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ORIGINAL ARTICLE

Evaluation of antibacterial activity of *Stenotrophomonas maltophilia* against *Ralstonia solanacearum* under different application conditions

K.M. Elhalag[1], N.A.S. Messiha[1], H.M. Emara[2] and S.A. Abdallah[2]

1 Bacterial Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt
2 Faculty of Science, Botany Department, Benha University, Benha, Qalubia, Egypt

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Correspondence
Kamel M. Elhalag, 3 Elamira Fatma Ismail St., Dokki, Giza, Egypt.
E-mail: kamel_moon_82@yahoo.com

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Abstract

Aim: The aim of this study was the monitoring of different mechanisms involved in the antibacterial activity of the biocontrol agent, *Stenotrophomonas maltophilia* (PD4560), against *Ralstonia solanacearum in vitro* and *in vivo*. Optimization of conditions that favour these mechanisms was the second target of this study.

Methods and Results: Proteolytic activity of *Sten. maltophilia* (PD 4560), was tested on skimmed milk medium. The biocontrol agent was able to produce an alkaline serine protease enzyme with a molecular weight of 40 KDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses. Spraying of salicylic acid (SA) led to an increase in the efficacy of *Sten. maltophilia* in controlling the *Ralstonia* potato wilt while spraying of ammonium sulphate (AmS) did not affect the biocontrol efficacy. The efficacy was correlated with the expression of protease enzyme genes; *Prt* genes (mainly *PrtP* and *Prt4*) and *PR* genes (mainly *PR-1* and *PRQ*) as evaluated using real-time polymerase chain reaction analysis.

Conclusions: The biocontrol activity of *Sten. maltophilia* can be attributed to the direct mechanism alkaline serine proteolytic enzyme production and through induction of host systemic acquired resistance as indirect mechanism. Tuber bulking was the most suitable physiological growth stage to apply either SA or the biocontrol agent.

Significance and Impact of the Study: Both SA and peat-moss as an organic carrier enhanced the antibacterial efficiency of the biocontrol agent. Application of *Sten. maltophilia* is more suitable under alkaline soil conditions.

Introduction

Potato is an important cash crop and represents economic value for Egypt. The gross production value during 2011 was 821 million US$ according to FAO statistics. Potato is representing the first vegetable export crop in Egypt, with an annual export rate of about 250 000 tonnes representing about 15% of the annual production. The Egyptian potatoes are exported to EU countries, Russia, Eastern European countries and many Arab countries. The value of potato export during the season 2012/2013 was 147·4 Million Euros, while, the value of imported EU potato seeds during the same season was 61·3 Million Euros, that is, the net value balance was 86·1 Million Euros. Potato brown rot (bacterial wilt or BW) is considered the most important obstacle which hinders the smooth flow of the Egyptian potatoes to different international markets. The causal agent is *Ralstonia solanacearum* race 3, biovar 2, Phyllotype II, sequevar 1 (Prior and Fegan 2005).

Ralstonia solanacearum is a soil-borne pathogen that invades the host through wounds and colonizes the xylem tissues reducing water transport and eventually leads to plant wilt and death. The pathogen grows in high

densities in the stem, but remains limited to the xylem (Denny 2006). Control of potato brown rot was tried for decades without much success. Breeding of tolerant potato varieties is not practical as they may not be adapted to different agro-ecological zones and may not be effective against different *R. solanacearum* strains (Lopez and Biosca 2004). Moreover, tolerant varieties could harbour the pathogen as latent infection (Priou *et al.* 1999).

Chemical control and use of antibiotics are unacceptable control methods nowadays due to the health and environmental hazardous effect of the chemical residues (Parrott and Kalibwani 2004), but cultural and biological control could be promising alternative methods (Ran *et al.* 2005a, b; Seleim *et al.* 2011). Observed lack of consistency of the biological control methods under field conditions may relate to abiotic factors or to soil microbial biodiversity which affects the establishment and colonization of the crops' rhizosphere by the biocontrol agents. Sufficient colonized biocontrol agents along with sustainable activity leads to successful plant protection (Latour *et al.* 2009). Potato is considered an excellent model in developing modern biological control strategies. Potato rhizosphere is rich in plant growth-promoting rhizobacteria and biocontrol agents with microbial siderophores, antibiotics, biosynthesis of surfactants, induced systemic resistance and other control strategies (Diallo *et al.* 2011). *Agrobacterium*, *Bacillus* and *Pseudomonas* are known to dominate the potato rhizobacteria species, to while a lesser extent, *Stenotrophomonas* spp were found to stimulate to potato growth and was antagonistic to many potato fungal pathogens (Diallo *et al.* 2011 and Kumar and Audipudi Amrutha (2015). *Stenotrophomonas maltophilia* is able to colonize in potato rhizosphere and persist in its endophytic tissues (Garbeva *et al.* 2001), this enhances the ability of this micro-organism to protect potato against the *R. solanacearum* pathogen. A wild strain of *Sten. maltophilia* was first described as an effective antifungal organism (Zhang and Yuen 1999). Xanthobaccines antibiotics A, B and C are antifungal compounds which were isolated from the culture filtrate of *Sten. maltophila* and were responsible to suppress *in vivo* the damping-off of beet seedlings caused by *Pythium* spp. However, *in vitro* trials made by Nakayama *et al.* (1999), showed that Xanthobaccines were not effective against many gram-negative and gram-positive saprophytic bacteria, *R. solanacearum* was not included in that study. Recently, *Sten. maltophilia* was proven to degrade the 3-hydroxy palmitic acid methyl ester (3OH-PAME), the main quorum sensing molecule, which govern the virulence of *R. solanacearum* (Achari and Ramesh 2015). Messiha *et al.* (2007) assumed that the antibacterial potential of *Sten. maltophilia* (PD3353 and PD 4560) is related to the proteolytic activity of these biocontrol agents. This protease may be active against *R. solanacearum* by attacking the Braun lipoprotein layer which is present next to lipopolysaccharide layer in the cell wall of gram-negative bacteria. The bactericidal effect of the serine protease was supposed by Gabay (1994). Dunne *et al.* (2000) related the improved biocontrol activity of *Sten. maltophilia* W81 against *Pythium ultimum* to overproduction of extracellular serine protease. The proteolytic activity may support the antagonistic potential of *Sten. maltophilia* indirectly by hydrolysing complicate proteins into simple available form of nitrogenous compounds. These simple compounds enhance the growth of the biocontrol agent and hence its antagonistic potential.

In this study *in vitro* and *in vivo* studies were conducted more deeply to evaluate the potential efficacy of *Sten. maltophilia* (PD 4560). This evaluation is based on studying the optimum conditions that favour the activity of protease enzyme as a direct antagonistic mechanism against *R. solanacearum*. Potato defence mechanisms through expression of pathogenesis-related proteins were also considered. The mechanisms of induced resistance against *R. solanacearum* invasion were investigated.

Materials and methods

Bacterial strains

Pathogen strains

A mixture of three strains of *R. solanacearum* race 3 biovar 2 was used as pathogens, K3, K10 and K16, which were originally isolated from diseased potato tubers showing typical brown rot symptoms by Elhalag (2008). K3 was isolated from the Meet Ghamr district, El-Dakahlia governorate, while K10 and K16 were isolated from Talia district, El-Monofya governorate.

Biocontrol agent strain

The wild type *Sten. maltophilia* strain (PD 3533) was isolated from the rhizosphere of eggplant (*Solanum melongena*; Black Beauty), grown in Kafr El-zayat, Gharbia governorate in the Nile Delta of Egypt, and screened for antagonistic potential by Messiha (2001). The *Sten. maltophilia* strain used in this study is a mutant strain resistant to rifampicin and chloramphenicol (100 μg ml^{-1} each) (PD 4560) (Messiha *et al.* 2007). Similar antagonistic characteristics for both the wild and the mutant strains were confirmed (Messiha *et al.* 2007 and Elhalag *et al.* 2015). Both the wild type and mutant strains were confirmed to be nonhuman pathogenic (Elhalag 2014; Elhalag *et al.* 2015). Since the reason of the antibacterial activity of *Sten. maltophilia* was unknown, the hypotheses of possible direct inhibition of the pathogen as a result of

proteolytic activity as well as indirect inhibition through induction of SAR were tested.

