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The Relation of Different Crop Roots Exudates to the Survival and Suppressive Effect of *Stenotrophomonas maltophilia* (PD4560), Biocontrol Agent of Bacterial Wilt of Potato

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Abstract

Experiments were performed under greenhouse conditions to control bacterial wilt of potato (potato brown rot), caused by *Ralstonia solanacearum* race 3 biovar 2, Phylotype II, sequevar 1 using various biocontrol strategies. These strategies involved the use of the bacterial biocontrol agent *Stenotrophomonas maltophilia* (PD4560), in clay or sandy soils, planted with cowpea, maize or tomato which was grown separately in different pots in the inoculated soils. After harvest, the soil derived from each cultivated crop was inoculated with a mixture of three virulent *R. solanacearum* strains (K3, K10 and K16) to achieve a final concentration of 5×10^8 cfu/g dry soil and used in pots under greenhouse conditions to cultivate potato seed tubers. The highest survival of *S. maltophilia* in soil (more than 160 days) coincided with a remarkable suppressing effect on disease incidence caused by *R. solanacearum* that expressed by wilt severity (up to 100% reduction), area under disease progress curve (AUDPC) (up to 99% reduction) and counts of the pathogen in soil (up to 75% reduction), rhizosphere (up to 80% reduction) and plant tissue (up to 97% reduction) of potato plants. The amino acid analysis of root exudates of crops under investigation revealed high percentages of asparagines (15.5–21%), glutamine (16–20%) and sulphur-containing methionine (7–9%) in both of the cowpea and maize, respectively. In tomato root exudates, high percentages of arginine (around 26%) and lysine (around 23%) were detected. Methionine is known to favour the growth of *S. maltophilia* suggesting that especially cowpea and maize are suitable for crop rotation with potato and will enhance the sustainability of the biocontrol agent *S. maltophilia*.

Introduction

Potato (*Solanum tuberosum*) is one of the most important staple crops in the world and also in Egypt. In Africa, Egypt is the largest potato producer producing annually approximately 1.5–2 million tons from approximately 100 000 ha. Potato crops are vulnerable to several diseases that seriously affect the quality and quantity of its national yield as well as the export to Europe. Potato brown rot (bacterial wilt) is a major disease caused by *Ralstonia solanacearum* (in the Mediterranean and Europe race 3 biovar 2, Phylotype II, sequevar 1) that may result in substantial yield losses. For many countries and also the EU, *R. solanacearum* is a quarantine organism with a zero tolerance for brown rot infection. The pathogen can persist for a long time in soil and in the infected host plant debris up to 2 years (Graham et al. 1979). Furthermore, alternative hosts and non-host plants can also be colonized by the *R. solancearum* pathogen (Akiew and Trevorrow 1994) and weed hosts for example *Solanum dulcamara*, *Solanum nigrum* and *Portulaca oleracea* in Europe (Elphinstone et al. 1998) and *Rumex dentatus* and *Solanum nigrum* in Egypt (Farag et al. 2004).

Plants can communicate extensively using secondary metabolites to express messages (Degenhardt et al. 2003; Dicke and Hilker 2003). Root exudates are chemicals released into the surrounding environment by healthy and intact plant roots (Kalburtji and Mosjidis 1993). Such chemicals secreted by plant roots in the form of low molecular weight compounds such as amino acids, organic acids, sugars and phenolics serve important roles as chemical attractants and repellants in the rhizosphere (Bais et al. 2001). Root exudates also play a signalling role to the plants which are subjected to invasion by pathogens and pests (Ryan and Delhaize 2001).

