker**				
SDW-R	Sterile Distilled Water; Negative Control	Drench	500 mL	48 h BPI
V 0.5%	V 3-2435	Drench	0.5% v/v	48 h BPI
ASM	BTH 50 WG; Positive control	Leaf spray	75 ppm	7 d BPI

2.4.3 *In vivo* experiment on kiwifruit plants (direct efficacy against Psa)

Twelve plants per treatment (3 plants of yellow fleshed kiwifruit grown in pot X 4 blocks) were distributed in randomized blocks; at the 5^{th} – 7^{th} leaf stage they were treated at the leaves with a sprayer. Treatments, concentrations and times of application are shown in Table 3. After treatment application the plants were experimentally inoculated by spraying a water suspension (OD = 0.01_{600nm}; *ca*. 10^7 CFU/mL) containing the Psa strain IPV-BO 9312. The plants were then sealed in polyethylene (PE) bags for 2 days (humid chamber, Figure 2). The greenhouse conditions were set as 16 h light at 23°C and 8 h dark at 17°C, maintaining a relative humidity (RH%) of 85 % to 95 % until the disease symptom evaluation. Streptomycin sulphate and SDW treated at leaves were used as positive and negative controls, respectively (Table 3).

2.4.4 *In vivo* experiment on kiwifruit plants (plant resistance induction against Psa)

Twelve plants per treatment (3 plants of *Actinidia chinensis* grown in pot X 4 blocks) were distributed in randomized blocks; at the 5^{th} – 7^{th} leaf stage they were treated by irrigating the pots. After treatment application the plants were experimentally inoculated by spraying a water suspension (OD = 0.01_{600nm}; *ca*. 10^7 CFU/mL) containing the Psa strain IPV-BO 9312. The plants were then sealed in polyethylene (PE) bags for 2 days (humid chamber). The greenhouse conditions were set as 16 h light at 23°C and 8 h dark at 17°C, maintaining a RH% of 85% to 95% until phytopathometric assessments. ASM and SDW treated at the root apparatus by irrigation were used as positive and negative controls, respectively (Table 3).

Figure 2. *Actinidia chinensis* plants experimentally inoculated with *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312 under greenhouse conditions: plants sealed in PE bags to promote the water congestion and the penetration of the pathogen.

Table 3. Treatments, concentrations, modes and times of applications before inoculation (bpi) with *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312.

Code	**TREATMENTS**			
	Details	**Application type**	**Volume** (mL/plant) **Concentration** (i.a. ppm; % v/v)	**Application timing** (hours or days)
Experiment on coverage applications on kiwifruit plants				
SDW-L	Sterile Distilled Water; Negative Control	Leaf spray	/	24 h BPI
V 0.25%	V 3-2435	Leaf spray	0.25% v/v	24 h BPI
V 0.5%	V 3-2435	Leaf spray	0.5% v/v	24 h BPI
V 1%	V 3-2435	Leaf spray	1% v/v	24 h BPI
Sm	Streptomycin sulphate; Positive control	Leaf spray	100 ppm	24 h BPI
Experiment on elicitation of defence responses on kiwifruit plants				
SDW-R	Sterile Distilled Water; Negative Control	Irrigation	500 mL	48 h BPI
V 0.5%-R	V 3-2435	Irrigation	0.5% v/v	48 h BPI
ASM	BTH 50 WG; Positive control	Leaf spray	100 ppm	14 d BPI

2.4.5 Disease assessments, pathogen reisolation and identification, and statistical analysis

For both pathosystems (Xv-tomato and Psa-kiwifruit), the disease severity assessments were carried out 3 weeks after pathogen inoculation by counting the spots on 4 and 10 leaves per tomato and kiwifruit plant, respectively. Selected symptomatic leaf samples were employed for the isolation and the identification of the pathogen by microbiological and molecular analyses.

2.4.6 Pathogen isolation and identification

Selected symptomatic tomato and kiwifruit leaf samples were used for Xv and Psa isolation and identification, respectively. Leaf surface was sterilized with 2% sodium hypochlorite. Necrotic lesions were aseptically collected and crushed into a mortar with 2 mL of SDW; 30 μL of the extract was plated on GYCA and NSA media (to isolate Xv and Psa, respectively); the plates were incubated up to 48-72 h. Xv-like and Psa-like colonies were subcultured and identified with molecular assays (Koenraadt *et al.*, 2009; Biondi *et al.*, 2013; EPPO, 2013; EPPO Bulletin, 2014).

2.5 Gene expression in tomato plants

2.5.1 Treatments and sample collection

Under controlled conditions, tomato plants (cv. Moneymaker, 2 plants x 2 blocks/treatment) at the 3rd-4th leaf stage were uprooted and treated (all treatments are shown in Table 4) by root drenching, after treatment the plants were put back into their pots. ASM and JA treatments (positive controls) were separated about 60 cm from the rest of the plants, to prevent influences by volatile gas spreading (Reinbothe *et al.*, 1994; Cellini *et al.*, 2015). Negative controls consisted in non-treated (NT) tomato plants and treated by drenching the root apparatus in sterile distilled water (SDW-R). Apical tomato leaves were collected at six time-points: 0, 7, 24, 48, 120 h and 8 d. The tissues were flash frozen in liquid nitrogen and stored at -80°C until the RNA extraction. The greenhouse conditions were set as 16 h light at 25°C and 8 h dark at 19°C, maintaining a RH% of approx. 85% until the last day of leaf collection.

Table 4. Treatments carried out under greenhouse conditions on tomato plants cv. Moneymaker for evaluation of defence related genes expression.

Code	TREATMENTS		
	Details	**Application Type**	**Volume** (mL/treatment) **Concentration** (i.a. ppm; % v/v; mM)
SDW-R	Sterile Distilled Water Negative Control	Drench	500 mL
V 0.5-R	V 3-2435	Drench	500 mL, 0.5% v/v
JA	Jasmonic Acid; Sigma cod. J2500	Drench	500 mL, a.i 100 mM
ASM-R	BTH 50 WG; Syngenta	Drench	500 mL, a.i. 75 ppm
NT	Non treated	/	/

(a.i.) active ingredient.

2.5.2 RNA extraction, reference/target gene sequencing, primer design and RT-qPCR assays

The RNA extraction, reference/target gene sequencing, and primer design, were carried out in tomato plants following the methods mentioned above (Chapter I, section "Gene expression analysis in tomato plants"). The evaluation of the gene expression with RT-qPCR was realized through the one-step method applying SYBR Green/ROX chemistry, the expression of *pr1*a, *pr4*, *pr5*, *pal*, *loxf* and *erf1* target genes was evaluated and normalized to the endogenous control *β-actin* following the methods described in Chapter I (RT-qPCR primer list is shown in Table 5, Chapter I).

3. RESULTS AND DISCUSSION

In *in vitro* experiments, the Valagro S.p.a. product V 3-2435 did not show any effect against Xv nor Psa (Table 5) at any concentration when tested with diffusion method, although the concentrations used were 10 times higher than those of the dilution method. ASM and SDW (negative controls) were not effective. Streptomycin sulphate inhibited the bacterial pathogens: inhibition halo diameters were approx. 2 and 2.5 cm for Xv and Psa, respectively.

Table 5. Effectiveness *in vitro* (diffusion method, LB-agar) of the biostimulant V 3-2435 against of Xv and Psa (mean of 3 replicates).

Treatments	**Concentration Active Ingredient** (% v/v, ppm)	***Xanthomonas vesicatoria*** IPV-BO 2684	***Pseudomonas syringae* pv. *actinidiae*** IPV-BO 9312
SDW	/	-	-
V 0.25%	2.5% v/v	-	-
V 0.5%	5.0% v/v	-	-
V 1%	10% v/v	-	-
ASM	75 ppm	-	-
Sm	10,000 ppm	+	+

(-) not inhibited growth, **(+)** inhibited growth

When V 3-2435 was assayed against Xv with dilution method (Figure 3), it showed an increasing of effectiveness in agreement with the increase of the concentration. At 0 h all the pathogen suspensions were *ca*. 10^6 CFU/mL, after 24 h, Xv population in the treatment V 0.25% was one order of magnitude lower (*ca.* 10^8 CFU/mL), when compared to negative controls SDW and ASM (*ca.* 10^9 CFU/mL). The Xv population was *ca.* 10^6 CFU/mL and *ca.* 10^4 CFU/mL when V 3-2435 was applied at 0.5% and 1%, respectively after 24 h. Results on Psa highlighted that this pathogen was more sensitive than Xv to the biostimulant: at 0 h the bacterial population was directly affected (*ca.* 10^5 CFU/mL; Figure 4) by all concentration used. At 24 h the Psa population was lowered to approx. 10^2 CFU/mL by V 0.25% and V 0.5%; at 1% the whole Psa population was eliminated. Streptomycin killed Psa population and lowered the Xv concentration to 10^4 CFU/mL at 0 h; after 24 h the pathogen population was eliminated (Figure 4). Indeed, in *in vitro* experiment, in particular when the dilution

method was applied, V 3-2435 showed a bacteriostatic effect against both Xv and Psa, this, due to the minimal copper percentage present in the biostimulant.

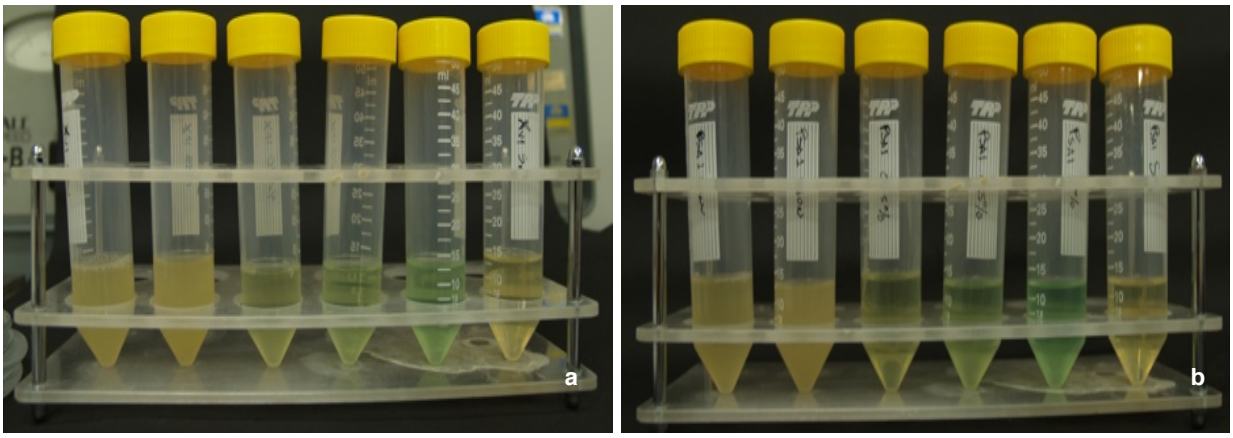

Figure 3. Efficacy *in vitro* (dilution method): a) *Xanthomonas vesicatoria* strain IPV-BO 2684; b) *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312. From the left to the right of each picture SDW, ASM, V 0.25%, V 0.5%, V 1% and Sm treatments.

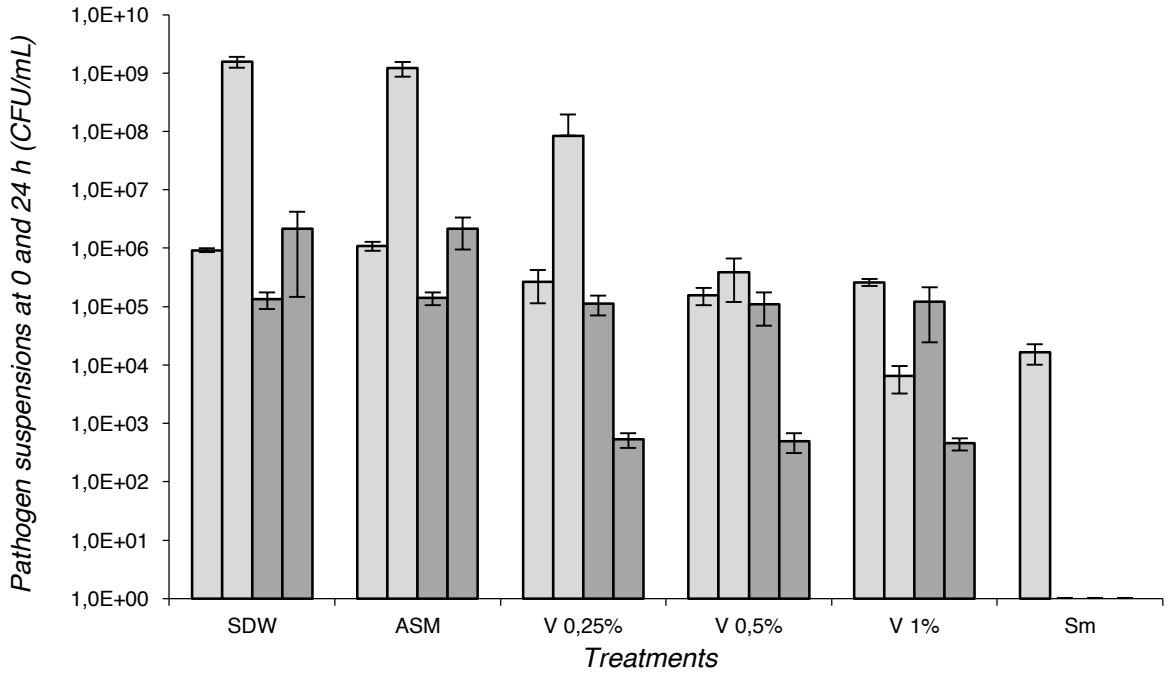

Figure 4. Efficacy *in vivo* (dilution method) of the biostimulant V 3-2435 against Xv (light grey histograms) and Psa (dark grey histograms) populations. The first column of the histogram per each colour represents the population at 0 h, the second at 24 h. Each column represents the mean of 3 replicates and standard deviations (±SD).

When V 3-2435 was applied at tomato leaves to evaluate its direct efficacy against the bacterial strain IPV-BO 2684 of Xv, it significantly reduced the disease severity (mean of number of spots/leaf). The mean of the bacterial leaf spots was approx. 16, 13 and 11 on the tomato plants treated with V 3-2435 at 0.25%, 0.5% and 1%, respectively; while the control plants (SDW-L) showed a significantly higher disease severity (mean of approx. 46 spots/leaf) (Figure 5). No statistical differences in the inhibition of disease severity was evaluated among the different percentages of V 3-2435 applied; moreover, tomato plants treated with streptomycin sulphate showed a disease severity which was significantly similar (approx. 5 spots/leaf) to those treated with the biostimulant (Figure 5).

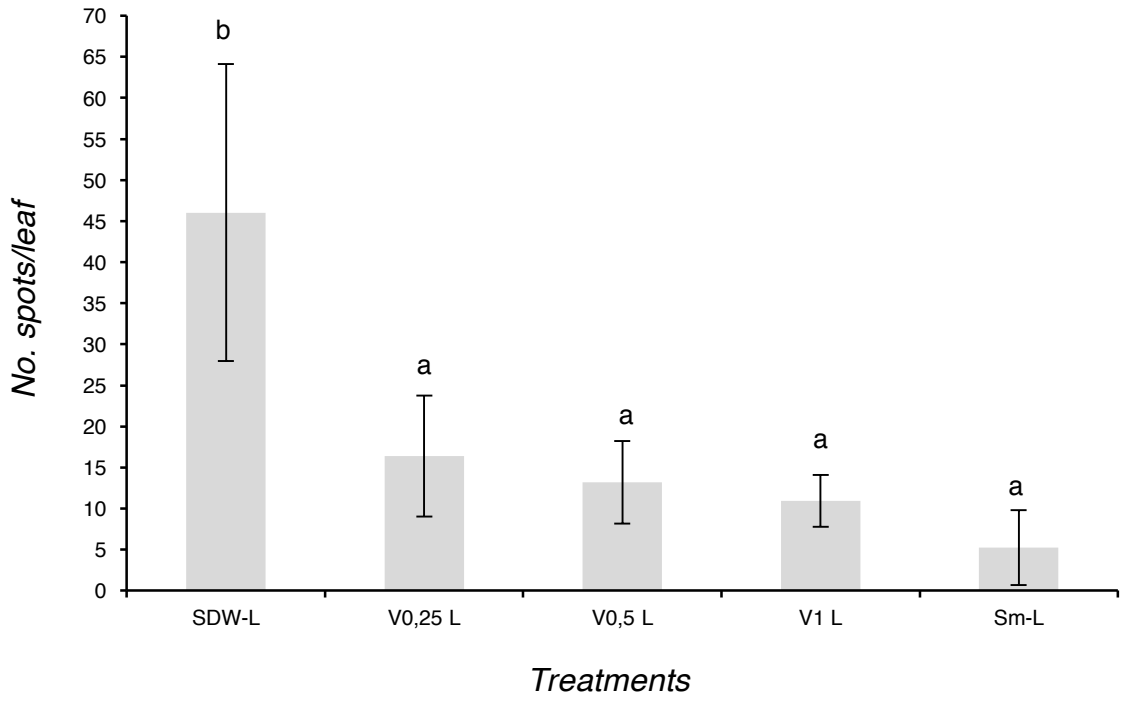

Figure 5. Effectiveness of the biostimulant V 3-2435, compared to negative and positive controls, by coverage applications against *Xanthomonas vesicatoria* strain IPV-BO 2684 inoculated on tomato plants cv. Perfect peel. Different letters indicate significant differences (Tukey's HSD, p 0.05).

The ability of the biostimulant V 3-2435 to directly reduce the disease severity of bacterial leaf spot of kiwifruit caused by Psa was also evaluated in order to verify the *in vivo* efficacy against the pathogen strain IPV-BO 9312. The biostimulant, applied at the kiwifruit leaves at different concentrations (0.25, 0.5 and 1%) reduced the ability of the pathogen in causing disease symptoms; the reduction of the disease severity, in fact, was directly related to the increase of biostimulant concentration (Figure 6). Kiwifruit plants treated with V 3-2435 at 0.5 and 1%, significantly reduced the mean number of spots per leaf (approx. 16 and 5, respectively) compared to water control plants (SDW-L, mean of approx. 62 spots/leaf). On the contrary, when the biostimulant was applied at 0.25%, an apparent disease severity reduction was highlighted (*ca.* 38 spots/leaf) in comparison to that of negative control (SDW-L), but the statistical discrimination between these two treatments was uncertain: this was due to the high data variability of disease severity that was detected during the phytopathometric assessments on the SDW-L treated plants. As expected, the *A. chinensis* plants treated with streptomycin sulphate showed the most significant reduction of disease severity (*ca.* 1 spot/leaf).