Proteolytic activity screening test (proteases) of Stenotrophomonas maltophilia strains *in vitro*

The proteolytic activity of *Sten. maltophilia* strain (PD 4560) was screened in an *in vitro* culture plate bioassay using medium containing skim milk (Chantawannakul *et al.* 2002). Disks of filter paper were saturated with a suspension of *Sten. maltophilia* (10^9 CFU ml^{-1}) and placed on to the centre of each plate (at least three replicates with consideration of negative control plates). The plates were incubated for 48 h at 28°C. The development of clear (halo) zones around the saturated disks on the opaque skim milk-containing plates proved the proteolytic activity of the strain. The test was repeated two times and the diameter of the clear zone was measured.

Extraction, purification and characterization of *Stenotrophomonas maltophilia* protease enzyme

In vitro *extraction of protease enzyme produced by* Stenotrophomonas maltophilia *(PD 4560)*

Broth medium with the previously described conditions, for producing the protease enzyme, was inoculated with 5% (v/v) suspension (5×10^6 CFU ml^{-1}) of *Sten. maltophilia* (PD4506) bacteria and incubated for 48 hrs. Protease enzyme was extracted according to Dubey *et al.* (2010).

Ammonium sulphate fractionation for protease enzyme produced by Stenotrophomonas maltophilia *(PD 4560)*

The cells of *Sten. maltophilia* were harvested from the inoculated broth after 48 h of incubation by centrifugation at 10,000 rev min^{-1} (8300 ***g***) for 15 min. The pellet was collected in a Falcon tube and stored at −4°C for further study while the supernatant was used for enzyme extraction by AmS saturation method (Padmapriya *et al.* 2012; Pallavi *et al.* 2011; Das and Prasad 2010; Miyaji *et al.* 2005). The protein fraction was precipitated with 85% AmS and was centrifuged at 10,000 rev min^{-1} (8300 ***g***) for 10 min. The precipitate was collected and resuspended in 25 mmol l^{-1} NaCl; 10 mmol l^{-1} Tris base buffer (Applied Biosystems, Carlsbad, CA).

Purification and characterization of molecular weight of proteases

Protease enzyme extracted from *Sten. maltophilia* strains was purified by filtration chromatography method using Sephadex G-75 gel according to Pallavi *et al.* 2011. Molecular weight of the purified enzymes was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method (Laemmli 1970) at the central lab of faculty of agriculture, Cairo University, Egypt. Standard molecular markers (Fermentas, life sciences, UK) consisted of a mixture of seven purified proteins that resolved into sharp bands in the range of 14·4–116 kDa.

Determination of factors affecting the production of the protease enzyme by *Stenotrophomonas maltophilia* (PD 4560)

Effect of pH on protease production

The effect of pH on protease production from *Sten. maltophilia* (PD 4560) was carried out using different pH (4·7, 5·5, 7·0, 8·5, 9·5 and 10·5). The medium used for optimized production of protease enzyme consisted of glucose 1% (w/v), casein 0·5%, yeast extract 0·55%, KH_2PO_4 0·2%, Na_2CO_3 1%, $MgSO_4$ $7H_2O$ 0·2% and 20 g agar per one liter (pH 8·0) (Das and Prasad 2010). Disks saturated with *Sten. maltophilia* were placed on the surface of the plates of the medium mentioned above with the different pH and the protease production was observed after 48 h at 28°C. The most suitable pH for proteolytic activities was confirmed by the development of the largest clear zone (halo) around the disks. The test was repeated two times and the results were the average of three replicates for each treatment.

Effect of nitrogen sources on enzyme production

The effects of different nitrogen sources on enzyme production by *Sten. maltophilia* (PD 4560) were investigated based on the experiments by Das and Prasad (2010) and Sevinc and Demirkan (2011) by using different organic nitrogen sources including skim milk, tryptone, casein, methionine, alanine, tyrosine as well as inorganic sources, $(NH_4)_2SO_4$ and KNO_3. The effect of different nitrogen sources were tested by replacing the peptone and yeast extract by each nitrogen source separately with the consideration of adding the same concentration of nitrogen to each growth medium. The most suitable nitrogen source was confirmed by the development of the largest zone of clearing (halo) around the colonies. The test was repeated two times and the results were the average of three replicates for each treatment.

Detection of genes (Prt4, Prt5 and Prt6) encoded for the production of serine protease enzymes of Stenotrophomonas maltophilia *(PD 4560)* in vitro *under different conditions by conventional PCR*

Detection of the cell wall-associated serine proteinases genes *(Prt4*, *Prt5* and *Prt6)* was done for *Sten. maltophilia* (PD 4560) isolates grown under above mentioned conditions (different pH and nitrogen sources). Culture pellets of *Sten. maltophilia* (PD 4560) were collected from 48-h

culture broth by centrifugation (10000 rev min^{-1}) and used for DNA extraction as described by Elhalag *et al.* (2015). PCR was performed using a Biometra, T-Personal thermal cycler according to Pastar *et al.* (2006).

Effect of foliar spraying of either SA or AmS on the efficiency of the biocontrol agent *Stenotrophomonas maltophilia* (PD 4560) in controlling potato wilt disease under greenhouse conditions

Expression of genes that may involve in the direct disease suppression through encoding the protease enzyme as well as genes that may involve in the induction of systemic acquired resistance (SAR) were studied under greenhouse conditions. Factors affect expression or repression of these genes were also studied.

The experiment was conducted using clay soil from El-Gharbia governorate after confirming the absence of the pathogen. Moderate mature potato cultivar Lady Rosette was used. Potato eyepieces were immersed for 5 min in 0·05% (aq.) NaOCl, washed in sterile distilled water, and air-dried for 48 h. Soil was infested with a mixture of the three virulent Egyptian strains of race 3 biovar 2 of *R. solanacearum*: K3, K10 and K16. The bacterial strains were grown for 48 h on nutrient agar (NA) plates at 28°C. The bacterial cultures were suspended in 0·01 mol l^{-1} phosphate buffer (PB) and the bacterial density was adjusted to $6{\cdot}1 \times 10^9$ CFU ml^{-1} using a 6300 spectrophotometer (Jenway, UK). The inoculum was added at ratio of (1:10 (v/v). Soil pots were filled with 3 kg soil, four replicates for each treatment with consideration of four more pots added to each treatment for gene expression studies. The inoculum density of the *Sten. maltophilia* (PD 4560) suspension was adjusted to approx. $4{\cdot}2 \times 10^9$ CFU and was mixed with the soil at a ratio of 1:10 (v/v). *Stenotrophomonas maltophilia* was either mixed directly with the soil or carried on peat-moss to achieve the same final concentration in soil, peat-moss was mixed with the soil at rate of 2% wt. The soil was mixed with the pathogen and the antagonist either amended with AmS, SA or left un-amended. Controls were considered, negative control, controls with pathogen only for different amendments and antagonist only. Sodium salicylate and AmS were sprayed onto upper and lower leaf surfaces of potato plants at 30 and 45 days from potato cultivation at a concentration of 0·6 g l^{-1} (Ji and Wilson 2003). The conditions of the greenhouse were adjusted to 25°C during the day and 20°C during the night, with a RH of 75–80% and a total of 14 h light.

Wilt severity was recorded daily for all different pots. Count of both the pathogen (*R. solanacearum*) and the biocontrol agent (*Sten. maltophilia*) were recorded in soil at 0, 40 and 80 days as well as at the end of the experiment; from soil, potato rhizosphere and potato endophytic tissues. The count of the pathogen was made on SMSA plating while the antagonist count was made on TSBA medium supplemented with 100 ppm chloramphenicol and rifampicin.

*Analysis of in planta gene expression of either pathogenesis-related protein genes (*PR *genes) in potato and genes encoded for proteases (*Prt *proteins) of the biocontrol agent*

For studying gene expression level at different plant growth stages, two-step RT-PCR was performed. One replicate from each treatment and as one replicate for each stage were used for gene expression of specific marker genes in plant which encoded for plant pathogenesis-related proteins, that is, *PR-1*, *PR-2* and *PR-Q* as well as genes encoded for proteolytic activities of *Sten. maltophilia* (*Prt* proteins), that is, *PrtM*, *PrtP*, *P1*, *Prt4*, *Prt5* and *Prt6*, during the different stages of potato growth. These stages included: vegetative growth (at 30 days after potato cultivation), tuber initialization (at 45 days after planting), tuber bulking (at 65 days after planting) and maturation (at 80 days after planting).