Biological control of pathogens in the soil is now considered the advanced alternative mechanism to synthetic pesticide use due to its safety to the environment and plant health as well as to human health (Brimmer and Boland 2003). In this context, a strain of *S. maltophilia* isolated from the rhizosphere of eggplant (*Solanum melongena*) in soil was first used as an antibacterial agent *in vitro* against *R. solanacearum* (Messiha et al. 2007). *S. maltophilia* (PD 4560) used in this study is a non-human pathogenic strain and has been tested for human pathogenicity according to Wolf et al. (2002) and (Attfield and Bell 2006) and found to be non-pathogenic and safe. *S. maltophilia* is usually associated with the rhizosphere of plants such as maize as well as cruciferous plants (Lambert and Joos 1989). The aim of this study was to determine the survival of the *S. maltophilia* biocontrol agent in the rhizosphere of cowpea, maize and tomato plants and to focus on the relation between the amino acids present in the root exudates of the crops under investigation and the survival of this biocontrol agent. In addition, the relationship between the survival of the *S. maltophilia* biocontrol agent and disease suppression was investigated.

Materials and Methods

Plant material

Tomato (*Lycopersicon esculentum* cv. Pinto), used as a test plant to confirm the virulence of stored *R. solanacearum* strains, was provided by potato brown rot project in Egypt. Potato seeds cv. Lady Rosetta used in this study were kindly provided by the Potato Brown Rot Project (PBRP), Agric. Res. Center (ARC), Giza, Egypt. Maize [cv. Single hybrid yellow 168 (S.H.Y. 168)] and cowpea (cv. Kaha1) seeds were provided by the Agricultural Research Centre (ARC) of the Egyptian Ministry of Agriculture and Land reclamation.

Source of different soil types

Clay soil samples collected from El-Gharbia governorate were examined for the absence of *R. solanacearum* by plating from a suspensions prepared from 50 ml soil sample (obtained by taking 5 g of soil sample in 45 ml phosphate buffer) on modified semiselective medium of South Africa (SMSA) according to Elphinstone et al. (1998), and positive colonies were confirmed by real-time PCR (Weller et al. 2000). Sandy soil samples were collected from El-Behira governorate from a PBRP approved pest free area and sampled and tested as described above.

Bacterial strains

In this study, three highly virulent strains of *R. solanacearum* race 3 biovar 2 (K3, K10, K16), originally isolated from diseased potato tubers and maintained in our culture collection in sterilized tab water, were used (Elhalag 2008).

Pathogenicity and virulence test

Each of the *R. solanacearum* strains used was tested for its virulence (aggressiveness) by pot inoculating tomato seedlings (3–5 leaves/seedling) grown under controlled greenhouse conditions (28–30°C, 16° at night, 75–85% RH and 16 h light) using the stem injection technique as described by Janse (1988). Disease severity was recorded using the scale adapted by Kempe and Sequeira (1983) as follows: 0, no symptoms; 1, 10% of the foliage wilted; 2, 11–30% of the foliage wilted; 3, 31–60% of the foliage wilted; 4, more that 60% but <100% of the foliage wilted and 5, all leaves wilted.

Biocontrol agent and bioassays antagonism

The spontaneous rifampicin- and chloramphenicol-resistant mutant *S. maltophilia* strain PD* = Plant Protection Service Netherlands, culture collection 4560, derived from wild-type strain PD 3533, was used in the experiments. The PD 3533 strain was isolated from the rhizosphere of eggplant (*Solanum melongena*; Black Beauty) grown in Egypt (Messiha 2001), and the antagonistic potential for the mutant strain was confirmed (Messiha et al. 2007).

Survival of the biocontrol agent in co-crop planted soils

In the first part of the experiment, two types of soils (clay and sandy) were used. These soils were examined previously for the absence of potato brown rot bacteria and were sent to the Soils, Water and Environmental Research Institute of ARC, Giza, Egypt for chemical analysis and detection of available micro- and macro-elements.

Three types of crops, viz. maize (cv. S.H.Y. 168), cowpea (cv. Kaha 1) and tomato (cv. Pinto) plants were used in this experiment. Non-cropped pots were included for both soils as control. Four replicates were designed for each treatment, that is, 16 pots for sandy soil and 16 pots for clay soil. Pots (15 cm height and 15 cm diameter), holding three kg non-sterilized soil, were used. The conditions of the quarantine glasshouse at the Potato Brown Rot Project (PBRP) at Dokki, Cairo were fixed at 28–30°C daytime, 16°C at night, 75–85% RH and 16 h light. The experiment was conducted twice.