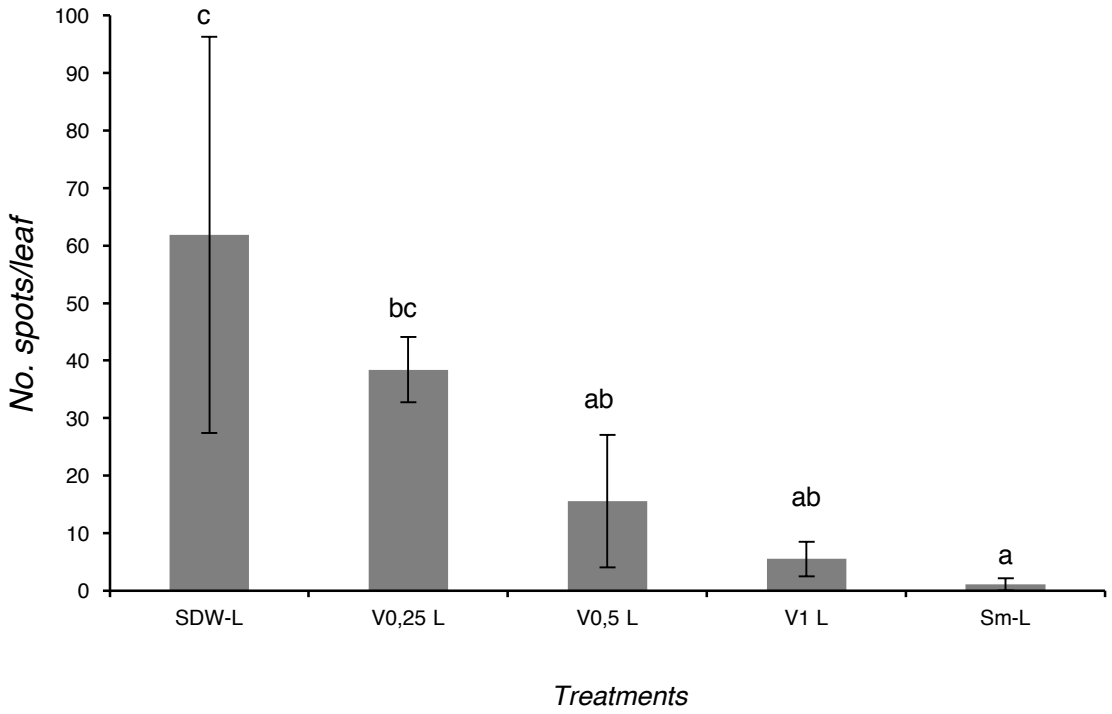

Figure 6. Effectiveness of the biostimulant V 3-2435, compared to negative and positive controls, by coverage applications against *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312 inoculated on *Actinidia chinensis* plants. Different letters indicate significant differences (Tukey's HSD, p 0.05).

These results confirmed the direct efficacy of the biostimulant, as evaluated in *in vitro* assays by dilution method, against both bacterial pathogens: V 3-2435 reduced the disease severity when applied at the leaves 24 h before the experimental inoculation with Xv and Psa (Figures 5 and 6).

The indirect effect of the biostimulant applied at the root apparatus, through the response mediated by the host, was evaluated on the two tomato cvs. Perfect peel and Moneymaker. On cv. Perfect peel (Figure 7), plants treated at the roots with V 3-2435 showed a significantly lower mean number of spots per leaf (approx. 11.5) than that present in the control plants (SDW-R, approx. 23 spots/leaf). In the tomato plants treated with ASM-R (positive control) at the root apparatus the number of leaf spots was approx. 1, significantly lower than that of those treated with SDW-R and the biostimulant (V 0.5-R) applied at the roots as well.

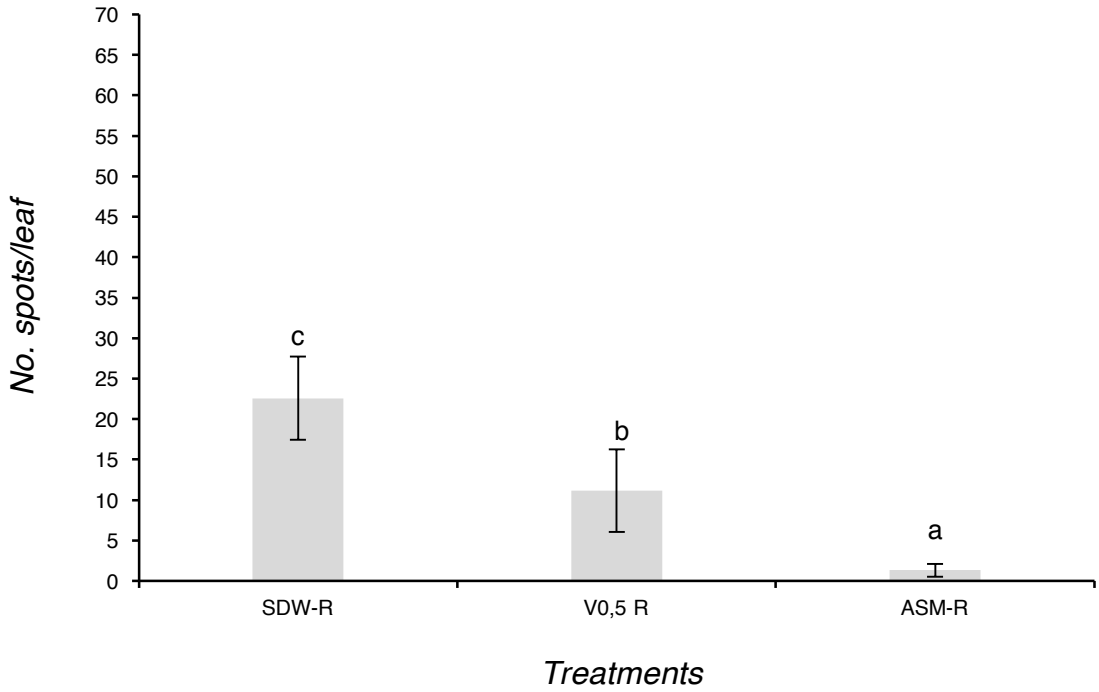

Figure 7. Elicitation of defence responses by the biostimulant V 3-2435 on tomato plants cv. Perfect peel against *Xanthomonas vesicatoria* strain IPV-BO 2684, compared to negative and positive controls. Different letters indicate significant differences (Tukey's HSD, p 0.05).

These results highlighted the ability of V 3-2435 to induce a plant defence response; in fact, when the product was applied at the roots, the reduction of the disease severity (leaf spots) implied a transduction of a signal for a defence response. When the experiment was repeated on cv. Moneymaker, the efficacy of the biostimulant as resistance inducer against bacterial leaf spot was confirmed; the disease severity of plants root treated with V 3-2435 at 0.5% was approx. 10 spots per leaf, significantly lower than that of control plants (SDW-R, approx. 22 spots/leaf) and statistically similar to that of positive control plants treated at the roots with ASM (approx. 3.5 spots per leaf) (Figure 8).

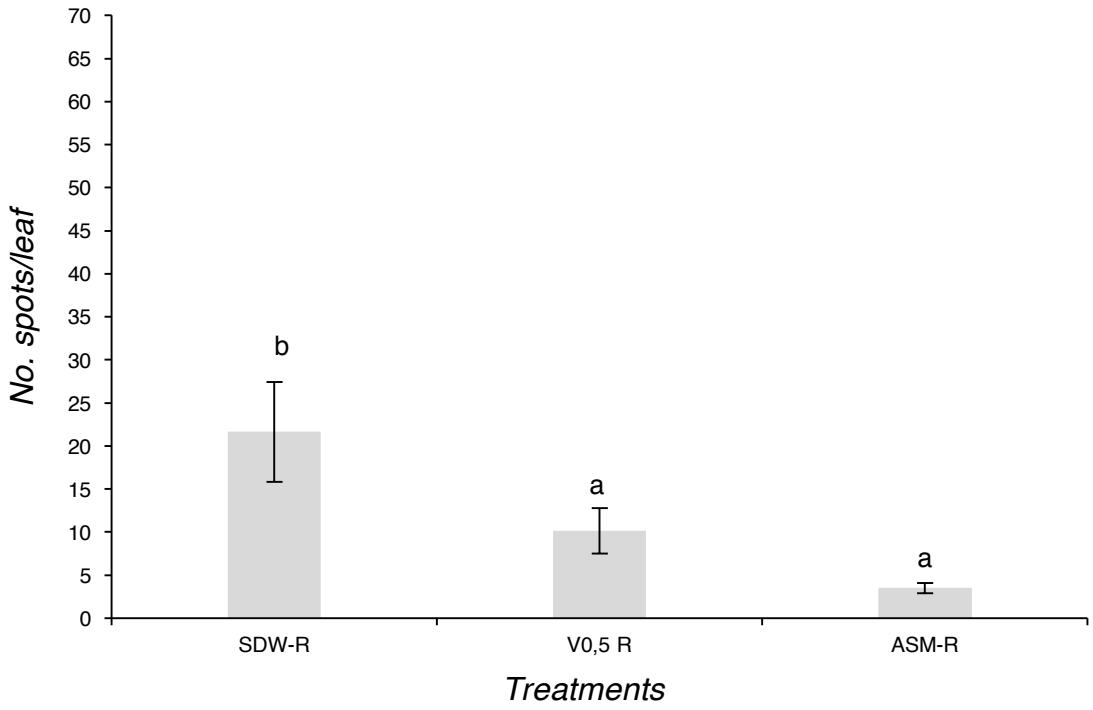

Figure 8. Elicitation of defence responses by the biostimulant V 3-2435 on tomato plants cv. Moneymaker against *Xanthomonas vesicatoria* strain IPV-BO 2684, compared to negative and positive controls. Different letters indicate significant differences among groups (Tukey's HSD, p 0.05).

The ability of V 3-2435 to induce plant defences by irrigating the root apparatus at a concentration of 0.5% was also confirmed in kiwifruit plants (approx. 29 spots/leaf), even though the disease severity was slight statistically reduced, in comparison to the

one of plants treated with water (SDW-R, *ca.* 37 spots/leaf), because of the high variability of phytopathometric data (Figure 9). The kiwifruits treated using ASM (positive control) resulted statistically less diseased (approx. 4 spots/leaf) when compared with those treated with V 3-2435 at 0.5% and with SDW-R; thus, the ability of ASM in reducing symptoms of bacterial canker is in agreement with results by Cellini *et al.* (2014) on green and yellow kiwifruit plants. Moreover, the results obtained in this experiment and shown in Figure 9, remarked the capacity of V 3-2435 to induce a defence response in plants; nevertheless, on kiwifruit-Psa pathosystem, they also highlighted that the method used for treatment application was significantly effective only when ASM, a pure resistance inducer, was applied.

The lower efficacy of the biostimulant - not observed in the tomato-Xv pathosystem - in inhibiting the disease severity through the activation of plant responses, compared to the efficacy of ASM-R control, could be due to the type and volume of treatment application (50 mL), which appeared not sufficient to cover all the volume occupied by the root apparatus.

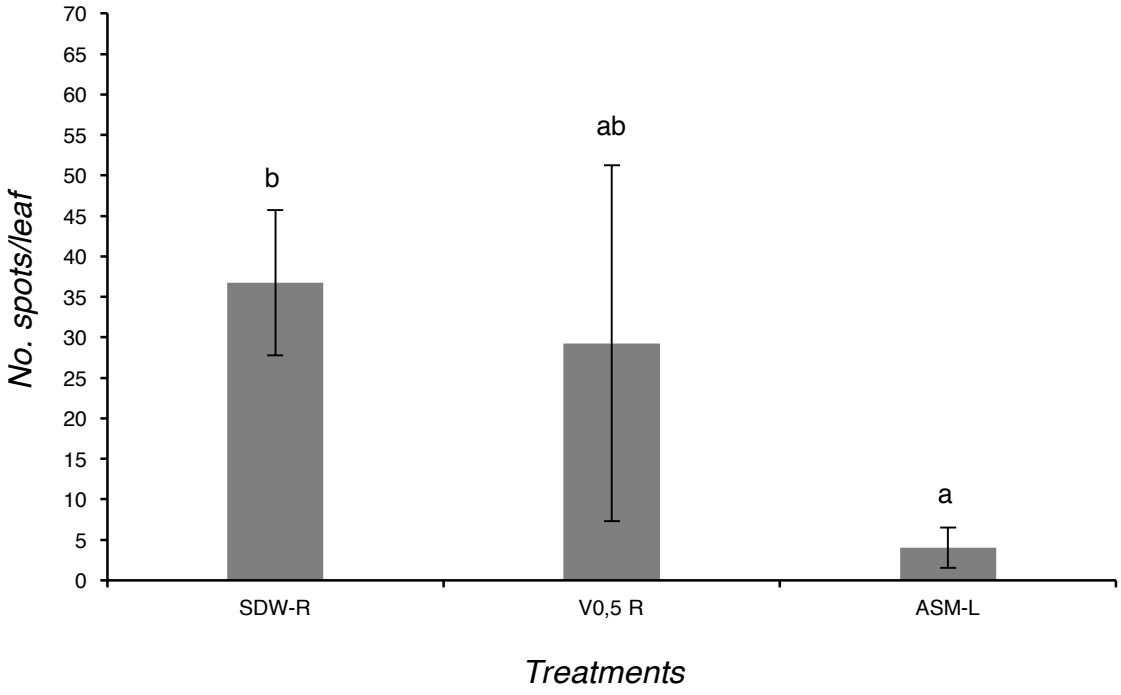

Figure 9. Elicitation of defence responses by the biostimulant V 3-2435 in kiwifruit plants against *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312, compared to negative and positive controls. Different letters indicate significant differences among groups (Tukey's HSD, p 0.05).

Finally, these results highlighted and confirmed the ability of V 3-2435 to induce a plant defence response; in both pathosystems, in fact, using different techniques of application (root drenching and irrigation), the reduction of the disease severity (mean of number of spots per leaf) was demonstrated, and this implies a transduction of a defence response signal.

The ability of the biostimulant to indirectly act against Xv through tomato plants defence responses was confirmed by the transcritpomic analyses; the change of *m*RNA abundance of 6 different genes, selected as markers of SA and JA pathways, was evaluated up to 8 days after root treatment with V 3-2435. In Figure 10 are shown samples of RNA extracts used in the transcriptomic analyses: the quality and quantity of RNA extracts are also reported in Appendix. As reported in Chapter I, *ß-actin* resulted reliable as reference gene: the RNA abundance in V 0.5%-R treatment, in fact, as in the negative (NT and SDW-R) and positive (ASM and JA) control plants, was similar.

The data presented show the change in transcript abundance of the studied genes in V 0.5%-R treatment in comparison to those in SDW-R (negative control treatment) and in ASM-R and JA treatments (positive controls). As in Chapter I, the use of the negative control treatment (SDW-R), whose type of application was similar (*e.g.* tomato plant uprooted and root drenched) to that of the other treatments (V 0.5%-R, ASM-R and JA), allowed to verify the real plant defence response to the biostimulant. Indeed, the SDW-R control was included in the gene expression kinetics to ensure that results accurately reflected the host response only to the treatment.

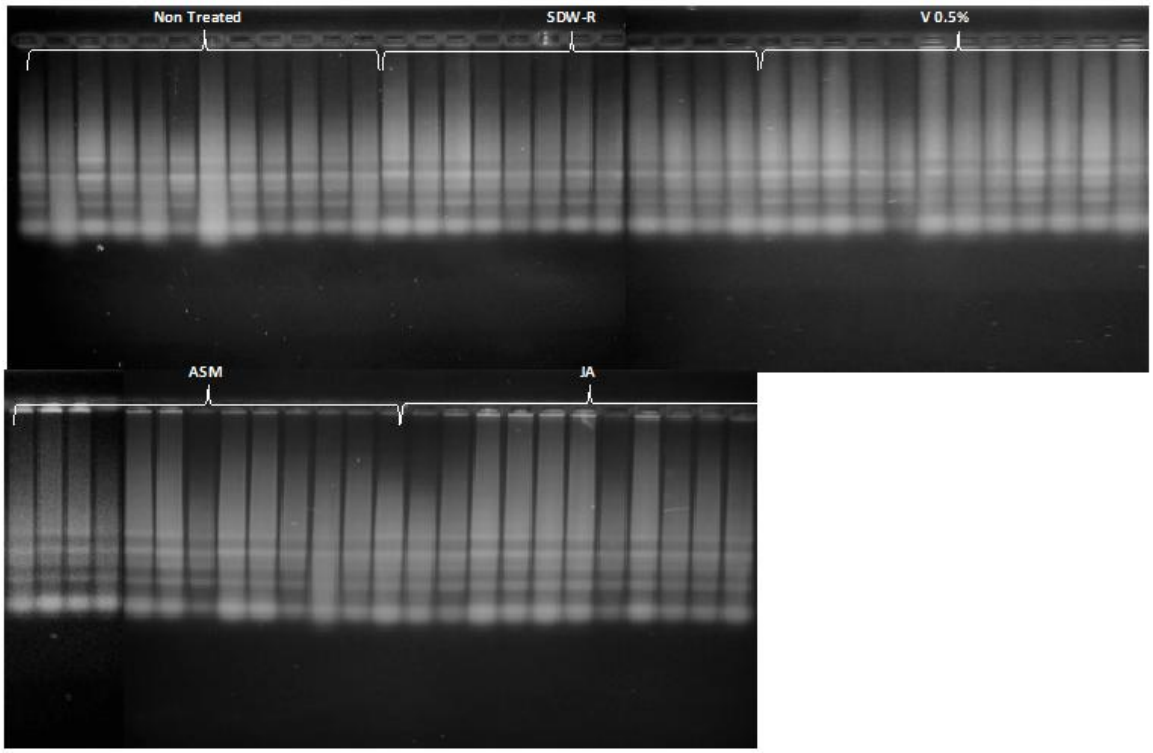

Figure 10. Total RNA quality run in 1.2% agarose gel, extracted from tomato plants.