The apices of the youngest three leaves of potato plants during different stages of growth were sampled from different treatments and stored at −30°C till analysis. The frozen leaves were immediately grinded thoroughly under liquid nitrogen to avoid thaw of the tissues. The formed powder was collected into separate micro-centrifuge tubes. Total RNA extraction was performed directly from the endophytic plant tissues using RNA easy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Concentrations of eluted RNA were determined by using a nanodrop spectrophotometer (Thermo scientific) at wavelength (280 nm), then stored at −4°C for (7 days) for further analysis.

Reverse transcription-polymerase chain reaction

Reverse transcription (mRNAs conversion into cDNAs) was carried out using the high-capacity cDNA reverse transcription Kits (Applied biosystem) according to the manufacturer's protocol. The input amount of total RNA for all samples was adjusted to 2 μg (20 ng μl^{-1}) of total RNA per 20-μl reaction. The cDNA RT reactions were prepared by pooling 10 μl of 2× RT master mix with 10 μl of sample RNA into each well of 96-well reaction plate. The total 20 μl reaction volume was loaded into the thermal cycler (Biomtera, T personal) and thermal cycling condition was programmed as follows: Step 1 (25°C for 10 min), Step 2 (37°C for 120 min), Step 3 (85°C for 5 min) and Step 4 (4°C on hold). The concentration of the (cDNA) yield was measured using nan-

odrop spectrophotometer (Thermo scientific) and stored at −30 for further work.

Analysis of gene expression level

Expression level of different genes were estimated using (7500, Applied biosystem). The products of cDNA from the previous step were used as templates and the amplification were carried out using specific primer sets. Primers *PR-1*, *PR-2* and *PR-Q* encoding for pathogenesis-related proteins were selected (Sarowar *et al.* 2005). *PR-1* and *PR-2* encode for harpin protein while *PR-Q* encodes for acidic chitinase or *β*-1- glucanase. Primers *PrtM* and *PrtP* encoding for Parvulin-like peptidyl-prolyl isomerase proteinase maturase protein and subtilisin-like serine protease respectively, were also selected (Wang *et al.* 2012). Primers *P1* (Sheng *et al.* 2006), *Prt4*, *Prt5* and *Prt6* encoding for cell wall-associated serine proteinases of *Sten. maltophilia* were selected (Vermeulen *et al.* 2005). Reaction mixtures for RT -PCR contained 12·5 μl (1×) of Quantimix with SYBR Green (Biotools). RT-PCR Master Mix, 1 μl (10 pm) of forward and reverse primers, 8 μl of RNAs-free H_2O, 2·5 μl cDNA was finally added as template. The master mix with samples of 25-μl reaction volume was assembled in a 100-μl micro-centrifuge tube on ice then cDNA for each sample was applied to the reaction master mix. In addition to the samples to be analysed, a negative control reaction without cDNA was included; Reference genes (internal control) with specific primers were included for each sample for both potato plant (cox gene) designed for the potato cytochrome oxidase gene sequence (Wilmer *et al.* 2006) and the *RMLA* gene specific for *Sten. maltophilia* that encodes for cell morphology (Huang and Wong 2007). The thermo-cycler program was performed according to Pérez-Osorio and Franklin 2008. The fluorescence data were acquired at the 72°C extension, SYBR Green binds all produced double stranded DNA emitting fluorescent signal with increasing signal intensity by increasing cycle number. The specificity and identity of the RT-PCR products were verified by performing a melt curve analysis: A melting curve analysis (Dissociation stage) was added that ramps from 72 to 99°C, raised by 1°C in each step, and waited for 30 s on the first step and 5 s each step afterward.

Statistical analysis

Data of area under disease suppressive curve (AUDPC), wilt severity as well as log transformed population of the pathogen and the antagonist (log (CFU + 1) were not normally distributed so analyses of variance could not be carried out and nonparametric analysis (Mann–Whitney test, asymptote significance (Asym. sig., 2-tailed) were conducted instead using SPSS Inc., Chicago, IL.

Results

Bacterial strains

The virulence of the *R. solanaceraum* used strains were proven using 3–5 leaves stage of tomato seedlings. The antagonistic potential of *Sten. maltophilia* (PD3533) and (PD4560) against the three used *R. solanacearum* strains was confirmed *in vitro*.

Extraction, purification and characterization of *Stenotrophomonas maltophilia* protease enzyme

Proteolytic activity of Stenotrophomonas maltophilia *strain (PD 4560) on skim milk agar medium*

Stenotrophomonas maltophilia (PD 4560) showed proteolytic activity and was able to produce protease spot on skim milk agar medium. The clear zone of proteolytic activity was 0·9 cm around *Sten. maltophilia* culture (data not shown).

Detection of protease enzyme produced by Stenotrophomonas maltophilia *(PD 4560) in broth medium using sodium dodecyl sulphate polyacrylamide gel electrophoresis*

The enzyme from *Sten. maltophilia* (PD 4560) purified by gel filtration method (Sephadex G75) appeared as a single band on SDS-PAGE corresponding to a molecular mass of approx. 40 kDa as compared to the protein marker used in this study Fig. 1.

The Effect of foliar spraying of either SA or AmS, individually on the efficiency of the biocontrol agent *Sten. maltophilia* (PD 4560) in controlling potato wilt disease caused by the pathogenic *R. solanacearum* under greenhouse conditions.

Determination of factors affecting the production of the protease enzyme by *Stenotrophomonas maltophilia* (PD 4560)

Effect of different pH and amino acids containing in the medium on protease enzyme production by Stenotrophomonas maltophilia *(PD 4560)*

Stenotrophomonas maltophilia showed the highest proteolytic activity as indicated by the clear zone around *Sten. maltophilia* spot as well as most vigorous growth at pH 8·5 followed by pH 9·5 and at pH 10·5. *Stenotrophomonas maltophilia* showed weak proteolytic activity and growth at pH 5·5 and 7·0. *Stenotrophomonas maltophilia*

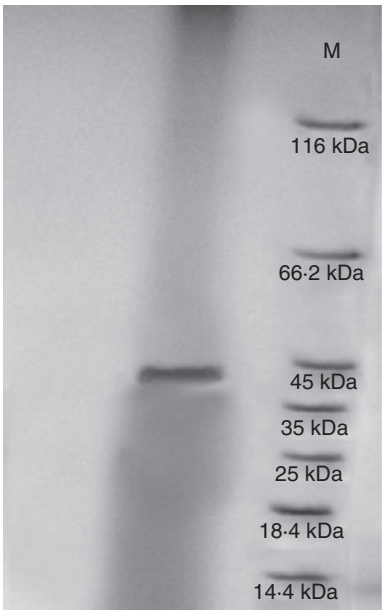

Figure 1 Detection of protease enzyme produced by the isolate of *Sten. maltophilia* (PD 4560) in broth medium after incubation for 48 h at 28°C, using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS page). M, protein marker (14.4–116) kDa. The single band represents the protease enzyme at 40 kDa. The test was repeated two times.

was unable to grow and hence no clear zone at pH 4·7 was detected (Data not shown).

Medium containing skim milk amino acid showed the best proteolytic activity but showed moderate growth of *Sten. maltophilia*. On the other hand, medium containing tryptone and methionine showed the highest growth without showing any proteolytic activity (Data not shown). Alanine and tyrosine showed only moderate growth without any proteolytic activity. Casein showed moderate growth of the antagonist and moderate proteolytic activity. AmS and potassium nitrate inhibited the growth of *Sten. maltophilia*. In conclusion, the most active proteolytic activity was shown under alkaline conditions (pH 8·5) and on skim milk growth medium followed by Casein growth medium.

*Detection of genes (*Prt4, Prt5 and *Prt6) encoded for the production of serine protease enzymes of* Stenotrophomonas maltophilia *(PD 4560)* in vitro *under different conditions by conventional PCR*

Prt4, *Prt5* and *Prt6* genes encoded the serine protease enzyme were detected for *Sten. maltophilia* isolates grown under all different conditions. The amplicon size was 120 bp compared to *Sten. maltophilia* control. The genes were not detected in any *R. solanacearum* strain used in this study.