Application of the biocontrol agent under greenhouse conditions

A suspension of the biocontrol strain was prepared in sterile distilled water and adjusted spectrophotometrically to approximately 4.3×10^9 cfu/ml. The inoculum was added to each pot at a rate of 1 : 10 (vol/wt) to achieve a final concentration of approximately 5×10^8 cfu/g dry soil and mixed thoroughly with the soil. The bacterial count for the biocontrol agent was observed along the experiment at time intervals of 0, 3, 5, 10, 14, 21, 30, 60 and 90 days from the day of inoculation. The experiment was conducted twice. The bacterial counts of *S. maltophilia* (PD4560) were performed on TSBA medium amended with 100 ppm rifampicin and chloramphenicol.

Root exudates and survival of *S. maltophilia*

Root exudates from the different crop cultivars were separately collected at a plant age of 80 days (maturation stage) according to Graham et al. (1981) and Neal et al. (2012). The counts of *S. maltophilia* (PD4560) in different plant root exudates were performed for all replicates of each crop by plating on to TSBA medium incorporated with 100 ppm rifampicin and 100 ppm chloramphenicol. After counting of *S. maltophilia* in root exudates, 1 ml of each plant root exudate was inoculated using a 50 μl of *R. solanacearum* suspension containing equal amounts of strains K3, K10 and K16 at a concentration of approximately 10^6 cfu/ml (Elhalag 2008). *R. solanacearum* cell counts were determined 0, 10, 20 and 40 days after inoculation by plating on SMSA medium (Elphinstone et al. 1998).

The collected root exudates were filter-sterilized under vacuum using a suction apparatus (Pall, Life sciences, Ann Arbor, MI, USA) and a 0.22-μm filter (Millipore, Minisart Sartorius Stedim Biotech GmbH, Goettingen, Germany) and stored at −20°C in the dark for further study. The experiment was conducted twice.

Analysis of amino acids in the root exudates of tested plants

Fractionation of total amino acid content in different root exudates was carried out at the central laboratory of Faculty of Agriculture at Cairo University, Egypt using an automatic amino acid analyser (AAA400; INGOS Ltd., Prague, Czech Republic), and hydrolysis for all samples was performed according to Block et al.(1958). The amino acids and their concentration in each sample were determined.

Pathogenicity and biocontrol efficiency on potato grown after co-crop plantation

Maize, cowpea and tomato plants were removed from the pots containing the respective soil type and inoculated with a suspension of *R. solanacearum* at a concentration of 1×10^9 cfu/ml. The ratio of inoculum/soil was 1 : 10 (vol/wt) to achieve a final concentration of approximately 5×10^7 cfu/g dry soil. After soil infestation, potato tubers (cv. Lady Rosetta) were cultivated in all pots, including the controls. Regular watering the plants was performed using tap water. Counts of both bacteria (*R. solanacearum* and *S. maltophilia*) were monitored in soil at 0, 40 and 70 days after soil infestation. These selected durations correspond to planting time, tuber initialization and maturation stage. Bacterial populations of both strains in the rhizosphere and roots were monitored only at the end of the experiment. Wilting progress of the potato plants was recorded daily till the end of the experiment, and area under disease progress curve (AUDPC) was calculated. The experiment was conducted twice.

Statistical analysis

A statistical analysis was performed to determine the survival of the *S. maltophilia* biocontrol agent and the