ASM application (positive control for SAR), as expected (Louws *et al.*, 2001; Graves and Alexander, 2002), provoked the induction of PRs expression in the considered time intervals. PR-1a, PR-5 and PR-4 gene expression kinetics showed a general significant increase of induction in tomato plants up to 8 days after ASM root application (Figures 11, 12 and 13): PR-1a, PR-4 and PR-5 expression levels were approx. 226-, 54- and 90-fold respectively. In the plants treated with JA (positive control for ISR), significant induction of PR-1a, PR-4 and PR-5 were not observed up to the 8th day. Only, a slight expression increase of these three PRs were recorded 7 hours after plant treatment.

After 120 h and 8 d the PR-1a induction was demonstrated, confirming the ability of ASM as an effective elicitor in inducing host resistance (Louws *et al.*, 2001). Such effectiveness was either confirmed by the *in vivo* experiments against bacterial leaf spot: the disease severity reduction was reported above.

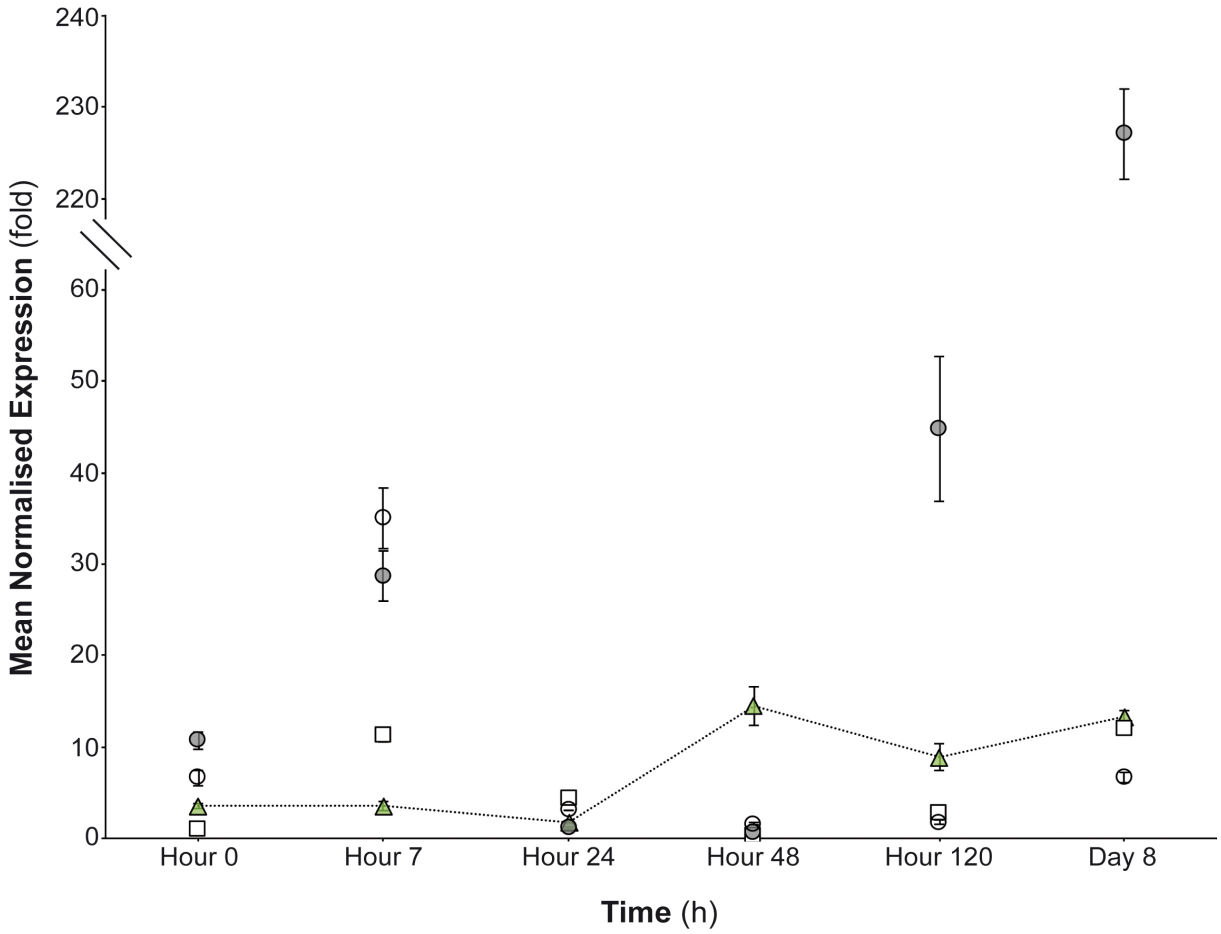

Figure 11. PR-1a expression kinetics in tomato leaves after root application of: V 3-2435 (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the PR-1a induction at each time point and the standard error (±SE).

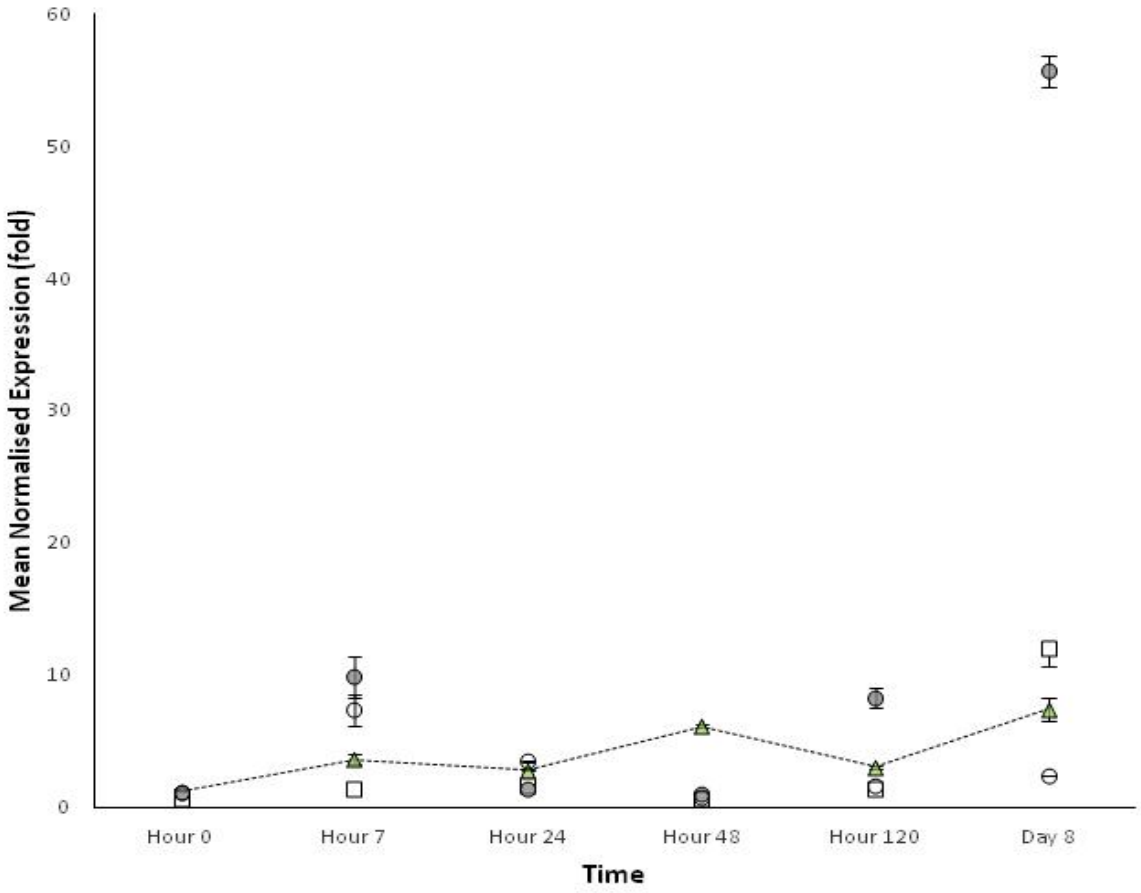

Figure 12. PR-4 expression kinetics in tomato leaves after root application of: V 3-2435 (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the PR-4 induction at each time point and the standard error (±SE).

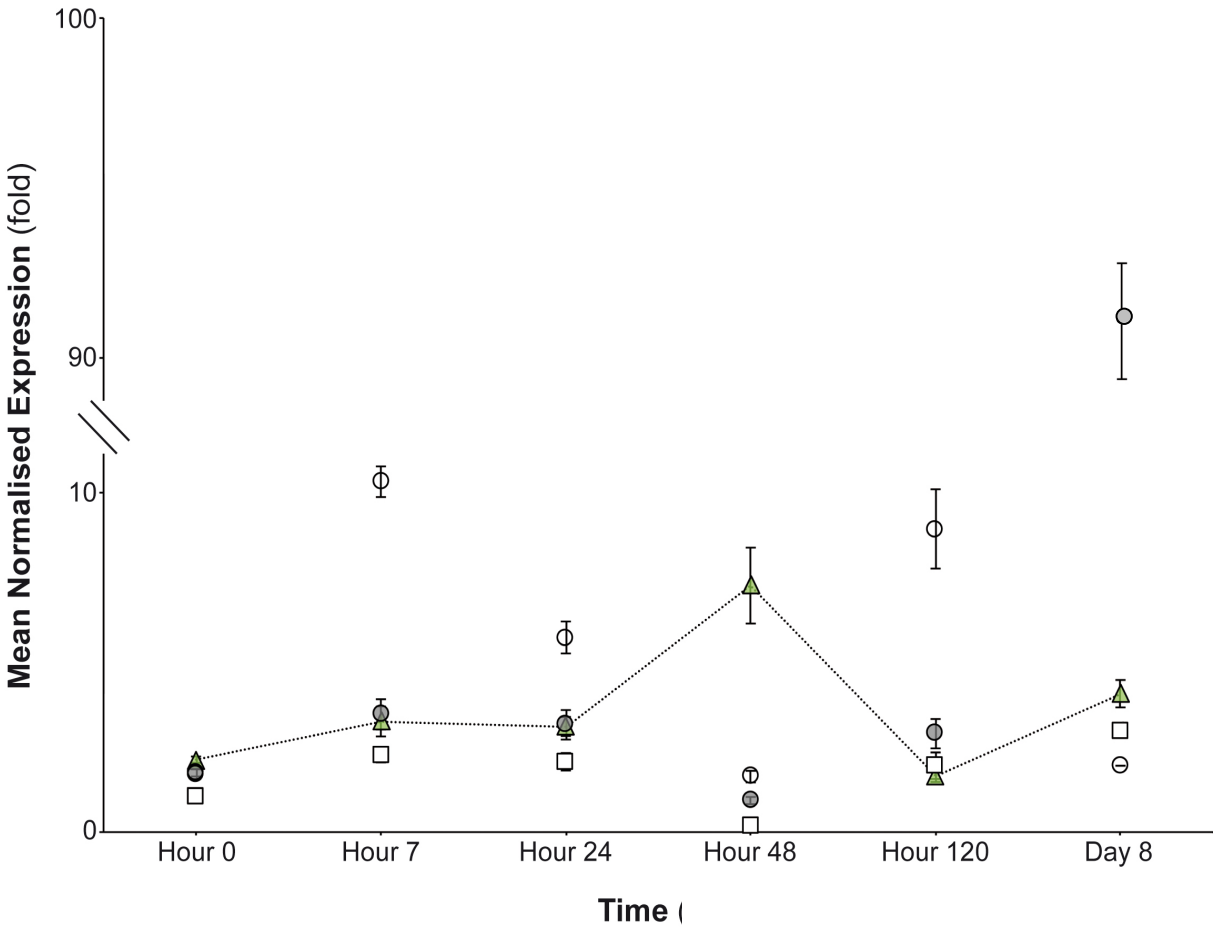

Figure 13. PR-5 expression kinetics in tomato leaves after root application of: V 3-2435 (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the PR-5 induction at each time point and the standard error (±SE).

A different expression pattern was observed for LOXF, PAL and ERF1 (Figures 14, 15 and 16); the plants treated with ASM showed a relevant induction of these genes at 120 h after treatment, but thereafter this induction constantly decreased. At the 8th day the LOXF, PAL and ERF1 expression was close to the baseline.

At 48 h after treatment with the fungicide V 3-2435, the *m*RNA abundance of all the selected genes resulted significantly higher than the one of the negative control (SDW-R). In particular, the PR-1a expression after 48 h from the treatment with the biostimulant was approx. 14.5-fold, while that of ASM-R treated plants was *ca.* 0.6-fold (Figure 11); the induction of PR-4 and PR-5 in the plants treated with V 3-2435, was as well significantly higher (approx. 6- and 7-fold, respectively) than that of those treated with positive controls (ASM-R and JA) (Figures 12 and 13).

Moreover, the expression of PAL in the plants treated with the fungicide was constantly and significantly higher in comparison with the one of the negative controls (SDW-R) up to 120 h after the treatment application. Per each time point up to 48 h, only tomato treated with JA (positive control) showed a significantly higher PAL induction in comparison with those treated with V 3-2435 (Figure 14). This represent an important result, since all the studies on tomato induced resistance remarked the crucial role of PAL expression against bacterial pathogens as already mentioned (Chapter I), the PAL upregulation brings to the phenylpropanoid pathway, and therefore to the production of lignin and phytoalexins (Dixon *et al.*, 1995).

The ERF1 expression, which is involved in the JA/ethylene defence response pathway (Lorenzo *et al.,* 2003; Lorenzo and Solano, 2005), was significantly higher than the one of SDW-R treated plants at 0, 24, 48, 120 h and 8 d time points. Only at 7 h after treatment application, ERF1 expression was statistically lower when compared to the expression of negative control (SDW-R), because the treatment application wounded the root apparatus enhancing the ethylene pathway of resistance responses (Tournier *et al.,* 2003) (Figure 16).

Forty eight hours after the fungicide application, also the higher LOXF expression compared to the expression of plants treated with water (negative control) was significant. As well as PAL, LOXF upregulation seems to be critical in the induced resistance against bacterial pathogens such as *P. s.* pv. *tomato* or Xv (Kavitha and

Umesha, 2008; Liu *et al.,* 2013). The abundance of *loxf* gene transcripts was constantly high per each time point from the beginning (0 h after treatment, approx. 5-fold), but at the 5th and 8th day from the treatment application it rapidly decreased slightly above the baseline.

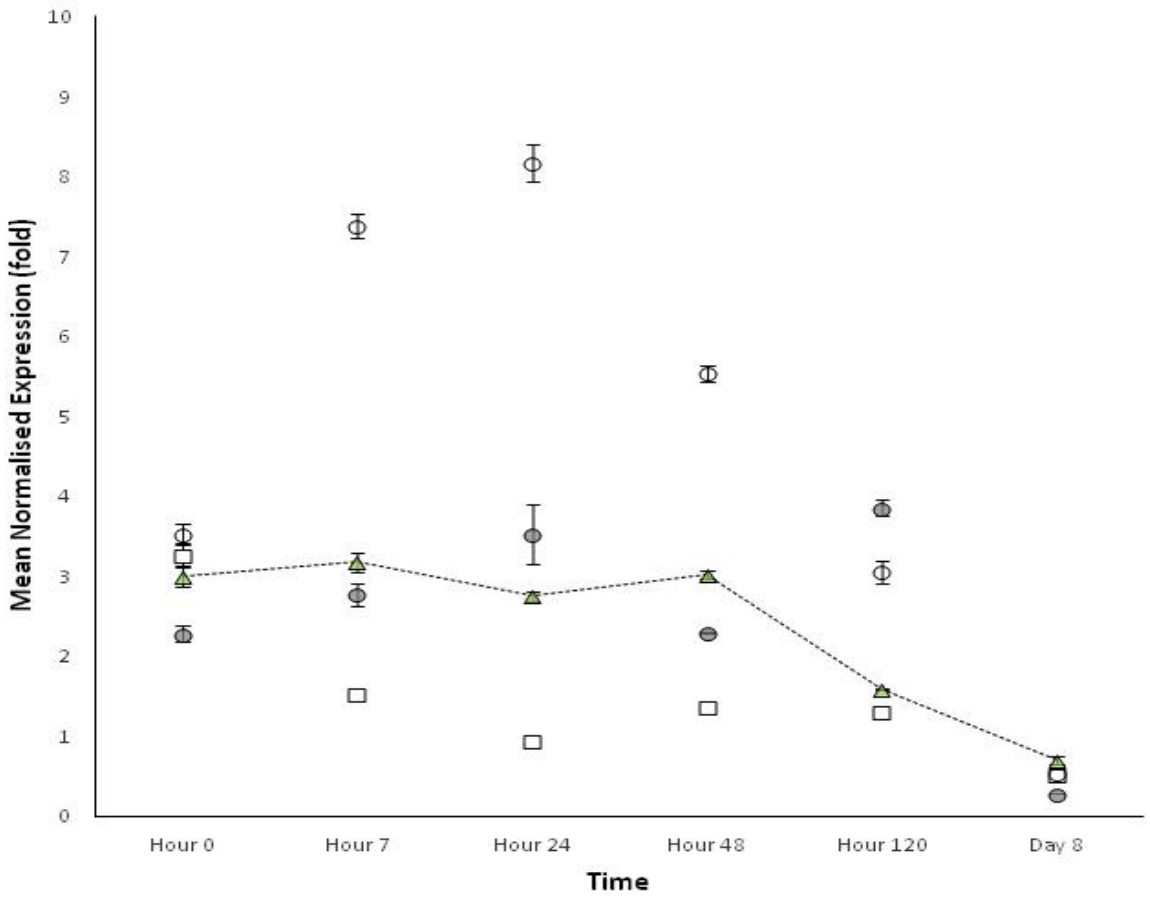

Figure 14. PAL expression kinetics in tomato leaves after root application of: V 3-2435 (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the PAL induction at each time point and the standard error (±SE).