Effect of foliar spraying of either SA or AmS on the efficiency of the biocontrol agent *Stenotrophomonas maltophilia* (PD 4560) in controlling potato wilt disease under greenhouse conditions

Disease incidence were assessed according to five indicators, disease severity, AUDPC, count of the pathogen in soil apart, potato rhizosphere and crown area of potato plants at the end of the experiment, after 80 days of pathogen inoculation (Table 1). Counts of *Sten. maltophilia* (PD4560) on its selective medium were done at the day of inoculation and at the end of the experiment, after 80 days of inoculation (Table 1). Data were not normally distributed so analyses of variance could not be carried out and nonparametric analysis (Mann–Whitney test, spss v16) were conducted instead. Foliar spraying of SA (SA concentration: 0·6 g l^{-1}) at potato tuber initialization and tuber bulking growth stage resulted in an increase in the suppressive effect of the biocontrol by *Sten. maltophilia* as indicated by 100% decrease in wilt severity and AUDPC as well as 18·6% decrease in count of *R. solanacearum* in potato rhizosphere $P = 0{\cdot}021$ (Table 1). SA alone was effective in disease suppression as indicated by 100% decrease in AUDPC and disease severity ($P = 0{\cdot}014$ and 0·08 respectively) as well as a decrease in the count of *R. solanacearum* in soil after 80 days from soil inoculation (5%, $P = 0{\cdot}043$), rhizosphere (37%, $P = 0{\cdot}021$) and crown area (7%, $P = 0{\cdot}043$) of potato plants as compared to positive control (soil inoculated with *R. solanacearum* only) (Table 1). *Stenotrophomonas maltophilia* increased the supressiveness of SA as indicated by 44% decreasing the count of *R. solanacearum* in soil, 19% in rhizosphere and 43% in crown area, after 80 days from soil inoculation with $P = 0{\cdot}021$ for all mentioned parameters according to the nonparametric statistical analysis (Mann–Whitney), asymptote significance (Asym. sig.) (2-tailed) (Table 1). Application of *Sten. maltophilia* alone was able to suppress the potato wilt disease as indicated by 75% decrease in disease severity ($P = 0{\cdot}04$), 84% decrease in AUDPC ($P = 0{\cdot}018$), decrease in count of *R. solanacearum* after 80 days soil inoculation (45% decrease in soil, 35% decrease in rhizosphere and 69% decrease in crown area of potato plants, $P = 0{\cdot}021$) (Table 1). Application of AmS alone as a nutritional element by foliar spraying, at the same concentration and the same potato growth stage for SA application, was unable to suppress the disease incidence with exception of 27% decrease in AUDPC ($P = 0{\cdot}021$). AmS spraying in the presence of

Table 1 Effect of AmS and SA on the efficiency of *Stenotrophomonas maltophilia* as biocontrol agent against potato bacterial wilt

					Pathogen (Mean ± SE)				Biocontrol (Mean ± SE)			
Treatments	*Sten. mph*	Peat-moss	Wilt severity	AUDPC	Log_{10} CFU in soil ($T0^4$)	Log_{10} CFU in soil ($T80^5$)	Log^{10} CFU RS. $Rhizo^6$ ($T80^5$)	Log_{10} CFU in $crown^7$ ($T80^5$)	Log_{10} CFU ($T0^4$) in soil	Log^{10} CFU soil ($T80^5$)	Log_{10} CFU in $Rhizo^6$ ($T80^5$)	Log_{10} CFU $crown^7$ ($T80^5$)
Tap water (control)	No *Sten. mph.*	No Peat-moss	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pathogen (*R.s.*)^0 (control)	No *Sten. mph.*	No Peat-moss	100 ± $0^{a,b,c}$	1143·9 ± $38^{a,b}$	7·73 ± 0·01	6·43 ± $0·05^a$	6·63 ± $0·04^{a,c}$	6·34 ± $0·05^a$	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Biocontrol (*Sten. mph.*[2] control)	*Sten. mph.*	No Peat-moss	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	7·69 ± 0·02	4·82 ± $0·1^a$	3·86 ± $0·1^a$	1·98 ± 0·06
R.s + *Sten. mph.*[2]	*Sten. mph.*	No Peat-moss	25 ± $25^{a,b}$	186·5 ± $186^{a,b}$	7·78 ± 0·01	3·54 ± $0·04^a$	4·15 ± $0·1^c$	1·95 ± $0·5^a$	7·79 ± 0·01	4·36 ± $0·01^{c,b}$	5·09 ± $0·01^{b,c}$	1·84 ± $0·1^{b,c}$
R.s + SA[1]	No *Sten. mph.*	No Peat-moss	0 ± 0^a	0 ± 0^a	7·69 ± 0·03	6·08 ± $0·1^{a,b}$	4·17 ± $0·06^{a,b}$	5·9 ± $0·1^{a,b}$	0 ± 0	0 ± 0	0 ± 0	0 ± 0
R.s + *Sten. mph.*+ SA[1]	*Sten. mph.*	No Peat-moss	0 ± 0^b	0 ± 0^b	7·71 ± 0·04	3·43 ± $0·03^{a,b,c}$	3·38 ± $0·03^{b,c}$	3·34 ± $0·03^{a,b,c}$	7·72 ± 0·01	2·96 ± $0·06^e$	5·27 ± $0·04^{b,e}$	2·6 ± $0^{b,e}$
R.s. + *Sten. mph.* + Peat-moss+ SA	*Sten. mph.*	Peat-moss	0 ± 0^c	0 ± 0	7·69 ± 0·004	2·39 ± $0·05^c$	1·79 ± $0·03^c$	2·29 ± $0·05^c$	7·71 ± 0·02	6·6 ± $0·02^e$	6·57 ± $0·02^e$	3·18 ± 0^e
R.s + AmS[3]	No *Sten. mph*	No Peat-moss	100 ± 0	843·2 ± 27^a	7·66 ± 0·04	6·39 ± 0·06	6·49 ± 0·03	6·29 ± 0·06	0 ± 0	0 ± 0	0 ± 0	0 ± 0
R.s + *Sten. mph.*+ AmS[3]	*Sten. mph.*	No Peat-moss	69·7 ± $2·2^d$	577·3 ± 19^d	7·64 ± 0·01	5·07 ± $0·07^{a,d}$	4·95 ± $0·01^{a,d}$	4·97 ± $0·07^{a,d}$	7·56 ± 0·1	3·76 ± $0·05^{a,b,d}$	3·54 ± $0·08^{a,c,d}$	1·9 ± 0^d
R.s + *Sten. mph.* + Peat-moss +AmS[3]	*Sten. mph.*	Peat-moss	0 ± 0^d	0 ± 0^d	7·66 ± 0·01	3·79 ± $0·1^d$	3·84 ± $0·07^d$	3·7 ± $0·1^d$	7·73 ± 0·01	5·04 ± $0·06^{c,d}$	5·14 ± $0·09^{,d}$	2·84 ± $0^{c,d}$

AUDPC, area under disease suppressive curve.

Similar letters (a, b, c, d, e) indicate significant difference between different treatments within the same parameter under investigation *P* value ranged from (0·043–0·014).

0, *R.s.* (Pathogen, *R. solanacearum*); 1, SA (Salicylic Acid); 2, the biocontrol agent *Sten. mph* (*Sten. maltophilia* (PD4560)); 3, AmS (Ammonium Sulphate); 4, 5 represent the log CFU count of both pathogen *R. solanacearum* and biocontrol *Sten. maltophilia* at zero time and 80 days respectively after inoculation of potato clay pots cv. Lady Rosetta with *R. solanacearum* and simultaneously with *Sten. maltophilia* at a concentration of 10^8 CFU ml^{-1} for each. Foliar spray of SA or AmS, at concentration 0·6 g l^{-1}, was made at two divergent development stages of potato tested (30-day-old and 45-day-old potato plants, that is, vegetative and tuber initialization respectively. Wilt severity was calculated at the end of the experiment (80 days). The experiment was repeated two times with similar results. The conditions of the greenhouse were adjusted to 25°C during the day and 20°C during the night, with a RH of 75 to 80% and a total of 14 h light.