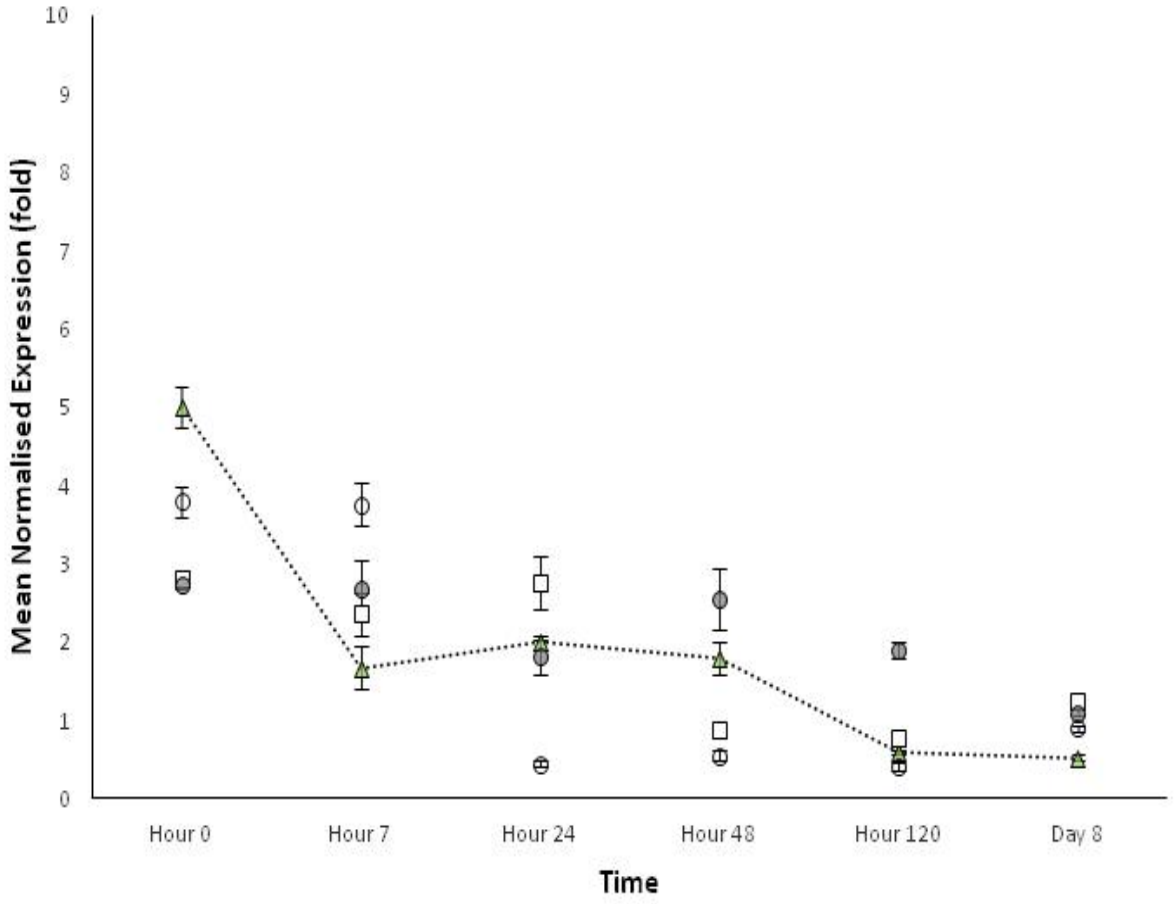

Figure 15. LOXF expression kinetics in tomato leaves after root application of: V 3-2435 (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the LOXF induction at each time point and the standard error (±SE).

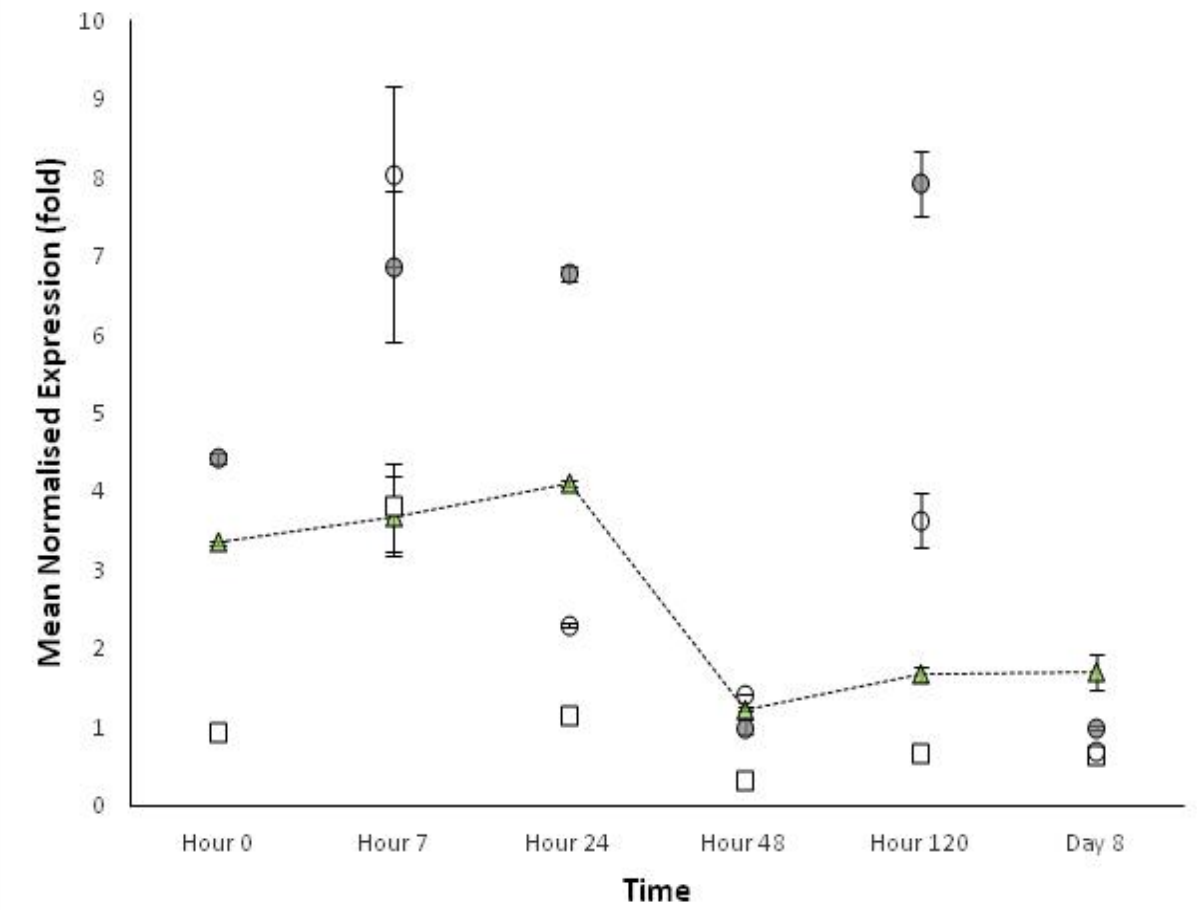

Figure 16. ERF1 expression kinetics in tomato leaves after root application of: V 3-2435 (green filled triangles), SDW-R (negative control, empty squares), ASM (positive control, grey filled circles), JA (positive control empty circles). The graph shows the ERF1 induction at each time point and the standard error (±SE).

In conclusion, V 3-2435 showed direct efficacy against the strains IPV-BO 2684 of Xv and IPV-BO 9312 of Psa when tested in *in vitro* assay using dilution method. This direct effect was confirmed in *in vivo* experiments, carried out under greenhouse conditions, against both bacterial pathogens. The ability of the fungicide in inhibiting bacterial growth (*in vitro*) and reducing disease severity (*in vivo*) of both Xv or Psa was directly related to the treatment concentration.

The product V 3-2435 showed also its capacity to trigger the plant immune system: when the fungicide was applied at the tomato and kiwifruit root apparatus the number of bacterial leaf spots caused by Xv and Psa, respectively, was reduced. Transcriptomic analyses, carried out on tomato plants, showed a general increase in PR-1a, PR-4, PR-5, PAL, LOXF and ERF1 expression at 48 h after root treatment with V 3-2435. It was indeed demonstrated that the compound activated both SAR and ISR pathways whose signal was translocated from the root apparatus to the leaves (Rasmussen *et al.*, 1991; Smith-Becker *et al.*, 1998; van Loon and van Strien, 1999; Chanda *et al.*, 2011; Chaturvedi *et al.*, 2012; Liu *et al.,* 2015). Since not all the selected defence response genes are related to the infection of Xv or other bacterial pathogens of tomato plants (Tieman and Klee, 1999; Ciardi *et al.*, 2000; 2001), the attention was focused to results of PAL expression, which is known to lead the biosynthesis of lignin and phytoalexins (Dixon *et al.*, 1995) and therefore to the reinforcements of pre-infection defences (structural changes that affect the pathogen penetration). In addition, also ERF1 and LOXF expression were upregulated, thus confirming the activation of JA pathway (ISR), which was demonstrated to be the 'first line' of tomato plant defences against bacterial pathogens.

The transcriptomic analyses were highly related to the experiments carried out *in planta* against Xv under greenhouse conditions. PAL, LOX and ERF expression, in fact, were significantly upregulated 48 h after treatment application; in *in vivo* experiments, the pathogen was experimentally inoculated two-days after the root application of the product V 3-2435.

Chapter III

EFFICACY OF *Bacillus* spp. AGAINST BACTERIAL CANKER OF KIWIFRUIT

1. INTRODUCTION

In the last decade, bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), provoked heavy losses calculated around several millions of euros. The pandemic spread of this bacterial pathogen occurred since 2008 and mainly in the species belonging to *A. deliciosa* and mostly to *A. chinensis* (Abelleira *et al.,* 2011; EPPO, 2016). Nowadays, 3 haplotypes, grouped by biochemical, genetic and pathogenicity characteristics, are referred to Psa (Vanneste *et al.,* 2013); in particular, haplotype 3, the most virulent among the reported haplotypes, is the responsible of the current global disease outbreak. Kiwifruit industries around the world are engaged in an effort to co-ordinate disease control strategies represented by the strict orchard hygiene practices, the research on breeding resistant varieties, the use of scheduled bactericidal compounds applications, and of elicitors activating the plant's immune system and, last but not least, the use of biological control agents/compounds (Scortichini *et al.,* 2012). Nevertheless, prophylaxis by diagnostic analyses on asymptomatic plant material is still the most effective method to reduce the number of the primary infection sources (Rees-George *et al*., 2010; Gallelli *et al*., 2013; Biondi *et al*., 2013; Loreti *et al*., 2014).

Chemical control applications against Psa are solely preventive and/or usually applied at early disease stages (Koh *et al.,* 1996; Vanneste *et al.,* 2011). The current chemical control applied in open field is highly dependent on bactericidal or bacteriostatic compounds such as streptomycin and/or copper formulations that are often used as scheduled treatments to prevent the disease (Koh *et al.,* 1996; Nakajima *et al.,* 2002; Lee *et al.,* 2005; Vanneste *et al.,* 2011). However while in Asia and Oceania continents the use of antibiotics to control pathogens in agriculture is allowed, in Europe and in particular, in Italy it is forbidden since 1971, therefore in Europe copper compounds are the most tools employed to control this disease (Balestra and Bovo, 2003; Lee *et al.,* 2005; Vanneste *et al.,* 2011). On the other hand both classes of compounds

present different consequences such as phytotoxicity, bacterial resistance and fruit residues (Goto *et al.,* 2004; Marcelletti *et al.,* 2011; Cameron and Sarojini, 2013).

An integrated approach, involving the use of multiple strategies is required for an effective management of the kiwifruit bacterial canker as, indeed, for the other bacterial diseases: as mentioned above the integrated control often implies the use of biocontrol agents and/or resistance inducers (Dong *et al*., 1999; Reglinski and Elmer, 2011).

The biological control is a preventive technique which utilizes the interactions among the microorganisms to lower the pathogen populations. Therefore, an antagonistic microorganism shall satisfy several characteristics, and must be non pathogenic or toxic for plants, animals, humans nor dangerous for the environment. A beneficial organism should be genetically stable, not demanding in the frame of nutritional needs and, of course, effective against plant pathogens. Usually, the biocontrol agents act directly against the pathogens, by two modes of action: i) production of antibacterial compounds (antibiosis), and ii) nutritional competition by occupying the pathogen penetration sites. Nowadays several bacterial strains are used as fungicides or bactericides, and they are also registered and employed to control different plant diseases. Most of those strains belong to the genus *Bacillus* and *Pseudomonas* whose species or strains are present in the market (De Meyer *et al.,* 1999; McSpadden Gardener and Fravel, 2002; Ryu *et al.,* 2003; McSpadden Gardener and Driks, 2004; Meziane *et al.,* 2005; Borriss, 2011), often registered and used as fungicides or bactericides against bacterial pathogens as *Erwinia amylovora* (Bazzi *et al*., 2006; Biondi *et al*., 2007; Chen *et al*., 2009), *Xanthomonas arboricola* pv. *pruni* (Biondi *et al*., 2006; 2009a) or *P. s.* pv. *tomato* (Fousia *et al*., 2016).

Only preliminary studies were carried out to evaluate the direct efficacy of different bacterial antagonistic species such as *B. subtilis, P. fluorescens* and *Pantoea agglomerans* (Stewart *et al*., 2011) against the bacterial canker of kiwifruit.

On the other hand, the resistance inducers represent an alternative insight in the context of an integrated pathogen control. Nowadays, different types of elicitors are known from both scientific literature and market: they are abiotic, obtained from chemical synthesis (Jakab *et al*., 2001), and biotic (Garcion *et al*., 2007), often molecules extracted from plants or microorganisms (antagonists or pathogens). In

particular, biotic elicitors do not share a common chemical origin, in fact, they belong to different chemical classes such as oligosaccharides, lipids, peptides or proteins. As mentioned in Chapter I, elicitors can induce a plant defense response at very low concentration (*e.g.* nanomoles) (Shibuya and Minami, 2001), being thus discriminated from toxins that has different mechanisms of action at high concentration (Boller, 1995).

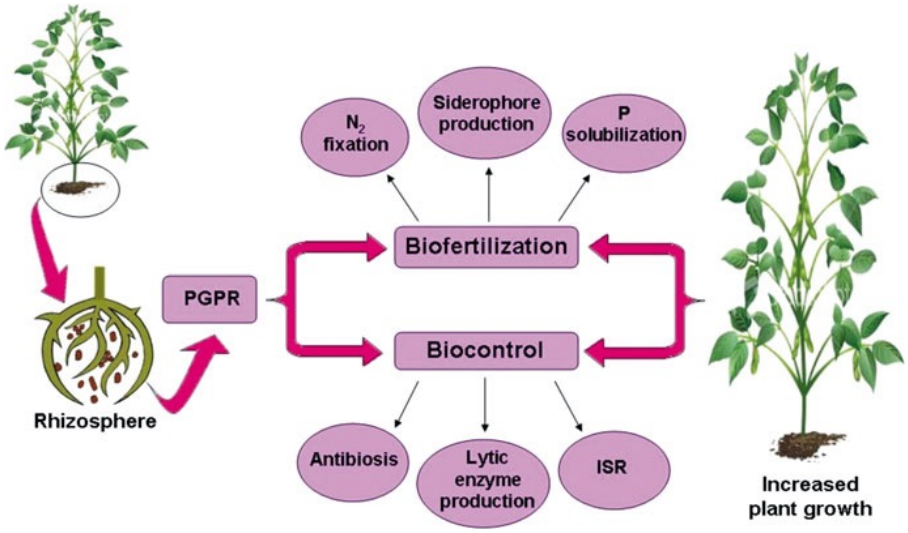

Figure 1. Known mechanisms of plant growth promotion or induced resistance by plant growth promoting rhizobacteria (PGPR) (Kumar *et al.,* 2011).

Beneficial bacteria may also act as biotic elicitors, and among them, rhizobacteria are the most known; they usually live in the rhizosphere feeding with radical exudates, without damaging the plant (symbiosis). Rhizobacteria, in fact, may also be beneficial to the plant stimulating the growth through the synthesis of growth promoting factors such as gibberellins and indole-acetic acid, extracellular phytase, chitinase and antifungal peptides (plant growth promoting rhizobacteria, PGPR), (Turner and Backman, 1991; Idriss *et al.,* 2002; Wang *et al.*, 2002; Pinchuk *et al.*, 2002; Kloepper *et al.*, 2004; Stewart *et al.,* 2011; Lahlali *et al.,* 2013). PGPRs together with different species of other antagonistic bacteria (*e.g. Bacillus*, *Pseudomonas*) are either known to induce plant defence responses (induced systemic resistance, ISR) against bacterial or fungal pathogens (van Loon *et al.*, 1998; Choudhary *et al.*, 2007; Berendsen *et al.*, 2012).

Also biological compounds extracted from different organisms such as bacteria, fungi and algae, and able to trigger the plant defense immune system, are enclosed in the group of biotic elicitors. One example is represented by Vacciplant whose active principle is laminarin (beta-1,3 glucan), extracted from the blue green algae (*Laminaria digitata*), that is able to elicit plant defence responses against different plant pathogens (Aziz *et al.,* 2003; Joubert *et al.,* 1998).

On the contrary, the abiotic elicitors that induce systemic acquired resistance (SAR) and are produced through chemical synthesis, should not be able to direct inhibit the pathogens, but they shall induce plant resistance against them. Therefore they must be able to trigger the expression of the same signals that are induced under the pathogen attack by mimicking the incompatibility interaction (Kessman *et al*., 1994).

The most known elicitor which showed a significant efficacy against the agent of bacterial canker of kiwifruit is the chemical compound Bion®, based on acibenzolar-S-methyl (ASM); this active principle belongs to benzothiadiazole (BTH) chemical class having the same action as the salicylic acid (SA), the transduction signal for SAR (Cellini *et al*., 2014).

In this chapter the strains D747 and QST 713, of *Bacillus amyloliquefaciens* (Bam) and of *B. subtillis* (Bas), respectively, were tested both in *in vitro* and *in vivo* experiments to evaluate their direct effect against Psa. In addition, the strain D747 and the biological product Vacciplant® were assayed for their ability to induce plant resistance against the bacterial canker of kiwifruit.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Pseudomonas syringae pv. *actinidiae* (Psa) strains NCPPB 3739 (haplotype I), CRA-FRU 3.1 (haplotype III) and IPV-BO 9312 (haplotype III) selected from the collection of the Phytobacteriology Laboratory (DipSA, University of Bologna) were employed and routinely grown at 27°C for 48-72 h on NSA or KB media.

Bacillus amyloliquefaciens (Bas, formerly *B. subtilis*, Chen *et al.*, 2009) strain QST 713 (Serenade®, Bayer CropScience S.r.l, Leverkusen, Germania) and *B. amyloliquefaciens* (Bam) strain D747 (Amylo-X®, Biogard®, CBC Europe S.r.l., Nova Milanese, Italia) were routinely grown on LPGA and BUG-agar media (see appendix) at 27°/36°C for 24 h and were used in *in vitro* and *in vivo* experiments.

2.2 Efficacy evaluation of biocontrol agents in *in vitro* experiment (diffusion method)

The production of antimicrobial compounds by Bas and Bam strains were assayed *in vitro* following the method reported by Vanneste *et al.* (1992). The axenic colonies of each antagonistic strain were transferred with a loop in the centre of Minimal Medium (MM, see appendix) Petri dishes (*ca.* 1 cm spot diameter); the plates were then incubated at 27°C for 48 h. The bacterial macro-colony was measured in diameter (XY axes mean) and then scraped off the plates with a lancet; the plates were exposed to chloroform vapours for 40-45 min. Then, the plates were homogenously covered with 5 mL MM soft-agar (see appendix) contaminated with Psa strains NCPPB 3739, IPV-BO 9312 and CRA-FRU 3.1 (*ca.* 10^6 CFU/mL). After 48-72 h at 27°C inhibition haloes were evaluated (inhibition of bacterial growth was visible around the spot where the antagonist strain had grown) by subtracting the mean of the diameters (XY axes mean) of the antagonist macro-colony, from the mean of inhibition halo diameters (XY axes mean). The assay was repeated three times and Psa strains, assayed as Bam and Bas, were used as negative control.

2.3 Efficacy evaluation of laminarin based product in *in vitro* experiment (diffusion and dilution method)

The *in vitro* experiments, using diffusion and dilution methods, were conducted following the procedures described in the Chapter I (Materials and Methods section). All the treatments are shown in Table 1. Sterile deionized water (SDW) and ASM were used as negative controls, streptomycin sulphate was used as positive control. The experiment was repeated three times and the data were processed with ANOVA test (p 0.05).

Table 1. Treatments and concentrations used in *in vitro* assays against Psa.

Treatments	Details	Diffusion method [%/ppm]	Dilution method [%/ppm]
SDW	Sterile deionized water; Negative Control	\	\
Vac 0.15	Elicitor; Vacciplant®, Goemar	0.15 % v/v	0.15 %
Vac 0.3	Elicitor; Vacciplant®, Goemar	0.30 % v/v	0.30 %
Vac 0.9	Elicitor; Vacciplant®, Goemar	0.90 % v/v	0.90 %
ASM	BTH 50 WG, Syngenta; Negative control	75 ppm	75 ppm
Sm	Streptomycin sulphate; Positive Control	10,000 ppm	100 ppm

2.4 *In vivo* experiment on kiwifruit plants (direct efficacy against Psa)

The efficacy of both biofungicides Amylo-X and Serenade against Psa was assayed under greenhouse conditions on *A. deliciosa* (cv. Hayward clone 8) and *A. chinensis* plants. Kiwifruit plants were grown in pots (5 L) and disposed in randomized replicates (3 plants X 4 replicates/treatment). The treatments and times of application are shown in Table 2; all treatments were applied at the leaves (*ca.* 100 mL/plant) using a sprayer before the inoculation with the pathogen. After treatment application the plants were experimentally inoculated by spraying a water suspension (OD = 0.01_{600nm}; *ca.* 10^7 CFU/mL) containing the Psa strain IPV-BO 9312. The plants were then sealed in polyethylene (PE) bags for 2 days (humid chamber). The greenhouse conditions were set as 16 h light at 23°C and 8 h dark at 17°C, maintaining the RH% between 85% and

95% until the phytopathometric assessments. Streptomycin sulphate and SDW were used as positive and negative controls, respectively (Table 2). The disease severity was evaluated 21 days after Psa inoculation by counting the number of leaf spots on 7 leaves/plant (approx. 84 leaves per treatment). The data collected were analyzed with ANOVA test (Duncan, p 0.05) by using the software SPSS 15.0 (SPSS Chicago, SPSS Inc.).

Table 2. Experiments on coverage treatments carried out under greenhouse conditions on yellow and green kiwifruit plants.

Code	TREATMENTS			
	Details	**Dose** (g/L, p/v)	**Concentration** (CFU/mL*, ppm)	**Application time**
Experiment on *Actinidia deliciosa* cv. Hayward				
SDW	Sterile Distilled Water	/	/	24 h BPI
Ser	Serenade (strain QST 713)	3 g/L	2,6 X 10^7	24 h BPI
AMX	Amylo-X (strain D747)	2 g/L	7,6 X 10^7	24 h BPI
Sm	Streptomycin sulphate (Sm)	0.01% p/v	100 ppm	24 h BPI
Experiment on *Actinidia chinensis* selection				
SDW	Sterile Distilled Water	/	/	24 h BPI
Ser	Serenade (strain QST 713)	3 g/L	3.3 X 10^7	24 h BPI
AMX	Amylo-X (strain D747)	2 g/L	6.3 X 10^7	24 h BPI
Sm	Streptomycin sulphate (Sm)	0.01% p/v	100 ppm	24 h BPI

(BPI) Before pathogen inoculation; (*) calculated after vitality test on LPGA medium

2.5 *In vivo* experiment on kiwifruit plants against Psa (plant resistance induction)

Twelve kiwifruit plants per treatment (*A. chinensis* selection) were distributed in randomized blocks (3 plants X 4 replicates). All treatments were applied at the 5^{th} – 7^{th} leaf stage (Table 2). Vacciplant was applied by spraying the leaf surfaces, the Bam strain D747 on the contrary, was irrigated (50 mL) with an aqueous suspension (*ca*. 10^8 CFU/mL). After treatment applications the plants were experimentally inoculated by spraying a water suspension (OD_{600nm} = 0.01; *ca*. 10^7 CFU/mL) containing the strain IPV-BO 9312 of Psa. The plants were then sealed in polyethylene (PE) bags for 2 days (humid chamber). The greenhouse conditions were set as 16 h light at 23°C and 8 h dark at 17°C until the disease evaluation. The positive control ASM (defence response

inducer) was used by spraying, whereas the negative control (SDW-R) by irrigating (Table 3).

Table 3. Experiments on induced resistance carried out under greenhouse conditions on *A. chinensis* plants.

Code	TREATMENTS			
	Details	**Dose** (g/L, p/v, v/v)	**Concentration** (UFC/mL*, ppm)	**Application time** (hours or days)
SDW-R	Sterile Distilled Water	/	/	48 h BPI
Bam-R	*Bacillus amyloliquefaciens* D747	2 g/L	7,6 X 10^7	48 h BPI
Lam-L	Laminarin; Vacciplant, Goemar	0.3% v/v	3,000 ppm	48 h BPI
ASM-L	BTH 50 WG, Syngenta	0.01% p/v	100 ppm	14 d BPI

(BPI) Before pathogen inoculation; **(L)** treatment applied on leaves by spraying. **(R)** treatment applied at roots by irrigation. (*) calculated after vitality test on LPGA medium

2.6 Psa Isolation and identification

Selected symptomatic kiwifruit leaf samples were used for Psa isolation and identification. Leaf surface was sterilized by washing with 2% sodium hypochlorite. Necrotic lesions were aseptically collected and crushed into a mortar with 2 mL of SDW; 30 μL of the extract was inoculated on NSA media; the plates were incubated up to 72 h. Psa-like colonies were subcultured and identified with molecular assays (Biondi *et al.,* 2013).

The PCR tests were performed in a final volume of 25 μL using the following chemical profile: buffer 1 x Taq Buffer (Promega), 1.5 Go Flexi mM MgCl2, 0.2 mM dNTPS, 0.5 U of *Taq* polymerase (Promega), 0.4 μM primer, 5% of dimethylsulfoxide (DMSO, Fluka) plus 5 μL bacterial suspension. The thermal profile consisted of an initial denaturing at 94°C for 5 min, followed by 35 cycles at 94°C for 20 s, 57°C for 20 s, 72°C for 30 s, and 10 minutes for final extension at 72°C. SDW and an aqueous suspension (*ca*. 10^8 CFU/mL) of Psa strain CRA-FRU 3.1 were used as negative and positive controls, respectively. All products were amplified by agarose gel electrophoresis (1.5%; see appendix) in 1 x TAE buffer (see appendix); the gel was stained in ethidium bromide (0.03%) and then visualized at transilluminator using UV light (312 nm) (Figure 4)

Figure 2. *Actinidia chinensis* plants inoculated with *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312 under greenhouse conditions.

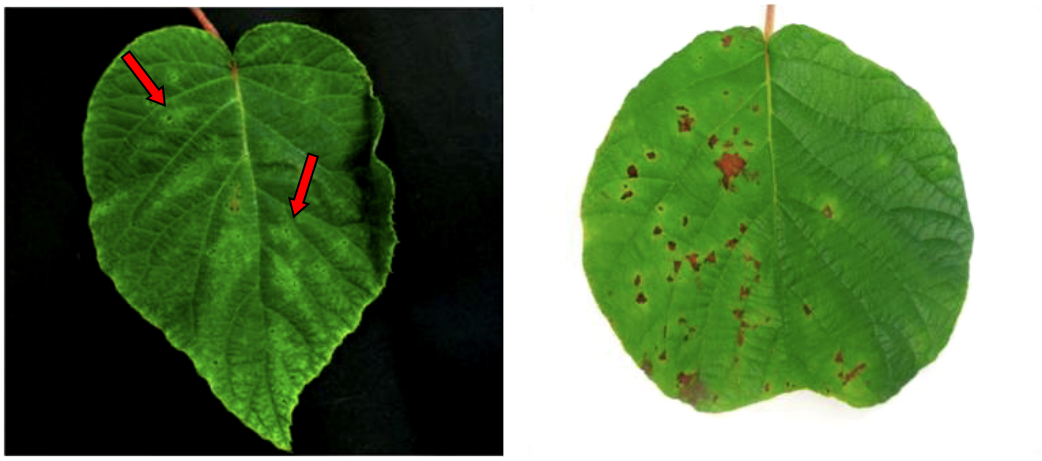

Figure 3. Leaf symptoms caused by *Pseudomonas syringae* pv. *actinidiae* strain IPV-BO 9312 on *Actinidia deliciosa* cv. Hayward (left) and *A. chinensis* selection (right).

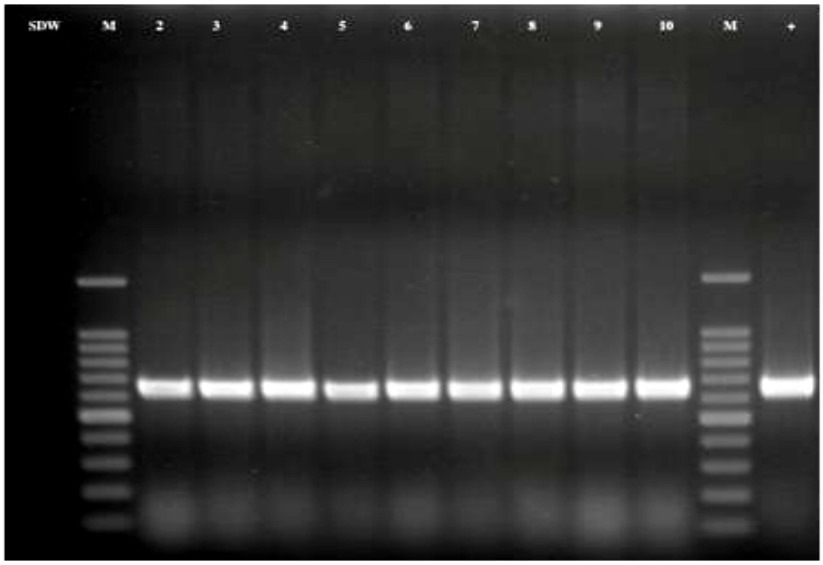

Figure 4. PCR assay on axenic colonies re-isolated from kiwi leaf spots. Lanes 2 and 3: samples of leaf spots collected from streptomycin treated plants; lanes from 4 to 6: samples of leaf spots collected from Amylo-X treated plants; lanes 7 and 8: samples of leaf spots collected from Serenade treated plants; lanes 9 and 10: samples of leaf spots collected from SDW treated plants; lane M: marker 100 bp (Promega, G190A, G210A); lane SDW: negative control; lane (+): positive control, strain CRA-FRU 3.1.

3. RESULTS AND DISCUSSION

The results of the *in vitro* experiments (diffusion method) with both *Bacillus* strains Bas and Bam are shown in Figure 5. Both antagonistic strains produced compounds able to inhibit the bacterial growth of the three Psa strains belonging to different haplotypes. Bas resulted significantly more effective in inhibition than Bam, showing means of inhibition haloes ranging from 2.8 to 3.0 cm (depending on Psa strain), significantly larger than those induced by Bam (from 2.1 to 2.3 cm) (Figure 5).

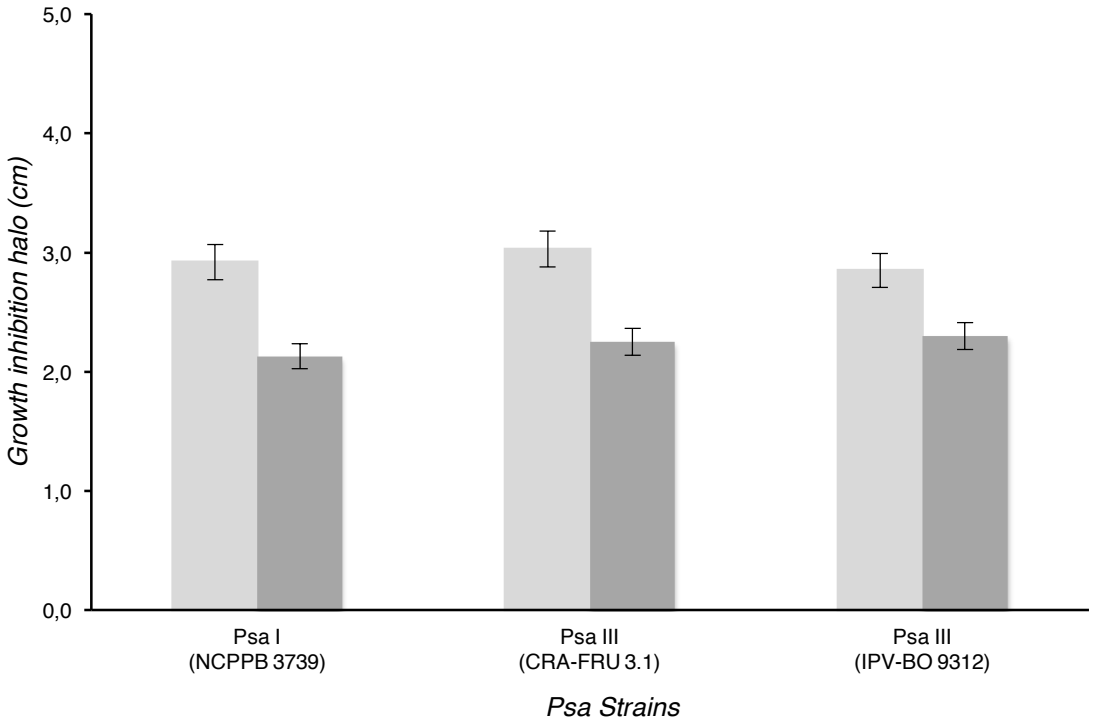

Figure 5. Results of *in vitro* experiment (diffusion method): inhibition halo mean diameters (cm) produced by the antibacterial compounds synthesized by the Bas (light grey histogram) and Bam (dark grey histogram) macro-colonies against different strains of Psa, belonging to haplotypes I and III.

The results obtained in *in vitro* experiments confirmed the capacity, expressed by both bacterial antagonistic strains, to produce antibiotic compounds, whose effectiveness, isolation and identification (*e.g.* polyketides, siderophores, polypeptides) was previously assessed (Reva *et al.*, 2004; Chen *et al.* 2006; 2007; 2009). On the contrary, in *in vitro* assays, the bioelicitor Vacciplant® resulted ineffective both using diffusion (data not shown) and dilution (Figure 6) methods in reducing Psa growth.

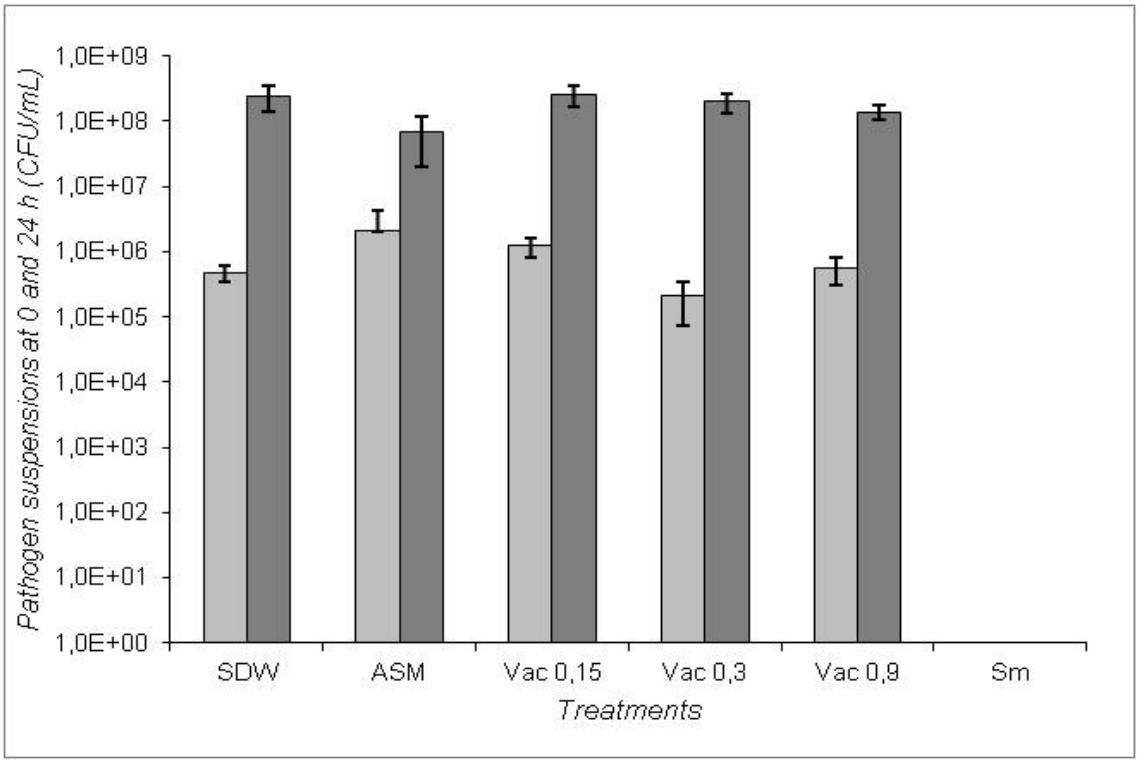

Figure 6. *In vitro* efficacy (dilution method) against Psa population of Vacciplant® at different concentrations at 0 h (light grey histograms) and 24 h (dark grey histograms). SDW and ASM were used as negative controls, Sm as positive.