the biocontrol *Sten. maltophilia* decreased the count of *R. solanacearum* in soil (21·1%, $P = 0{\cdot}021$) at 80 days from soil inoculation as well as the count of the biocontrol agent (21·9%, $P = 0{\cdot}043$) (Table 1). The addition of peat-moss in clay soil as a biocontrol carrier increased the suppressive effect of *Sten. maltophilia* in combination with foliar spraying of SA as indicated by decreasing the count of *R. solanacearum* after 80 days from soil inoculation in soil (30% decrease), rhizosphere (47% decrease) and crown area (30% decrease) where $P = 0{\cdot}021$ for all parameters as compared to absence of the peat-moss (Table 1). Addition of peat-moss also increased the suppression effect of *Sten. maltophilia* in combination with foliar spraying of AmS as indicated by decreasing disease incidence as indicated by 100% decrease in AUDPC and wilt severity ($P = 0{\cdot}041$ and 0·013 respectively), decrease in count of *R. solanacearum* ($P = 0{\cdot}021$) in soil (25% decrease), rhizosphere (22% decrease) and crown area (26% decrease) after 80 days from soil inoculation. SA spraying increased the count of *Sten. maltophilia* after 80 days from soil inoculation in rhizosphere (3·5% increase, $P = 0{\cdot}021$) and in crown area (41% increase, $P = 0{\cdot}021$). Addition of peat-moss in the presence of SA increasing the count of *Sten. maltophilia* in soil (123% increase), rhizosphere (25% increase) and crown area (22% increase) $P = 0{\cdot}021$ for all mentioned parameters (Table 1). On the other hand, AmS decreasing the count of *Sten. maltophilia* in soil (14% decrease, $P = 0{\cdot}021$) and suggests a trend decrease in count in the rhizosphere (30% decrease, $P = 0{\cdot}083$). Addition of peat-moss as biocontrol carrier in combination with foliar spraying of AmS increased the count of *Sten. maltophilia* in rhizosphere (45% increase) and in the crown area (49%), where $P = 0{\cdot}021$ for two parameters and a trend increase for count in soil where $P = 0{\cdot}083$ (34% increase). In conclusion, neither application of SA nor AmS significantly increased the efficiency of *Sten. maltophilia* in disease suppression except in the rhizosphere area for SA application. Peat-moss increased the survival of the biocontrol agent and hence the efficiency of the biocontrol agent.

Expression of pathogenesis-related protein genes involved in SAR in potato leaves at divergent potato growth stages with different combination systems

The three protein-related pathogenesis genes namely, *PR-1*, *PR-2* (encoding for harpin protein); *PR-Q* (encoding chitinases and 1- β glucanase); were evaluated at divergent potato growth stages (vegetative, tuber initialisation, tuber bulking and maturation stage) with different combination systems as described previously in the greenhouse experiment. The Cox gene encoding for cytochrom oxidase of potato was used as reference gene in this study. Gene expression of these genes was determined using real-time PCR (RT- PCR). Real-time PCR (applied bio system, 7500) showed different cycle threshold (C_t) values with all treatments at variant growth stages of potato. According to the manufacture protocol, $C_t \leq 30$ with typical curve plateau was considered a positive result (gene was expressed). The Cox gene (reference gene) was expressed in all variant treatments studied at all of the four potato growth stages. Melting temperature for the Cox gene was 79·8°C. ΔC_t = C_t value for the target gene- C_t value for reference gene). The first protein-related pathogenesis (*PR-1*) gene was expressed at the tuber bulking growth stage of potato plants when infected potato plants were treated with either SA or *Sten. maltophilia* separately (alone) ($\Delta C_t = 8{\cdot}96$ and 8·63 respectively). The *PR-1* gene was also expressed at the vegetative growth stage when pathogen-inoculated plants were treated with SA in combination with *Sten. maltopilia* in the absence or presence of the peat-moss, The C_t value for the target gene was around 25 ($\Delta C_t = 2{\cdot}97$ and 4·55) respectively. The *PR-1* gene was repressed by AmS and was totally unexpressed in the absence of the biocontrol agent and/or SA. The melting temperature for *PR-1* gene was 75·8°C.

The second protein-related pathogenesis gene (*PR-2*) showed expression only at the tuber initialization growth stage with pathogen alone treatment (positive control), where its C_t was 25·25 ($\Delta C_t = 0$). Melting temperature for PR2 gene was 69·9°C.

The third protein related to pathogenesis gene (*PR-Q*) was the most abundant expressed gene for pathogen inoculated plants treated with SA alone, SA in combination with the biocontrol agent in the absence or presence of peat-moss and for the biocontrol agent in combination with AmS only (Table 2). The *PR-Q* gene was expressed when SA was applied alone for pathogen inoculated plants at vegetative ($\Delta C_t = 5{\cdot}31$), tuber bulking ($\Delta C_t = 7{\cdot}49$) and maturation ($\Delta C_t = 5{\cdot}87$) potato growth stage. When SA was applied in combination of the biocontrol agent *PR-Q* gene was expressed at tuber initialization ($\Delta C_t = 5{\cdot}18$), tuber bulking ($\Delta C_t = 5{\cdot}45$) and maturation ($\Delta C_t = -1{\cdot}21$) potato growth stage (Table 2). When SA was applied in combination of the biocontrol agent and peat-moss as a carrier, *PR-Q* gene was expressed at tuber bulking ($\Delta C_t = 2{\cdot}48$) and maturation ($\Delta C_t = 4{\cdot}56$) potato growth stages (Table 2). The *PR-Q* gene also showed expression at similar potato growth stages (tuber bulking ($\Delta C_t = 6{\cdot}34$) and maturation ($\Delta C_t = 5{\cdot}93$) Thus, the level of expression of this gene was higher specifically at either tuber bulking or maturation stages with the positive peat-moss treatment. The melting temperature for *PR-Q* gene was

Table 2 Expression of the pathogenesis-related protein (PRQ), expressed *in planta* at four divergent growth stages* of potato leaves cv. Lady Rosetta with different combination systems used in this study under greenhouse conditions

Details/Primers	CT PRQ[2]											
Growth stage	Vegetative* (30 days)			Tuber initialization* (45 days)			Tuber Bulking* (65 days)			Maturation* (80 days)		
Primer name	C_t (target gene) PRQ	C_t (reference gene) Cox[a]	ΔC_T (sample)	C_t (target gene) PRQ	C_t (reference gene) Cox[a]	ΔC_T (sample)	C_t (target gene) PRQ	C_t (reference gene) Cox[a]	ΔC_T (sample)	C_t (target gene) PRQ	C_t (reference gene) Cox[a]	ΔC_T (sample)
Tap water (control)	32·26	**23·1**	Und.	34·7	**20·55**	Und.	38·68	**21·56**	Und.	Undetected	**22·05**	Und.
Pathogen *R. solanacearum* (*R.s*) (control)	Und.	**25·03**	Und.	35·01	**25·94**	Und.	31·5	**23·02**	Und.	Undetected	**22·78**	Und.
Biocontrol *Sten. maltophilia* (*Sten. mph)* (control)	32·83	**22·29**	10·54	35·54	**26·64**	Und.	30·49	**20·28**	Und.	31·65	**24·37**	Und.
R.s + SA	**28·33**	**23·02**	5·31	39·84	**25·29**	Und.	**28·51**	**21·02**	7·49	28·42	**22·55**	5·87
R.s + *Sten. mph* + SA	34·71	**23·18**	Und.	**28·63**	**23·45**	5·18	**28·95**	**23·5**	5·45	**28·66**	**29·87**	−1·21
R.s + *Sten. mph* Peat-moss +SA	Und.	**21·14**	Und.	33·54	**24·19**	Und.	**24·25**	**21·77**	2·48	**25·77**	**21·21**	4·56
R.s + AmS	31·26	**19·26**	Und.	35·03	**20·11**	Und.	32·38	**20·64**	Und.	33·46	**22·11**	Und.
R.s + *Sten. mph*+ AmS	31·7	**21·44**	Und.	35·98	**20·85**	Und.	**29·01**	**22·67**	6·34	**28·45**	**22·52**	5·93
R.s + *Sten. mph.*+ Peat-moss+ A	33·51	**18·84**	Und.	35·67	**22·2**	Und.	Undetected	**22·73**	Und.	33·54	**23·54**	Und.
Negative internal control (USDW)[3]							36·12					
Melting temperature							76·9°C					

*, Vegetative, tuber initialization, tuber bulking and maturation growth stages of potato plants; 2, PRQ (pathogenesis-related protein gene in plant encoding for glucanase); 3, ultra-pure sterilized distilled water; a, cox, housekeeping gene of potato.

Fluorescent signal was generated during real-time PCR by SYBR Green and was indicative to the amount of PCR product produced using the specific primer for the PRQ gene-encoding glucanase (Dong *et al.* 1999). $C_t > 30$ (or undetected) were considered negative result while $C_t < 30$ considered positive result (bold font cells in the table). PRQ, expressed at divergent stages of potato cv. Lady Rosetta as a result of inoculating the potato in clay pot soil by *R. solanacearum* isolates race 3 biovar 2 and simultaneously inoculated with the biocontrol agents *Sten. maltophilia* (PD 4560) at a concentration of 10^8 CFU ml^{-1} for each of the isolate used and thereafter foliar sprayed with either of SA and AmS separately in greenhouse conditions at a concentration of 0·6 g l^{-1}. C_t values shown are representing one replicate out of three replicates run by real-time PCR and the whole experiment was conducted twice.

76·9°C. *Stenotrophomonas maltophilia* alone was unable to induce *PR-Q* expression. AmS alone was unable to induce *PR-Q* expression, while combination of AmS with *Sten. maltophilia* induced *PR-Q* expression at late potato growth stages and this expression was absent in the presence of the peat-moss. SA alone or in combination with *Sten. maltophilia* showed the most expression of *PR-Q* gene while addition of peat-moss increased this expression at the late maturity stages.

Expression of genes involved in the proteolytic activity of the biocontrol agent Stenotrophomonas maltophilia *(PD 4560) in potato leaves at divergent potato growth stages with different combination systems were used in this study* (*in planta and* in vitro)

Expression of genes encoding for the protease enzyme from *Sten. maltophilia* was determined *in planta* and in culture (*in vitro*) using six different primer sets for the *P1*, *PrtM*, *PrtP*, *Prt4*, *Prt5* and *Prt6*. *Prt4*, *Prt5* and *Prt6* genes are cell wall-associated serine protease, *P1* and *PrtP* genes are exo serine protease and *PrtM* gene encoded for parvulin-like peptidyl-prolyl isomerase proteinase maturase protein. They showed different C_t values by real-time PCR. The *RM1A* gene encoding for d-glucose-1-phosphate thymidyl transferase responsible for bacterial cell morphology was used as reference gene for *Sten. maltophilia* with all combination systems used in this study. It was expressed with all treatments and at all potato growth stages. The melting temperature of *RM1A* was 78·1°C. In *in vivo* experiments, the first gene *P1*, encoded for substilin-like serine protease showed expression at both tuber bulking and maturation growth stage in both treatments involved in AmS-positive treatment in which AmS was applied at a concentration of 0·6 g/L by spraying on the leaves at initiation of vegetative stage, and the potato tubers were grown in the clay soil that were inoculated with the pathogen *R. solanacearum* simultaneously along with the biocontrol *Sten. maltophilia*, that is, *R. solanacearum* + *Sten. maltophilia* + AmS or positive peat-moss treatment as a carrier for *Sten. maltophilia* (*R. solanacearum* + *Sten. maltophilia*- peat-moss + AmS), where C_t was around 30 (ΔC_t = 12·45 and 16·0) respectively. Moreover, *P1* gene was expressed at maturation stage with SA combination treatment

Treatments				
Negative control (water control)				
Positive control (*R. solanacearum*, (*R.s.*))		*PR2*		
R.s + *Sten. maltophilia (Sten. mph)*		*PrtP*	*PR1*	*P1, Prt5*
R.s.[a] + SA			*PR1, PRQ*	*PRQ*
R.s. + *Sten. mph.* + SA	*PR1, Prt4*	*PRQ, Prt4, Prt6*	*PRQ, PrtP*	*PRQ, P1, PrtP, Prt4*
R.s. + *Sten. mph.* - Peat-moss + SA	*PR1, Prt4*	*PrtP, Prt4*	*PRQ, Prt4*	*PRQ, PrtP, Prt4, Prt5*
R.s.+ AmS				
R.s. + *Sten. mph.* + AmS			*PRQ*	*PRQ, PrtP, Prt4, Prt5*
R.s. + *Sten. mph*- Peat-moss + AmS	*PrtP*	*PrtP*		

Figure 2 The expressed genes related to both pathogenesis-related protein (*PR1*, *PR2* and *PRQ*) derived from potato plant and protease enzyme (*P1*, *Prt4*, *Prt5*, *Prt6* and *PrtP*) derived from the biocontrol agent *Sten. maltophilia* inside potato leaves at divergent stages of potato cv. Lady Rosetta under different treatments. Potato were grown in clay soil and inoculated by the pathogen *R. solanacearum* isolates race 3 biovar2 and simultaneously inoculated with the *Sten. maltophilia* (PD 4560) at a concentration of 10^8 CFU ml^{-1} for each of the isolate used and thereafter foliar sprayed with either of SA and A. sulphate separately under greenhouse conditions at a concentration of 0·6 g l^{-1}. Four pots were used for each treatments in the greenhouse located at PBRP. Data were collected three replicates run by real-time PCR and the whole experiment was conducted twice and the results were highly similar. *Sampling date.

(*R. solanacearum* + *Sten. maltophilia* + SA), where C_t was 20 (ΔC_t = 11·2). Hence these results revealed that *P1* gene expression was higher with SA-positive combination than with AmS positive combination treatment. The results of *in vitro* analysis of *P1* gene expression in synthetic growth culture, specifically used for monitoring the protease production was performed with variant pH and amino acids, showed different C_t values (Data not shown). *P1* gene was expressed with both pH 8·5 and 10·5 (ΔC_t = 5·41 and 8·42), respectively, while it was not expressed with pH 5·5 and 7·0 as well as with the presence of casein or alanine in the growth medium tested. The melting temperature of *P1* was 78·8°C. In *in vivo* experiments, the second *PrtM* gene encoded for parvulin-like peptidyl-prolyl isomerase proteinase maturase protein was never expressed neither in any of the treatments *in planta* at any of different potato growth stages nor *in vitro* synthetic growth medium of variant pH and amino acids treatments of the growth medium (Data not shown).

In the *in vivo* experiments, *PrtP* gene encodes for subtilisin-like serine protease was the most abundant gene expressed among the other tested genes in all different treatments and at all different potato growth stages (Fig. 2). It was expressed at vegetative stage in AmS-positive treatment, that is, pathogen + biocontrol - peat-moss + AmS where, C_t was around 29(ΔC_t = 4·95). It also was expressed at tuber initialization stage in three other treatments i.e. *Sten. maltophilia* alone where, C_t was 28·6 (ΔC_t = 3·34); with the AmS positive treatment, that is, pathogen+ biocontrol peat-moss+ AmS where, C_t was 23·4 (ΔC_t = 3·75) and the third one with positive peat-moss, that is, *R. solanacearum* + *Sten. maltophilia* peat-moss+ SA, where, C_t was 28·9(ΔC_t = 2·4). But at tuber bulking stage its expression was found and it was of a lesser level than at tuber initialization stage in positive SA treatment, that is, *R. solanacearum* + *Sten. maltophilia* + SA, where C_t was 28·6 (ΔC_t=7·17). However, at the maturation stage, it was expressed in *R. saloanacearum* + *Sten. maltophilia* + AmS (ΔC_t = 9·1); *R. saloanacearum* + *Sten. maltophilia* + SA (ΔC_t = 3·54) and *R. saloanacearum* + *Sten. maltophilia* peat-moss + SA (ΔC_t = 8·85). The highest expression level of *PrtP* gene was detected at maturation stage with *R. saloanacearum* + *Sten. maltophilia* + SA treatment (ΔC_t = 3·54). The *in vitro* analysis of the expression of the *PrtP* gene in synthetic growth culture with variant pH and amino acids showed that it was expressed at pH 5·5, 7·0, 8·5 and 10·5(ΔC_t = 5·4, 7·87, 3·81 and 7·95) but, it was not expressed with casein and alanine amino acids present in the growth medium used. The melting temperature of *PrtP* was 71·9°C. The genes encoded for cell wall-associated serine proteinases, that is, *Prt4*, *Prt5* and *Prt6* of *Sten. maltophilia* possess different expression levels with all treatments at all of divergent potato growth stages (Fig. 2). In the *in vivo* experiments, the first gene (*Prt4*) among the cell wall-associated protease listed above, was expressed at all four potato growth stages in *R. solanacearum* + *Sten. maltophilia* peat-moss + SA. While, it was expressed at vegetative, tuber initialization and maturation growth stages in negative peat-moss combination, that is, *R. solanacearum* + *Sten. maltophilia* + SA (Fig. 2). *Prt4*, was highly expressed with positive peat-moss treatment at either the vegetative or tuber bulking stage than the other stages (ΔC_t = 1·83 and −0·3) respectively. The *in vitro* analysis of the expression of the *Prt4* gene in synthetic growth culture with variant pH and amino acids showed similar trend of response with *PrtP* gene for either pH or amino acids. It was expressed at pH 5·5, 7·0, 8·5 and 10·5 (ΔC_t = 6·97, 6·48, 4·5 and 5·25), but, it was not expressed with casein and alanine amino acids present in the growth medium used. The melting temperature of prt4 was 73·4°C. However, *in vivo* experiments, the second gene (*Prt5*) was only expressed at maturation growth stage in *R. solanacearum* + *Sten. maltophilia* peat-moss + SA (Fig. 2). Furthermore, the analysis of *in vitro* expression of the *Prt5* gene in synthetic growth culture with variant pH and amino acids showed that it was expressed with pH (8·5) and casein amino acid. The melting temperature of prt5 was 77·6°C. In the *in vivo* experiments, the third gene (*Prt6*) was only expressed at tuber initialization growth stage in positive SA with the biocontrol along with the pathogen, that is, *R. solanacearum* + SA (Fig. 2). The *in vitro* analysis of the expression of the *Prt6* gene in synthetic growth culture with variant pH and amino acids showed that it was only expressed with pH 8·5 (Data not shown). The melting temperature of *Prt6* was 81·5°C.