The experiment performed on *A. deliciosa* under greenhouse conditions highlighted of the ability of Amylo-X based on D747 strain to reduce the disease severity (bacterial leaf spots) caused by Psa IPV-BO 9312 strain. The disease severity on plants treated with Bam (*ca*. 2 spots/leaf) was statistically similar to that evaluated on the plants treated with streptomycin sulphate (positive control: mean of approx. 0.3 spots/leaf), and significantly lower than that of negative controls (SDW, approx. 4 spots/leaf). The plants treated with Serenade, on the contrary, were significantly similar to the negative control plants (*ca.* 4 spots/leaf) (Figure 7).

In the second experiment, carried out on *A. chinensis*, the results confirmed the ability of Amylo-X to reduce the number of bacterial leaf spots caused by IPV-BO 9312 strain. The disease severity evaluated on plants treated with Amylo-X (*ca.* 14 spots/leaf), in fact, was significantly lower than that of control plants (SDW, *ca*. 62 spots/leaf). As in the first experiment, plants treated with Amylo-X showed a disease severity statistically similar to those treated with streptomycin sulphate (approx. 1 spot/leaf). The number of bacterial spots per leaf was also lowered in the plants treated with Serenade (*ca.* 32 spots/leaf) (Figure 8).

The host plants - *A. deliciosa* - used in the first experiment resulted less susceptible to Psa than the *A. chinensis* ones employed in the second assay. In previous studies it was demonstrated and confirmed the intermediate susceptibility of the genotypes belonging to *A. deliciosa* compared to several accessions of *A. chinensis* (EPPO, 2016; Perez *et al.*, 2015). However the lower susceptibility of plants used in the first experiment, and therefore the resulted low disease severity evaluated in control plants, did not allow a precise evaluation of the different treatments against Psa. In the second experiment, in fact, the plants treated with Serenade showed a lower disease severity in comparison to the control plants treated with SDW, even though the disease reduction was slight significant.

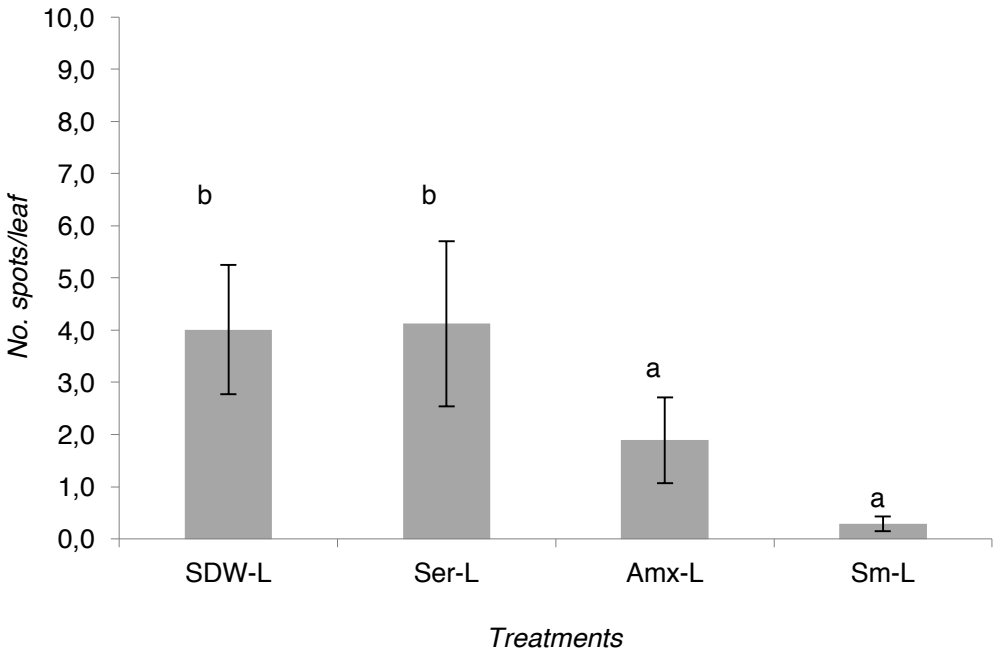

Figure 7. Efficacy of Serenade and Amylo-X against *Pseudomonas syringae* pv. *actinidiae* on *A. deliciosa*. Different letters indicate significant differences (Duncan, p 0.05).

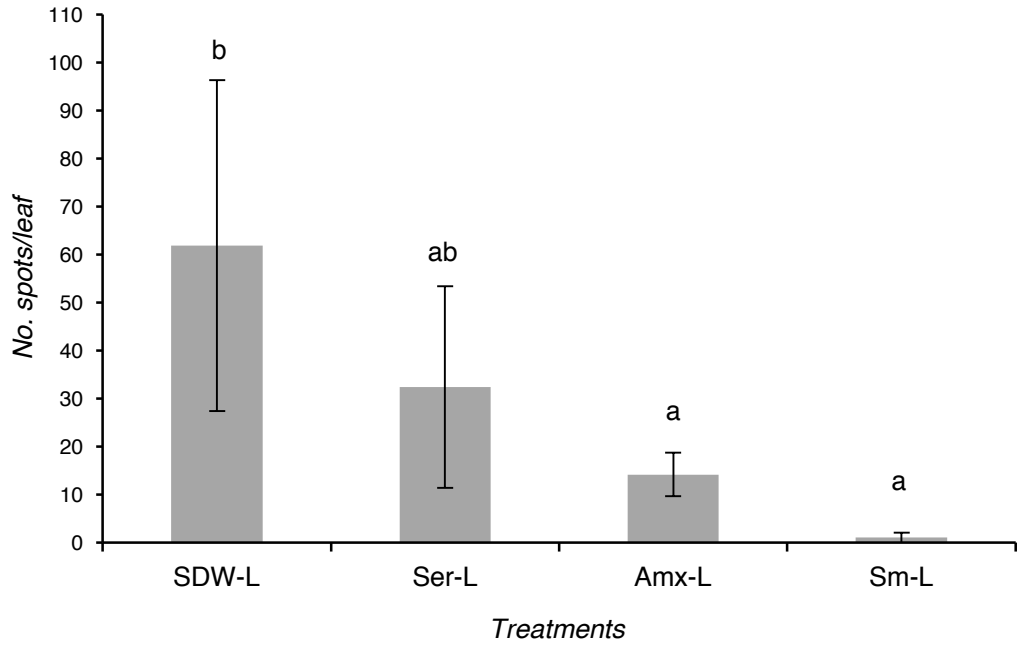

Figure 8. Efficacy of Serenade and Amylo-X against *Pseudomonas syringae* pv. *actinidiae* on *A. chinensis* plants. Different letters indicate significant differences (Duncan, p 0.05).

In both experiments, Amylo-X confirmed its effectiveness in significantly reducing the number of bacterial spots per leaf caused by Psa. *Bacillus* sp. based products are already known for their ability to control several bacterial diseases such as fire blight of pomaceous plants caused by *Erwinia amylovora* (Laux *et al.,* 2003; Broggini *et al.,* 2005; Bazzi *et al*, 2006; Biondi *et al.*, 2006; 2007; 2010; 2012;Kunz *et al*, 2012), crown gall of grapevine caused by *Agrobacterium vitis* (Biondi *et al*, 2009a), bacterial spot/canker of stone fruits (Biondi *et al*, 2009b) and tomato bacterial speck caused by *P. s.* pv. *tomato* (Fousia *et al.*, 2016). In *in vivo* experiments, Amylo-X was more effective than Serenade in reducing symptom severity both in green and yellow kiwifruit plants, whereas in *in vitro* assays Serenade was the more effective against Psa. Thus, the different efficacy resulted between the two bioproducts could not be connected solely to the antibiotic compounds produced by the bacterial antagonists, but also to their population level. In previous studies, carried out on *A. deliciosa* plants, it was demonstrated that D747 strain can survive on the leaves for some weeks at high population level (Biondi *et al.*, 2012). The antagonist ability to occupy the penetration sites (competition), in combination with the antibacterial compounds, could play a crucial role in suppressing the epiphytic Psa population. In other pathosystems, in fact, using as antagonists, different *Pseudomonas* strains, it was demonstrated that the different effectiveness against *E. amylovora* is related to the colonization or survival ability at high population level and for a long time on the different plant organs (Pusey, 1997; Alexandrova *et al.,* 2000). The need for further studies of population dynamics on each biofungicide tested are therefore necessary to improve their efficiency and to study their optimal application rates.

Moreover, when the antagonist strain D747 of Amylo-X was applied at kiwifruit root apparatus before the experimental inoculation with the pathogen, it lowered the disease severity (*ca.* 22 spots/leaf) with respect to plants treated with SDW-R (negative control, approx. 37 spots/leaf); the difference in disease severity between these two treatments was slightly significant because of the high data variability in the number of bacterial leaf spots evaluated in the plants treated with Bam (Figure 9). As described in Chapter II, the treatment volume (50 mL) containing the antagonist suspension applied by irrigation, could be not adequate for the wide root volume since the treatment was represented in this case by living organisms and not by a chemical compound (see also Chapter II).

The plant immune system induction by root application of a suspension containing Bam, was evident: a significant reduction of disease severity, still with high data variability, was observed. Recent studies conducted on *Bacillus* sp. strains, which also reported the efficacy of strain QST 713, supported the hypothesis of ISR plant elicitation through bacterial metabolites (Krause *et al.,* 2003; Chowdhury *et al.,* 2015; Fousia *et al.*, 2016).

The *A. chinensis* treated with Vacciplant®, which, as expected, resulted unable to directly inhibit the Psa growth when tested in *in vitro* experiments, showed a disease severity statistically similar to the one of the negative control plants (approx. 26 spots/leaf) and statistically different from those treated with ASM (positive control) that showed a significantly lower disease severity (*ca.* 4 spots/leaf). Since the number of bacterial leaf spots of Vacciplant® treated plants was lower than that of negative controls, the defence response mediated by the host was not significantly effective against Psa (Figure 9).

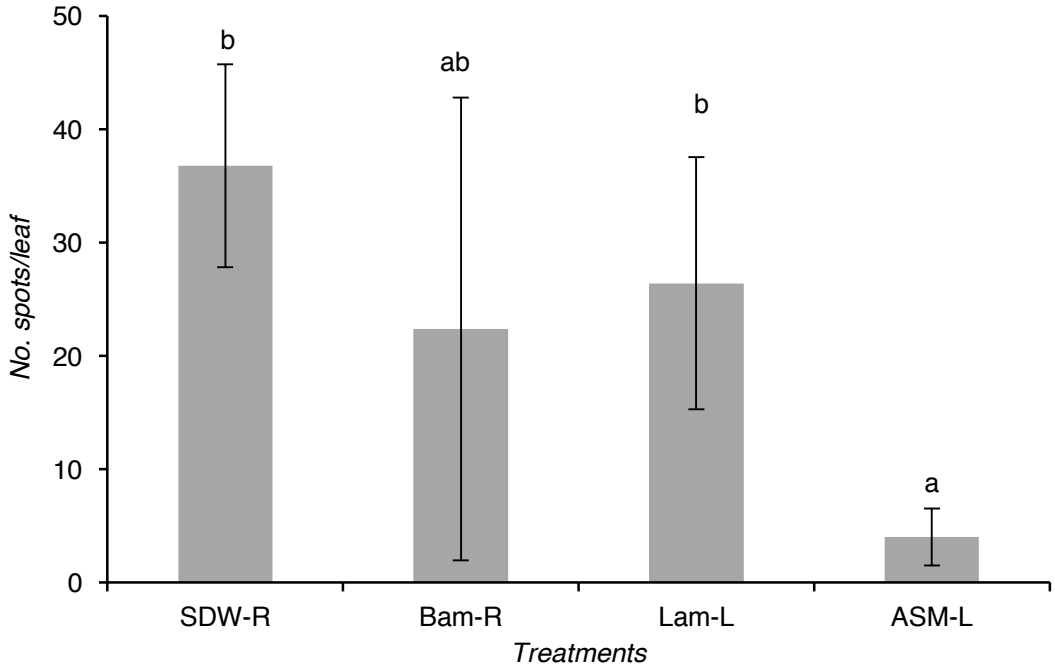

Figure 9. Efficacy of *Bacillus amyloliquefaciens* against *Pseudomonas syringae* pv. *actinidiae* on *A. chinensis* by elicitation of host defence response. Different letters indicate significant differences (Duncan, p 0.05).

In conclusion, *in vitro* experiments highlighted and confirmed the capacity of both *Bacillus* sp. strains to produce antimicrobial compounds able to inhibit Psa growth. The *in vitro* efficacy of Bas resulted significantly higher than that of Bam; whereas in the *in vivo* experiments, the direct efficacy of Serenade against the pathogen was significantly lower than that shown by Amylo-X. These results were consistent in both experiments carried out in green and yellow kiwifruit plants, thus suggesting a combined effect of both antibacterial compounds production and nutritional competition acted by the biopreparate Amylo-X. In addition, the bacterial suspension of Bam, applied at the root apparatus, triggered a plant defence response against Psa confirmed by a disease severity reduction on leaves.

As expected, instead, the resistance inducer Vacciplant®, when tested in *in vitro* experiments, was not able to directly reduce the bacterial growth. Moreover, Vacciplant® resulted ineffective against Psa also when tested on plants under greenhouse conditions.

Chapter IV

SUSCEPTIBILITY OF *ACTINIDIA* SPP. ACCESSIONS TO BACTERIAL CANKER

1. INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa), causal agent of bacterial canker of kiwifruit, was isolated for the first time in 1984 in Japan (Serizawa *et al.*, 1989; Takikawa *et al.*, 1989) and in China (Fang *et al.*, 1990) from *Actinidia deliciosa* symptomatic plants; in 1990 the pathogen was also identified in Korea (Koh *et al.*, 1994). In Italy, Psa was isolated and identified in 1992 (Scortichini, 1994) again from symptomatic plants of *A. deliciosa*. Almost two decades after this first report, a rapid epidemic spread, that affected the kiwifruit cultivations in all continents, started in *A. chinensis* and *A. deliciosa* plants. This pandemic event was due to the apparently higher susceptibility to Psa of *A. chinensis* cultivars, compared to the one of *A. deliciosa* cv. Hayward, coupled to the emerging of a new and more aggressive haplotype of the pathogen, most probably originated in China (Butler *et al.*, 2013). This strain is differentiable from those identified before the pandemic spread and significantly more virulent (Ferrante and Scortichini, 2010; Vanneste *et al.*, 2011; Chapman *et al.*, 2012; Scortichini *et al.*, 2012; Biondi *et al.*, 2013).

Nowadays, the control of this important disease, that significantly affects the kiwifruit production in Italy and in the major cultivation areas of the five continents, is limited to copper compounds and to antibiotics - where this is permitted - (Koh *et al.*, 1996), increasing the risk and the probability of selection of Psa strains resistant to these compounds (Nakajima *et al.*, 2002; Marcelletti *et al.*, 2011). A recent study highlighted the ability of the elicitor acibenzolar-S-methyl in reducing the disease severity of experimentally inoculated plants (Cellini *et al.*, 2014), and other studies carried out on biocontrol agents as *Bacillus* spp. or bacteriophages indicate this as a promising sustainable tool to control the disease (Biondi *et al.*, 2012; Di Lallo *et al.*, 2014; Frampton *et al.*, 2014).

Nevertheless, the prevention of Psa spreading by pollen (Stefani and Giovanardi, 2011; Tontou *et al.*, 2014) and the diagnostic control of asymptomatic plant material (Gallelli *et al.*, 2011; Biondi *et al.*, 2013; Gallelli *et al.*, 2013), are still the most useful method to decrease the *inoculum* sources. Studies on kiwifruit germplasm from different *Actinidia* species are ongoing and breeding programmes aimed to develop Psa resistant germplasm are underway worldwide (Beaston, 2012; Cameron and Sarojini, 2013; Wei *et al.*, 2013; Donati *et al.*, 2014).

In this study, the susceptibility of five *Actinidia* species and of different accessions of *A. chinensis* infected by Psa experimentally inoculated was studied and compared.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Bacterial pathogenic strain IPV-BO 9312 from the collection of the Phytobacteriology Laboratory of Department of Agricultural Sciences (DipSA, University of Bologna) was employed and routinely grown at 27°C for 48-72 h on NSA or KB (both amended with 1.5% boric acid, 80 mg/L of cephalexin sulphate and 120 mg/L of cycloheximide) (see Appendix).

2.2 Preliminary experiments

The study was carried out from 2013 to 2015. Twenty six kiwifruit accessions and one control cultivar (*A. chinensis* 'Belen' used as the most susceptible accession) were tested (Table 1). Six *A. deliciosa* accessions (including 'Hayward' clone 8, as intermediately susceptible cultivar), and 17 *A. chinensis* accessions were assayed under greenhouse and climatic chamber conditions. To also evaluate the susceptibility to bacterial canker of *Actinidia* species different from the common kiwifruit, one accession for each of the following species *A. arguta*, *A. eriantha* and *A. hemsleyana* was tested as well.

The plants were experimentally inoculated using the virulent strain IPV-BO 9312 of Psa isolated in Veneto region in 2013 from bleeding sap of asymptomatic kiwifruit plants (Biondi *et al.*, 2014), selected as the most virulent after comparison to different reference strains (*e.g.* CRA-FRU 3.1, OMP-BO 1875.3, CFBP 7286) (data not shown).