Discussion

The production of proteolytic enzyme (s) by different strains of *Sten. maltophilia* isolated from soil and rhizosphere were reported earlier by Dunne *et al.* (1997) and Miyaji *et al.* (2005). This protease enzyme is suggested to play an important role in supporting *Sten. maltophilia* growth and consequently antagonism against the pathogen through hydrolysing complicated proteins into simpler nitrogenous compounds increasing the nutrient availability for the antagonist.

It has been reported by Gupta *et al.* (2002) that optimization of culture medium components plays a significant role in enhancing the production of proteases. Successful biocontrol strategy requires good understanding of conditions affecting the antagonistic activities of the biocontrol agent. Growth of *Sten. maltophilia* as well as its proteolytic activity was favoured under alkaline condition (pH 8·5) proving that the protease enzyme is

an alkaline protease. Casein, as organic sources of nitrogen were approved to be the optimal nitrogen source for proteolytic activity of *Sten. maltophilia* (PD4560). This was in agreement with previous finding by Dunne *et al.* (2000) who related the improved biocontrol activity of *Sten. maltophilia* W81 against *Pythium ultimum* to overproduction of extracellular serine protease. The most suitable amino acids for growth of *Sten. maltophilia* were methionine and tryptone. Methionine was found to be an important growth factor for many *Stenotrophomonas* strains and was found in all *Sten. maltophilia* strains (Van Den Mooter and Swings (1990). Meanwhile, AmS and potassium nitrate as inorganic sources of nitrogen, did not support neither proteolytic activity nor growth. In other study, skim milk and casein also favoured protease production by *Bacillus* sp (Sharma *et al.* 2015).

The purified protease enzyme showed a molecular mass of approx. 40 kDa. This is in accordance with previous researchers (Miyaji *et al.* 2005).

One of the main objectives of this study was to select the most efficient foliar spraying that may fortify the biological control activity of *Sten. maltophilia* as well as the most appropriate growth stage of potato for application of the biocontrol agent. Messiha *et al.* (2007) found that *Sten. maltophilia* (PD 4560) was able to decrease the disease incidence of potato plants in Egyptian clay soil. In this study application of SA alone to pathogen-inoculated soil grown with potato plants cv. Lady Rosetta was found to suppress the disease. The potato wilt suppressive effect of SA on the *R. solanacearum* race 3 biovar 2 was earlier discussed by Farag (2013). The disease suppression was accompanied by an increase in photosynthetic pigments, exogenous ascorbic acid (AA), proline accumulation, endogenous superoxide dismutase (SOD) and peroxidase activity as well as decrease in phenols. Lamb and Dixon (1997) have reported that the disease suppressive effect of SA is accomplished by accumulation of H_2O_2 which act as a signal in induction of hypersensitive response (HR) and SAR, against pathogens. SA was found to increase the suppressive effect of *Sten. maltophilia* increasing the population size of *Sten. maltophilia* in the potato rhizosphere and crown area. In this context, Ji and Wilson (2003) obtained similar findings but for another plant–pathogen interaction system, in which foliar spray with SA significantly increased the population size and the efficacy of the biological control agents *Pseudomonas syringae* Cit7 (pNAH7) as well as induced host SAR. Also, exogenous application of the carbon source, salicylate, increased the population size of *Pseudomonas putida* strain in the phyllosphere of beans (Wilson and Lindow 1995). Moreover, its role in the induction of SAR against several bacterial pathogens in different host–pathogen interaction systems has been previously reported by several researchers; Wilson and Lindow (1995); Palva *et al.* (1994). This might be due to the induction of sets of signalling genes as biological inducers of SAR in potato plants studied.

Addition of AmS did not support the population count of *Sten. maltophilia* under greenhouse conditions. It did not support the proteolytic activity as well as the growth of *Sten. maltophilia in vitro*. These findings are in accordance with previous work by Ji and Wilson (2003) for other host–pathogen interaction system, that is, tomato-*Ps. syringae*. In conclusion, AmS is proved to be not the favourable source of nitrogen supply to either pathogen or the biocontrol agent, in agreement with, Puri *et al.* (2002); Sangeetha *et al.* (2008). Addition of peat-moss in general found to increase the count of *Sten. maltophilia* in soil, potato rhizosphere and crown area hence increased the suppressive effect of *Sten. maltophilia* in combination with either SA or AmS (Table 1). Amendment with external carbon source was found to selectively increase the population of the biocontrol agent (Pingsheng and Wilson 2003). The used *Sten. maltophilia* strain could survive for 10 months in the used peat-moss originating from the Netherland (Elhalag 2014). The carrier may favour the conditions for the survival of the biocontrol agent. The used peat-moss contains a high amount of organic nitrogen which enhanced the population growth of *Sten. mlatophilia*. The count of *R. solanacearum* was decreased in soil, rhizosphere, and crown area after 80 days from soil inoculation. From previous work (Messiha *et al.* 2007) we found a positive correlation between organic matter content and pH and survival of *Sten. maltophilia*. On the other hand, negative correlations were found between same parameters and the survival of *R. solanacearum.*

Successful plant infection by a pathogen is accompanied by the pathogen ability to penetrate and colonize host tissues and overcome active plant defence responses to finally induce the set of events that lead to disease symptoms (Poueymiro *et al.* 2009). Plant lines of defence involve preformed physical or chemical barriers as well as inducible responses like cell-wall strengthening, release of antimicrobial compounds or a rapid localized and programmed cell death at sites of infection known as the HR (De Wit 2007; Mur *et al.* 2008). *Ralstonia solanacearum* survives in the soil, enters the plant root system, and colonizes the xylem vessels (Poueymiro *et al.* 2009). As in many other bacterial pathogens, a major determinant of *R. solanacearum*'s pathogenicity is its type III secretion system (T3SS), encoded by *hrp* genes (Van Gijsegem *et al.* 2002). *Ralstonia solanacearum* produces a variety of extracellular products that contribute to its ability to colonize host plants and cause disease symptoms (Genin and Boucher 2002). One of

the most important of these products is an acidic, high molecular mass extracellular polysaccharide (EPS I). Denny and Baek (1991) and Kao *et al.* (1992) assumed that EPS I is the cause of wilting in infected plants, as it blocks the vascular system and thereby alters water movement. The EPS I-deficient mutants of the pathogen *R. solanacearum* were totally non-pathogenic as these mutants poorly colonized the stem of infected plants Araud-Razou *et al.* 1998). They also suggested that EPS I may contribute to minimizing or avoiding the recognition of bacterial surface structures by plant defence mechanisms. In addition to EPS, *R. solanacearum* secretes several plant cell wall-degrading enzymes. These includes three polygalacturonases (PglA, PehB and PehC) (Schell *et al.* 1988; Huang and Allen 1997), an endoglucanase (Egl) (Roberts *et al.* 1988) and a pectin methylesterase (Tans-Kersten *et al.* 1998). PhcA represses expression of *hrp*B, which encode T3SS. It was proved active only in the early stages of the pathogenesis and it was not needed when wilt developed (Yoshimochi *et al.* 2009). Several primary metabolic pathways were highly expressed during pathogenesis. These pathways included sucrose uptake and catabolism, and components of these pathways were encoded by genes in the *scrABY* cluster (Jacobs *et al.* 2012). Induced resistance in plants is regulated by complex signal transduction pathways that respond to infection by pathogens and specific abiotic inducers of resistance.