Every year, from late spring to advanced autumn, one-year-old kiwifruit plants per accession were grown in 2-2.5 L pots - containing a peat and vermiculite soil mix - and pruned to stimulate the new shoots and young leaves; all the plants were inoculated at the same phenological stage with highly similar leaf age. From 7 to 15 plants were then distributed in 4 randomized blocks and experimentally inoculated by spraying young leaves with a calibrated water suspension containing the Psa strain IPV-BO 9312 (0.01 OD_{660nm}, *ca.* 10^7 CFU/mL). The plants were sealed in polyethylene (PE) bags for 48 h to favor the water congestion and allow the pathogen penetration (Figure 1). The controlled conditions, hold until disease assessment, were 16 h of day light and

8 h of darkness, 23°C and 17°C during day and night, respectively; moreover, during the day the relative humidity was maintained at 85%, while at night it was set at 97%. Under greenhouse conditions the light intensity was set to a threshold of 30,000 lux to avoid induced resistance linked to light stress (Stael *et al.*, 2015). Plants sprayed with sterile deionized water (SDW) and non-inoculated ones were used as negative controls.

The disease severity was evaluated 21 days after Psa inoculation by counting the number of leaf spots on 10 leaves/plant (70 to 150 leaves per treatment). The collected data were related to positive control 'Belen', and were analyzed with ANOVA test (Duncan, p 0.05) by using the software SPSS 15.0 (SPSS Chicago, SPSS Inc.). The pathogen was reisolated on NSA amended with boric acid, cephalexin and cycloheximide (Gallelli *et al.*, 2011; Stefani and Giovanardi, 2011) from selected leaf samples, previously externally sterilized with 5% sodium hypochloride; the Psa-like colonies were identified with molecular assay (Biondi *et al.*, 2013).

Table 1. *Actinidia* spp. accessions used for preliminary susceptibility experiments.

No. accession	Species	Gender	Kind of plants
A050	*A. hemsleyana*	/*	Seedling
A096	*A.eriantha*	/*	Seedling
A140-39	*A. chinensis*	♂	Grafted plants
Autari	*A. deliciosa*	♂	Grafted plants
Belen	*A. chinensis*	♂	Own rooted
Hayward clone 8A	*A. deliciosa*	♀	Own rooted
Jincui OP A212	*A. deliciosa*	♀	Grafted plants
Matua	*A. deliciosa*	♂	Own rooted
NPK 1	*A. chinensis*	♀	Own rooted
NPK 2	*A. chinensis*	♀	Own rooted
NPK 3	*A. chinensis*	♀	Own rooted
NPK 5	*A. chinensis*	♀	Own rooted
NPK 6	*A. chinensis*	♀	Own rooted
NPK 7	*A. chinensis*	♂	Own rooted

No. accession	Species	Gender	Kind of plants
Soreli	*A. chinensis*	♀	Own rooted
Qin-Mei	*A. deliciosa*	/*	Grafted plants
T92	*A. arguta*	♀	Own rooted
Tomuri	*A. deliciosa*	♂	Grafted plants
Jinfeng A 204	*A. chinensis*	♀	Grafted plants
Lushan A164 C1	*A. chinensis*	♀	Grafted plants
A166 C3	*A. chinensis*	♀	Grafted plants
A139 M3	*A. chinensis*	♂	Grafted plants
A125_93	*A. chinensis*	♂	Grafted plants
A134_41	*A. chinensis*	♂	Grafted plants
A 326_46	*A. chinensis*	♂	Grafted plants
A 332_29	*A. chinensis*	♂	Grafted plants
A 172 C9	*A. chinensis*	♀	Grafted plants

* Seedlings of unknown gender

Figure 1. On the left are shown leaves after polyethylene bags removal, water drops are visible at the end of the leaf veins (hydatodes), on the right, *Actinidia* sp. accessions under greenhouse conditions.

2.3 Extended experiment

Selected *Actinidia* spp. accessions from preliminary susceptibility experiment were employed in an extended experiment of susceptibility to bacterial canker in which the number of plants for each accession tested was increased (30 plants divided in 4 replicates); as positive control, the cv. Belen of *A. chinensis* was used.

The experimental conditions of the previous experiment (method of pathogen inoculation, concentration of Psa suspension, temperature and humidity) were maintained.

The phytopatometric assessments were carried out on 10 leaves/plant by counting the number of spots per leaf. From the spots of uncertain etiology, the bacterium isolation was performed on semi-selective medium (NSA amended with 1.5% boric acid, cephalexin sulphate, 80 mg/L and cycloheximide; 120 mg/L), and the identification was assessed using the molecular assay (Biondi *et al.*, 2013, Figure 6) on subcultured Psa-like colonies (Figure 5).

The data were analyzed with ANOVA test (Duncan's, p 0.05) by using the software SPSS 15.0 (SPSS Chicago, SPSS Inc.). The data of disease severity (mean of number of spots/leaf), collected from the preliminary experiment of susceptibility, were normalized to the cv. Belen (*A. chinensis*) considered as 100%; to obtain values of relative susceptibility (%, RS) related to the positive control.

3. RESULTS AND DISCUSSION

None of the accessions of *Actinidia* sp. assayed resulted resistant to the bacterial leaf spot caused by Psa. The accession which resulted less susceptible to the pathogen was the T92 of *A. arguta*; the disease severity was evaluated as a mean of 0.12 spot per leaf, 'Qinmei' was the significantly most diseased (approx. 21 spots/leaf).

Almost all assayed selections and accessions have shown a significantly lower susceptibility compared to that of positive control; only in one case (cv. Qinmei), the disease severity resulted significantly higher in comparison to that of cv. Belen of *A. chinensis* (Figure 2). The accessions A050 (*A. hemsleyana*) and A096 (*A. eriantha*) and the selections NPK3, A326_46 and A166 (*A. chinensis*), have shown an apparently similar susceptibility to that of T92; the average number of spots per leaf, in fact, was 0.13, 0.22, 0.28, 0.36 and 0.49, respectively.

The cultivars Hayward, Matua, Soreli, Tomuri and Autari, and the accessions A140 and A172 showed an intermediate susceptibility (mean of leaf spots ranging from 2 to 2.5, Figure 2).

In Figure 3, the histograms show the relative susceptibility (RS) to the positive control represented by cv. Belen, considered as 100%; as already anticipated, the cv. Qinmei resulted to be significantly more susceptible (200%) with respect to the positive control.

The accessions T92, A050, A096 and NPK3 resulted to be apparently less susceptible to the bacterial canker when compared to all the tested accessions and showed a significantly lower disease severity in comparison to cv. Belen: their RS was calculated as 1%, 1%, 2% and 3%, respectively (Figure 3).

Different accessions (A204, A164, A139, A125_93, A134_41, A332_29), obtained by grafted material, were characterized by high mortality percentage of the plants; the number of plants assayed for their susceptibility resulted therefore too low, indeed, the variability of the disease severity data that were collected resulted too high, affecting a correct discrimination of some genotypes in specific statistical classes. Those accessions were not considered in the final elaboration, that allowed the selection of few of the most promising accessions.

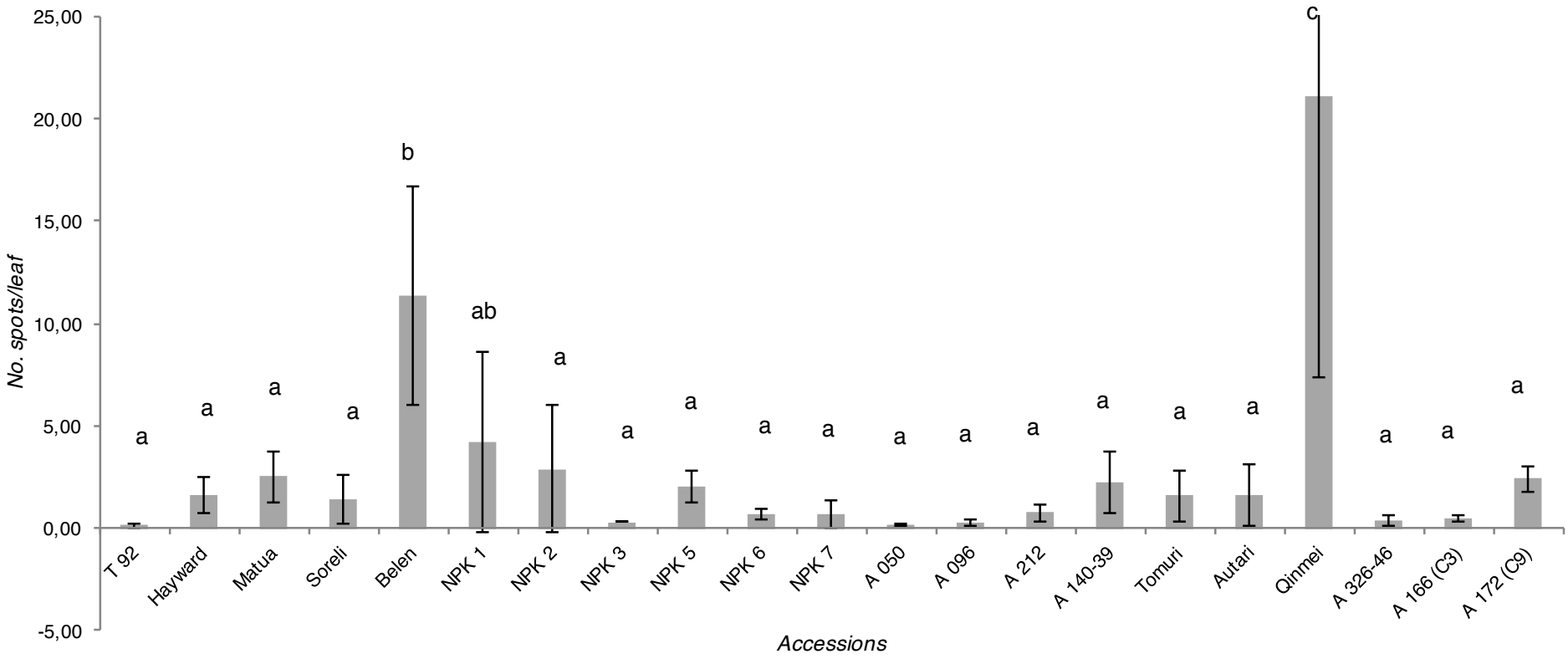

Figure 2. Results of the preliminary susceptibility experiment carried out on several *Actinidia* sp. accessions: the disease severity was represented by the mean of spots per leaf caused by the strain IPV-BO 9312 of *Pseudomonas syringae* pv. *actinidiae*. Letters indicate the different statistical classes whose the accessions belong to (Duncan's, p 0.05). In figure are also shown the standard deviations.

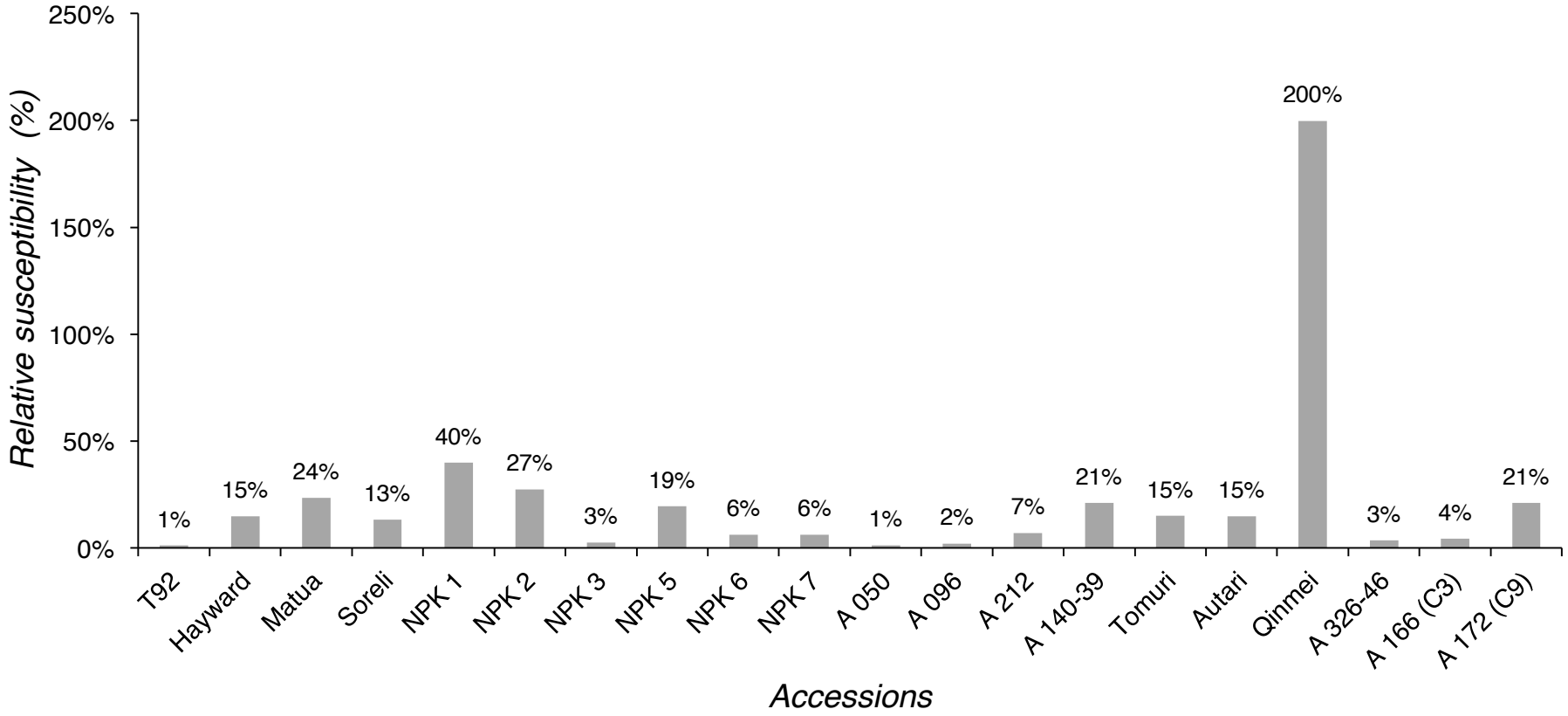

Figure 3. Relative susceptibility of the different *Actinidia* sp. accessions related to the disease severity (mean of No. spots/leaf) of cv. Belen (positive control, considered as 100%).

In the extended experiment of susceptibility, the selected accessions NPK3 and A166 confirmed to be significantly less diseased when compared to the positive control (14 spots/leaf); in fact, the mean of number of leaf spots was 1.12 and 0.83, respectively (Figure 4).

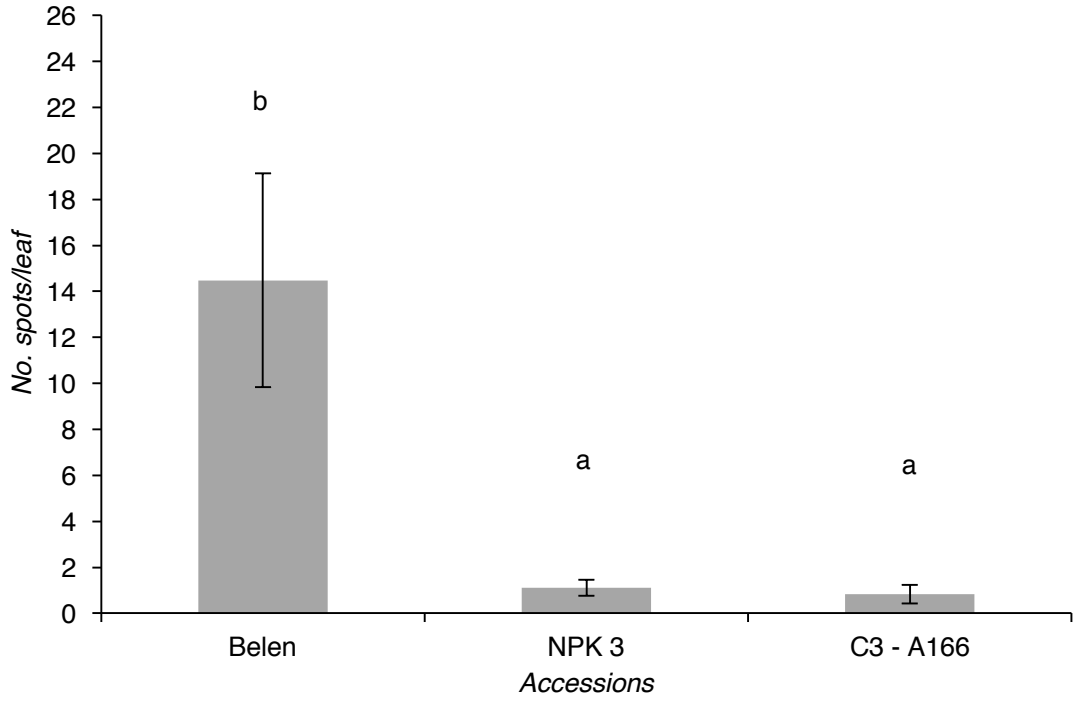

Figure 4. Results of the extended susceptibility experiment carried out on several *Actinidia* sp. accessions: the disease severity was represented by the mean of spots per leaf caused by the strain IPV-BO 9312 of *Pseudomonas syringae* pv. *actinidiae*. Letters indicate the different statistical classes of the accessions (Duncan's, p 0.05). In figure are also shown the standard deviations.

Figure 5. On the left is shown an example of isolation on NSA (amended with boric acid, cephalexin and cycloheximide) from leaf spots caused by *Pseudomonas syringae* pv. *actinidiae* (leaf on the right).

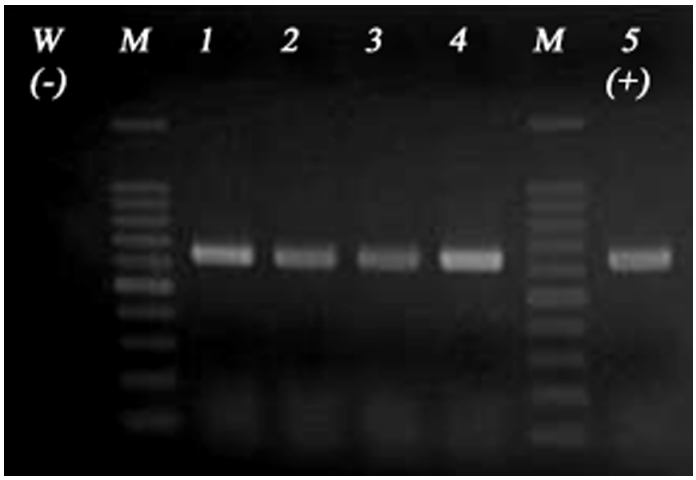

Figure 6. Molecular assay results carried out on axenic cultures isolated on semi-selective medium from leaf spots of different kiwifruit accessions. W: sterile deionized water (negative control); Lanes 1-2: *Pseudomonas syringae* pv. *actinidiae* (Psa) from cv. Belen leaf spots; lane 3: Psa from cv. Hayward spots; lane 4: Psa from cv. Matua spots; Lane 5: strain IPV-BO 9312 of Psa (positive control).