Pathogen-related proteins (PR) proteins are induced and accumulated in host plants as a result of pathogen infection and often associated with SAR against wide range of pathogens (Kim and Hwang 2000 and Durrant and Dong 2004). Examples of these genes are *PR-1*, *PR-2* and *PR-Q*. Harpin is one of the know inducers of SAR to some bacterial and fungal plant pathogens and is accomplished by expression of protein-related genes *PR-1* and *PR-2* (Dong *et al.* 1999). Zhang *et al.* (1999); Kim and Delaney (1999) hypothesized that expressions of PR genes are regulated by NPR1 & NIM1 proteins through interaction with the transcription factors. *PR-1* is a dominant *PR* group induced by pathogens, SA or other factors and it is commonly used as a marker for SAR (Sarowar *et al.* 2005). The induction of the SAR-specific *PR-1* marker gene depends on SA accumulation. *PR-1* activates both the SA and the Jasmine/Ethylene (JA/ET) defence pathway. Both pathways are required for the induction of resistance (Kariola *et al.* 2003).

From this finding we can conclude that both the biocontrol agent and SA-induced SAR and combination of them increase the expression of *PR-1*gene two to three times as well as led to earlier expression of the gene which was accompanied by better disease suppression.

PR-Q is one of the defence-related genes induced upon infection and used as a marker for sugar-mediated defence (Herbers *et al.* 1996). The *PR-Q* tested encoded for acidic chitinase and extracellular β-l, 3-glucanase (Ryals *et al.* 1996). *PR-Q* gene was the most abundant gene under different treatments. A positive correlation was previously observed between nitrogen content in the peat-moss and the survival of *Sten. maltophilia* (data not shown), therefore it can be speculated that the nitrogen element contained in the peat-moss may have a role regulatory the *PR-Q* expression in this treatment. Nevertheless, the role of phosphorus and potassium contained also in the peat-moss cannot be excluded in the regulatory mechanism of *PR-Q* gene expression. The *PR-Q* expression was found to be induced by peat-moss and SA, but sugar-mediated regulation was independent of SA (Herbers *et al.* 1996). Expression of *PR-1* and *PR-Q* were highly affected by potato physiological development stage.

Stenotrophomonas maltophilia is also known to persist in the entophytic plant tissues (Garbeva *et al.* 2001) as it was detected in the potato crown area cv. Lady Rosetta under investigation.

The proteolytic activity was optimum at pH 8·5. Casein enhanced the proteolytic activity while tryptone and methionine supported the growth but not the proteolytic activity. AmS and potassium nitrate inhibited the growth of *Sten. maltophilia*. A positive correlation was observed between survival of *Sten. maltophilia* and organic matter content as well as pH (Messiha *et al.* 2007). This was proven in this study by the optimum growth of the biocontrol agent as well as higher proteolytic activity under alkaline conditions. Also, survival of the biocontrol agent when applied on peat-moss carrier was longer than nonamended soil (Elhalag *et al.* 2015).

P1 and *PrtP* genes are subtilisin-like serine proteases. *P1* gene was expressed at pH 8·5 and 10·5 and it was suppressed at pH 5·5 and 7. Casein and alanine suppressed the expression of *P1* gene *in vitro* (data not shown). *In vivo in planta*, this gene was expressed only at late maturity stages of the biocontrol agent, *Sten. maltophilia* treatment either alone or in combination with SA (Fig. 2). SA promotes the expression of *P1* gene as compared to the AmS. The peat-moss and AmS repressed the *P1* gene expression at all of the potato maturity stages (Data not shown). Also, AmS did not support the protease enzyme production by *Sten. maltophilia in vitro* bioassay test (data not shown). AmS is known to decrease soil pH (acidify soil) (Sturz *et al.* 2004) which may repress genes responsible for production of the alkaline protease activity. Salting out of proteolytic enzyme by AmS that inactivate proteolysis can be expected. In addition,

Sten. maltophilia preferred to utilise the organic source (nitrous source) than inorganic one (Elhalag 2014). These findings are compatible with the *in vitro* proteolytic activity of the biocontrol agent *Sten. maltophilia*, confirming the possible contribution of this gene for the proteolytic activity of the biocontrol agent. The suppression of the gene (lack of induction) at the early potato growth stages may be as a result of unsuitable conditions for gene expression. This is regarded as an important mechanism of defence which is operated actively and directly against the potato wilt disease caused by *R. solanacearum*, via its direct lysis of the pathogen cell wall. Nevertheless, this mechanism is evident to operate along with other defense mechanisms such as phytoalexin production (Elhalag 2014). It should be noted that *PrtP* is 'a key enzyme in the proteolytic system' as it initiates the degradation of caseins in the culture plate bioassay. Also, *PrtP* produces peptides and amino acids from casein in cooperation with intracellular peptidase (Marugg *et al.* 1996). The *PrtP* gene encodes the proteinase precursor that has a homology to the substilin family of the serine proteases (Vos *et al.* 1989). The serine protease has an important role in pathogenesis and in the biocontrol activity (Sharma *et al.* 2011). *PrtP* gene encodes for the production of exo-protease enzyme by *Sten. maltophilia* (PD 4560) was the highest expressed gene among the other detected genes with all treatments and at all potato growth stages studied, Furthermore, in comparison of its level of expression comparable with *P1* gene. It is evident that it is expressed at higher concentration than the *P1* gene at same growth stage and treatments. Hence, it seems that this particular gene is the most important gene in countered with the other detected genes in the defence mechanism against the *R. solanacearum* pathogen. The divergent *PrtP* activities at different treatments under different potato physiological stages may be correlated with the plant conditions such as the presence of certain amino acid at particular potato growth stage. The more frequent expression of the *PrtP* gene as compared to the *P1* gene may be correlated with the wider pH range suitable for the activity of the *PrtP* gene as compared to the *P1* gene.

Prt4, *Prt5* and *Prt6* are sets of primers (molecular markers) referred to as cell wall-associated serine proteinases. *Prt4*, *Prt5* and *Prt6* regarded as surface molecules signals for other inducible previously mentioned integrated networking mechanism (hypersensitivity), protein-related defences and precursor for phytoalexin production (Elhalag 2014). This could be used as a biochemical and molecular markers for the detecting the defence mechanisms in certain potato cultivars intended to plantation. The *Prt4* gene encoded for putative vacuolar protease (Bryant 2005). The highest activity of the gene *in vitro* was at pH 8·5 and 10·5. It was almost expressed during all of the potato growth stages but only with the *Sten. maltophilia* combined with the SA in the presence and the absence of peat-moss and AmS repressed it (Fig. 2). *Prt5* genes encode putative extracellular proteins belong to the proteinase K subfamily 1 (Bryant 2005). It was only expressed at maturation stage of potato growth stage with the application of *Sten. maltophilia* alone or combined with SA and the peat-moss. The *Prt6* genes encoded for the putative extracellular proteins belonging to the proteinase K subfamily 2 (Bryant 2005).

We could conclude that the antibacterial activity of the *Sten. maltophilia* depends on the proteolytic activity of the serine protease which was proven by the expression of *PrtP* gene and cell wall-associated serine proteinases (*Prt4*, *Prt5* and *Prt6*). Also the correlation between expression of these genes and disease suppression were proven as the highest genes expressions were accompanied by best disease suppression. The other antagonistic activities of the biocontrol agent, *Sten. maltophilia* correlated with the induction of SAR through the expression of *PR-1* and *PR-Q* genes. The expression of these genes was enhanced in the presence of SA. Most of the proteinases genes as well as the SAR genes studied in this work were expressed by *Sten. maltophilia* (PD 4560) with SA in the presence and the absence of peat-moss. In conclusion, to strengthen the efficiency of *Sten. maltophilia* as a biocontrol agent; it is recommended to be applied in an organic carrier such as peat-moss with a high percentage of nitrogen with alkaline pH along with application of SA (spraying at vegetative, tuber initialization and tuber bulking stages). The best potato growth stage for application of the biocontrol agent is at the tuber initialization stage. Fertilizers which may acidify the soil should be avoided.

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Conflict of Interest

No conflict of interest declared.

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