In conclusion, the *A. chinensis* accession NPK3 highlighted a comparable susceptibility to that of the species *A. arguta*, *A. hemsleyana* and *A. eriantha*; moreover, NPK3 resulted to be less susceptible among all *A. chinensis* and *A. deliciosa* selections.

Although more experiments are needed to increase the number of data and indeed confirm the robustness of the analysis, the results pointed out the lower susceptibility shown by NPK3 to Psa experimental infections, carried out using the most virulent Psa strain present in the bacterial collection of the University of Bologna.

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APPENDIX

1. BACTERIOLOGICAL MEDIA

BUG agar

BUG agar	57.0 g
H_2O	1,000 mL

GYCA

Yeast extract	5.0 g
Glucose	5.0 g
Calcium carbonate	40.0 g
Bacto agar	15.0 g
H_2O	1,000 mL

King's medium B (KB) (King *et al*., 1954)

Proteose Peptone No. 3	20.0 g
K_2HPO_4	1.5 g
$MgSO_4$ x $7H_2O$	1.5 g
Glycerol (87%)	8.16 mL
Bacto agar	15.0 g
H_2O	1,000 mL
pH adjusted at 7.2	

KBC (semi-selective KB)

Proteose Peptone No. 3	20.0 g
K_2HPO_4	1.5 g
$MgSO_4$ x $7H_2O$	1.5 g
Glycerol (87%)	8.2 mL
Bacto agar	15.0 g
H_2O	950 mL
pH adjusted at 7.2	

After sterilization (121°C for 15 min), amended with:

Boric acid (1.5 %)	100 mL
Cycloheximide (25 mg/mL)	8.0 mL
Cephalexin sulphate (10 mg/mL)	8.0 mL

LB-agar (Luria and Bertani medium)

Yeast extract	5,0 g
Bacto Tryptone	10.0 g
NaCl	10,0 g
H_2O	1,000 L
Bacto agar	15.0 g
pH adjusted at 7.0	

LB agar + ampicillin + IPTG + X-Gal

Yeast extract	5,0 g
Bacto Tryptone	10.0 g
NaCl	10,0 g
Bacto agar	15.0 g
H_2O	1,000 mL
pH adjusted at 7.0	

(Continue)

After sterilization (121°C for 15 min), amended with:

Ampicillin (1,000x)	1 mL
IPTG (100x)	10 mL
X-Gal (1,000x)	1 mL

LB-broth

Bacto Tryptone	25.0 g
Yeast extract	5.0 g
NaCl	10.0 g
H_2O	1,000 mL

pH adjusted at 7.0

LPGA (Ridè *et al.*, 1983)

Yeast extract	5.0 g
Bacto peptone	5.0 g
D-glucose	10.0 g
Bacto agar	20.0 g
H_2O	1,000 mL

pH adjusted at 7.2

Minimal Medium (MM) or GA medium (Vanneste *et al.*, 1992)

Bacto Agar	18.0 g
H_2O	950 mL

After sterilization (121°C for 15 min), amended with 50 ml of salts stock solution 20 X.

MM-Medium (Soft agar) (Vanneste *et al.*, 1992)

Bacto Agar	7.0 g
H_2O	950 mL

After sterilization (121°C for 15 min), amended with 50 ml of salts stock solution 20 X.

NSA (Lelliot *et al.*, 1966)

Bacto Nutrient-Broth	8.0 g
Sucrose	50.0 g
Bacto agar	15.0 g
H_2O	1,000 mL

pH adjusted at 7.0

NSABC (semi-selective NSA)

Bacto Nutrient-Broth	8.0 g
Sucrose	50.0 g
Bacto agar	15.0 g
H_2O	900 mL

pH adjusted at 7.0

After sterilization (121°C for 15 min), amended with:

Boric acid (1.5 %)	100 mL
Cycloheximide (25 mg/mL)	8.0 mL
Cephalexin sulphate (10 mg/mL)	8.0 mL

SOC broth

Tryptone	20.0 g
Yeast Extract	5.0 g
NaCl	0.5 g
$MgSO_4 \times 7H_2O$	5.0 g
Dextrose x H_2O	3.96 g
H_2O	1,000 mL

2. SALT SOLUTIONS AND BUFFERS

Ampicillin 1000x

Ampicillin	1 g
H_2O	10 ml

Stored at -20°C.

Boric acid (1.5%)

Boric acid	15.0 g
H_2O	1,000 mL

IPTG 100x (Isopropyl-β-D-1-thiogalactopyraniside, 0.05 M)

IPTG	120 mg
H_2O	10 mL

Filtered with Millipore 0.45 μm and aliquot in 1.5 mL Eppendorf tubes. Stored at 4°C.

Na-Lauroyl Sarcosine (30%)

Na-Lauroyl Sarcosine	30.0 g
H_2O	100 mL

RNA Extraction Buffer

Guanidine Iso-thyocianate	23.6 g
CH_3COO-Na+ (pH 5)	0.82 g
NaEDTA (pH 8)	0.46 g
PVP 40	1.25 g

Make up to 50 ml of volume with distilled water. Stored at 4°C.

Salt Stock Solution 20x

K_2HPO_4	35.1 g
KH_2PO_4	15.1 g
L-Asparagine	15.0 g
$(NH_4)_2SO_4$	10.0 g
Nicotinic acid	2.5 g
D-Glucose	20.0 g
$C_6H_5Na_3O_7 \times 2H_2O$	2.5 g
$MgSO_4 \times 7H_2O$	0.05 g
H_2O	500 mL

Stored at -20°C.

TAE 50x

TRIS (Tris[Hydroxymethyl]aminomethane)	242.0 g
Glacial acetic acid	57.1 mL
NaEDTA 0,5 M pH 8	100 mL
H_2O	1,000 mL

X-Gal 1000x (5-bromo-4-Chloro-3-indolyl-β-D-galactopyranoside)

Stock solution	80.0 mg
N, N-dimethylformammide	1.0 mL

Stored at -20°C

3. RNA SEQUENCES

RNA sequences cloned and used for RT-qPCR primers.

ACTIN <320 bp>

GTTTGGATCTTGCTGGTCGTGATTTAACTGATAACCTGATGAAGATCCTCACCGA
GAGAGGTTACATGTTCACCACCACTGCTGAACGGGAAATTGTCCGCGACATGAA
GGAAAAGCTTGCCTATGTGGCTCTTGACTATGAGCAGGAGATTGAAACTGCCAG
GAGCAGCTCCTCCATTGAAAAGAACTATGAATTGCCTGATGGACAAGTTATTACC
ATTGGTGCTGAGAGGTTCCGTTGCCCAGAGGTCCTCTTCCAGCCATCCATGATT
GGTATGGAAGCTGCAGGTATCCACGAGACTACCTACAACTCTATTATG

PR1a <429 bp>

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CTGGTGCTGGGGAGAACCTTGCCAAGGGTGGTGGTGACTTCACGGGGAGGGC
AGCCGTGCAATTGTGGGTGTCCGAGAGGCCAGACTATAACTACGCTACCAACC
AATGTGTTGGTGGAAAAATGTGTGGACATTATACTCAAGTAGTCTGGCGCAACT
CAGTCCGACTAGGTTGTGGTCGGGCTCGTTGCAACAATGGGTGGTGGTTCATTT
CTTGCAACTACGATCCTGTAGGCAACTGGGTTGGAGAACGTCCTTATTAAAGTA

PR4 <354 bp>

TTTGTCATCAACATGATGATGGCGGTGGCCGCAGCGCAAAGCGCTATGAACGTT
AGGGCAACGTATCATTTGTACAATCCGCAAAACATAAACTGGGATTTAAGAACTG
CTAGCGTTTACTGCGCTACCTGGGATGCTGACAAGCCTCTGGAGTGGCGCCGG
AGGTATGGCTGGACCGCTTTTTGCGGTCCAGCTGGACCTACGGGCCAAGCTTC
ATGCGGTAGATGCTTGAGGGTGACCAACACAGGAACAGGAACACAAGAAACAG
TGAGAATAGTAGATCAATGCAGAAATGGAGGGCTTGATTTGGATGTAAACGTTTT
CAACCGATTGGACACTAATGGATTGGGCTAT

PR5 <561 bp>

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CATGGGTCATCAATGCACCGAGGGGCACTAAGATGGCACGTATATGGGGTCGT
ACGAATTGCAACTTTGATGGTGCTGGTAGAGGTTCATGTCAGACTGGTGATTGT
GGTGGGGTCTTGCAATGTACCGGGTGGGGCAAACCACCAAACACCCTGGCCGA
GTACGCCTTGGACCAGTTTAGCAACCTAGATTTCTGGGACATTTCTTTAGTCGAT
GGATTTAATATTCCAATGACTTTCGCCCCGACCAATCCTAGTGGAGGGAAATGC
CATGCAATTCATTGTACGGCTAATATAAATGGTGAATGTCCTGGTTCACTTAGGG
TACCCGGAGGATGTAACAATCCTTGTACCACGTTCGGAGGACAACAATATTGTT
GCACACAAGGTCCATGTGGCCCTACTGATTTGTCGAGATTTTTCAAACAAAGAT
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PAL <501 bp>

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ATTGTTGTAATAGTCGTTGACAAGTTCCGAAAATTGGGCAAACATCAATTTCCCA
ATAGATGCAAGGGCCAATCTTGTATTATCCATGGACACACCAATAGGGGTACCT
TGGAAGTTTCCACCGTGTAAGGGCTTGTTTCTTGAAACATCGATCATGGGTTGT
CGTCACTGAGTTAATCTCTCTCTCATCATCTTAGTTGCTGCACGGATGACTTCAA
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LOXF <179 bp>

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GGCCATTGAAGAGCAAGCTAGACCCTGAGGTATATGGACCTCCTGAATCAGCAA
TTACAAAAGAGCTAATTGAAGAAGAAATTGGAGGATTTATGACTGTTGAAGAGGC
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ERF1 <287 bp>

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CATCGGTATCATCAGCTTCGGAAAGTGGCTCGCCTAAGAGGAGGAGAAAGGGT
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TTAAAATATGGAGCTAAGAACTCAATGGCTAGGGCTTGTTTGGCTTTGAAGTGG
ACAGAAAATAGCAAATCAT

4. QUALITY AND PURITY RNA

Quality and quantity of total extracted RNA evaluated with Tecan Infinit® 200 Pro NanoQuant instrument. T0: 0 h; T1: 7 h; T2: 24 h; T3: 48 h; T4: 120 h; T5: 8 d.

1-12	260	280	Conc ng/µl	Ratio	Sample ID (1)	replicate
1	1,82839999	0,819600027	1462,72	2,23	**NT-T0**	a
2	1,895799998	0,846099984	1516,64	2,24	**NT-T0**	b
3	2,545100063	1,158000033	2036,08	2,2	**NT-T1**	a
4	1,483900003	0,666299991	1187,12	2,23	**NT-T1**	b
5	1,399499971	0,628399972	1119,6	2,23	**NT-T2**	a
6	0,392199989	0,177300002	313,76	2,21	**NT-T2**	b
7	1,728499975	0,773199979	1382,8	2,24	**NT-T3**	a
8	1,06150005	0,478300005	849,2	2,22	**NT-T3**	b
9	0,49920002	0,225500014	399,36	2,21	**NT-T4**	a
10	0,724200003	0,32819999	579,36	2,21	**NT-T4**	b
11	0,624300003	0,281900018	499,44	2,21	**NT-T5**	a
12	0,777199976	0,349299997	621,76	2,23	**NT-T5**	b
13-24	260	280	Conc ng/µl	Ratio	Sample ID (2)	
13	2,36000007	1,039700028	1888	2,27	**SDW-R-T0**	a
14	1,791600004	0,802900031	1433,28	2,23	**SDW-R-T0**	b
15	1,407600004	0,63389999	1126,08	2,22	**SDW-R-T1**	a
16	0,914900009	0,41069999	731,92	2,23	**SDW-R-T1**	b
17	0,466300022	0,210800011	373,04	2,21	**SDW-R-T2**	a
18	0,650599994	0,292100005	520,48	2,23	**SDW-R-T2**	b
19	0,924399976	0,413200013	739,52	2,24	**SDW-R-T3**	a
20	1,053600006	0,476799984	842,88	2,21	**SDW-R-T3**	b
21	1,652700000	0,739700016	1322,16	2,23	**SDW-R-T4**	a
22	1,38340003	0,623500023	1106,72	2,22	**SDW-R-T4**	b
23	0,834500004	0,376200005	667,6	2,22	**SDW-R-T5**	a
24	1,690499995	0,75820002	1352,4	2,23	**SDW-R-T5**	b
25-36	260	280	Conc ng/µl	Ratio	Sample ID (3)	
25	1,73110006	0,757100023	1384,88	2,29	**PAW-R-T0**	a
26	1,626699973	0,72750001	1301,36	2,24	**PAW-R-T0**	b
27	2,244199984	1,011999942	1795,36	2,22	**PAW-R-T1**	a
28	0,962900028	0,432899989	770,32	2,22	**PAW-R-T1**	b
29	0,403700005	0,183800001	322,96	2,2	**PAW-R-T2**	a
30	2,087200075	0,939900026	1669,76	2,22	**PAW-R-T2**	b
31	1,630599953	0,733100023	1304,48	2,22	**PAW-R-T3**	a
32	1,349700015	0,606499977	1079,76	2,23	**PAW-R-T3**	b
33	0,970599953	0,438900001	776,48	2,21	**PAW-R-T4**	a
34	1,453099947	0,652899988	1162,48	2,23	**PAW-R-T4**	b
35	0,979700025	0,441599987	783,76	2,22	**PAW-R-T5**	a
36	1,605200049	0,721299998	1284,16	2,23	**PAW-R-T5**	b
37-48	260	280	Conc ng/µl	Ratio	Sample ID (4)	
37	2,030999966	0,8904	1624,8	2,28	**PAW-RI-T0**	a
38	2,059900086	0,921299975	1647,92	2,24	**PAW-RI-T0**	b

39	1,133499987	0,510600023	906,8	2,22	**PAW-RI-T1**	a
40	1,596000053	0,714399993	1276,8	2,23	**PAW-RI-T1**	b
41	1,597700045	0,715600006	1278,16	2,23	**PAW-RI-T2**	a
42	1,277099982	0,577100009	1021,68	2,21	**PAW-RI-T2**	b
43	0,32289999	0,148500007	258,32	2,17	**PAW-RI-T3**	a
44	1,140699979	0,515299998	912,56	2,21	**PAW-RI-T3**	b
45	1,022599984	0,461800031	818,08	2,21	**PAW-RI-T4**	a
46	0,442199986	0,201500006	353,76	2,19	**PAW-RI-T4**	b
47	0,671000019	0,303599995	536,8	2,21	**PAW-RI-T5**	a
48	0,672599971	0,30419999	538,08	2,21	**PAW-RI-T5**	b
49-60	260	280	Conc ng/µl	Ratio	Sample ID (5)	
49	1,379500035	0,599799994	1103,6	2,3	**VAL-R-T0**	a
50	0,947700035	0,42569999	758,16	2,23	**VAL-R-T0**	b
51	0,995500032	0,446700025	796,4	2,23	**VAL-R-T1**	a
52	1,919499949	0,858899988	1535,6	2,23	**VAL-R-T1**	b
53	1,971499935	0,883900009	1577,2	2,23	**VAL-R-T2**	a
54	1,807999954	0,81370002	1446,4	2,22	**VAL-R-T2**	b
55	1,766300049	0,793299988	1413,04	2,23	**VAL-R-T3**	a
56	0,421299998	0,191399992	337,04	2,2	**VAL-R-T3**	b
57	1,557399988	0,700800005	1245,92	2,22	**VAL-R-T4**	a
58	0,727799974	0,329599995	582,24	2,21	**VAL-R-T4**	b
59	0,852800004	0,387300003	682,24	2,2	**VAL-R-T5**	a
60	0,791900009	0,35730001	633,52	2,22	**VAL-R-T5**	b
61-72	260	280	Conc ng/µl	Ratio	Sample ID (9)	
61	1,487500016	0,68540002	1190	2,17	**ASM-T0**	a
62	2,492000043	1,137199946	1993,6	2,19	**ASM-T0**	b
63	1,622700054	0,731300026	1298,16	2,22	**ASM-T1**	a
64	1,68329997	0,760800015	1346,64	2,21	**ASM-T1**	b
65	0,974499948	0,43909999	779,6	2,22	**ASM-T2**	a
66	1,798400044	0,810000017	1438,72	2,22	**ASM-T2**	b
67	1,787099987	0,803799994	1429,68	2,22	**ASM-T3**	a
68	1,124400053	0,512299981	899,52	2,19	**ASM-T3**	b
69	1,02379996	0,460999992	819,04	2,22	**ASM-T4**	a
70	1,112000048	0,49589999	889,6	2,24	**ASM-T4**	b
71	1,594699994	0,71920003	1275,76	2,22	**ASM-T5**	a
72	1,428899944	0,642800011	1143,12	2,22	**ASM-T5**	b
73-84	260	280	Conc ng/µl	Ratio	Sample ID (10)	
73	1,012000069	0,433200046	809,6	2,34	**JA-T0**	a
74	2,124199886	0,956800003	1699,36	2,22	**JA-T0**	b
75	1,261999998	0,566599984	1009,6	2,23	**JA-T1**	a
76	1,784000013	0,801599976	1427,2	2,23	**JA-T1**	b
77	0,915599968	0,415499989	732,48	2,2	**JA-T2**	a
78	1,347700037	0,606299989	1078,16	2,22	**JA-T2**	b
79	0,748099975	0,340500008	598,48	2,2	**JA-T3**	a
80	0,663899995	0,302199993	531,12	2,2	**JA-T3**	b
81	0,378800001	0,173299998	303,04	2,19	**JA-T4**	a
82	0,346000001	0,157699998	276,8	2,19	**JA-T4**	b
83	0,770500015	0,347999997	616,4	2,21	**JA-T5**	a
84	0,683200024	0,309200007	546,56	2,21	**JA-T5**	b

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