R. solanacearum pathogen in the two types of soil tested (clay and sandy), cultivated with different crops (cowpea, maize and tomato) or non-cultivated. Survival of *R. solanacearum* in the root exudates of the three tested crops was compared to its survival in sterilized distilled water, as a control. Log-transformed counts of the two bacteria were fitted to the standard logistic model with an asymptote as described by Franz et al. (2005),

$$C_t = a_{\mathrm{m}}/(1 + \exp(-d * (t - c)))$$

where C_{t} = log-transformed number of bacteria at time *t* (days), a_{m} = upper asymptote (level of inoculation), *d* = the slope at the inflection point (per days) which is referred to as decline rate (the absolute decline rate is given by $[a_{\mathrm{m}}*d]/2$) and *c* = the position of the inflection point (days) which is referred to as 50% reduction time. The goodness of the data fitting to the model (R^2) was then calculated. The estimated parameter values *c* and *d* for the different managed substrates were calculated from the analytical analysis using SAS v. 9.1 (SAS Institute Inc., Cary, NC, USA) and then subjected to multivariate analysis of variance (MANOVA, using the SPSS v 16, SPSS Inc., Chicago, IL, USA) to detect the differences between the survival of *S. maltophilia* in different soil types cultivated with the different tested crops. Descriptive and NPar test (Mann–Whitney *U*-test) analyses were conducted, using SPSS v16 for comparison between disease severity, area under disease progress curve (AUDPC) and log-transformed cfu count of *S. maltophilia* and *R. solancearum* in the two utilized soils, the rhizosphere and the plant tissues of potato plants, 70 days postsoil inoculation (T70). The descriptive and NPar tests were performed according to the soil types as well as to the crop types. For comparison of amino acid percentages in the root exudates of the different tested crops, a paired *t*-test was conducted, using SPSS v16. Correlation analyses were conducted for the survival (decline rate per days (*d*) and 50% reduction time in days (*c*) for both of the *S. maltophilia* and *R. solanacearum* and for amino acid percentages found in all of the root exudates in SPSS V16.

Results

Pathogenicity and virulence test of the pathogen strains (K3, K10 and K16)

The three *R. solanacearum* strains (K3, K10 and K16) used showed high percentages of infection and disease severity on tomato plants based on the scale equation by Kempe and Sequeira (1983). The most virulent strain as determined 2 weeks after seedling inoculation was K3, with 83.8% infection, followed by K10 (78.4%) and K16 (76.6%). Virulence of the strains was comparable to that determined for the strains when freshly isolated (Elhalag 2008). Also on potato plants cv. Lady Rosetta, *R. solanacearum* strain K3 proved to be the most virulent strain when determined by wilt severity and per cent of infection (Data not shown).

Antagonistic properties of *S. maltophilia* (PD4560)

Stenotrophomonas maltophilia (PD 4560) showed remarkable antagonistic activities and was able to nearly suppress 100% of the growth of *R. solanacearum* on King's medium as compared to the untreated control.

Survival of *S. maltophilia* in grown in two soil types cultivated with three different crops

Stenotrophomonas maltophilia survived well in soil cultivated with cowpea and maize with a slight decline 160 days after soil inoculation (from 7.92 to 5.66 and 5.68 cfu/g) for both crops, respectively (Fig. 1). On the other hand, the lowest survival was found in non-cultivated soil (control) and those cultivated with tomato, with sharp declines after 160 days (from 7.92 to 1.26 and 2.49 cfu/g) for non-cultivated and tomato soil, respectively. There was no significant difference between survival of *S. maltophilia* in clay soil and sandy soil. The goodness of the data fitting to the model (R^2) values was around 0.95 for clay soil and 0.97 for sandy soil. Survival of *S. maltophilia* in different crop soils was significantly different in all soils: (MANOVA: Wilk's lambda (1.9×10^2) for crop, (17.1) for soil: $P < 0.001$ for both), ANOVA for the 50% reduction time (*c*): $F = 1.74 \times 10^3$, $P < 0.001$ while for the decline rate (*d*): $F = 152.1$, $P < 0.001$. There was a significant difference in survival of *S. maltophilia* in clay and sandy soil. The decline rate (*d*) was slightly higher in sandy as compared to clay soil and the ANOVA for (*c*), $F = 0.87$, $P > 0.05$ and for (*d*), $F = 6.1$, $P < 0.02$. The experiment which was conducted twice showed in both cases the same results.

Survival of *R. solanacearum* in three different crops, cultivated in two types of soil

Decline in log-transformed cfu count of *R. solanacearum* was highest in soils cultivated with cowpea and

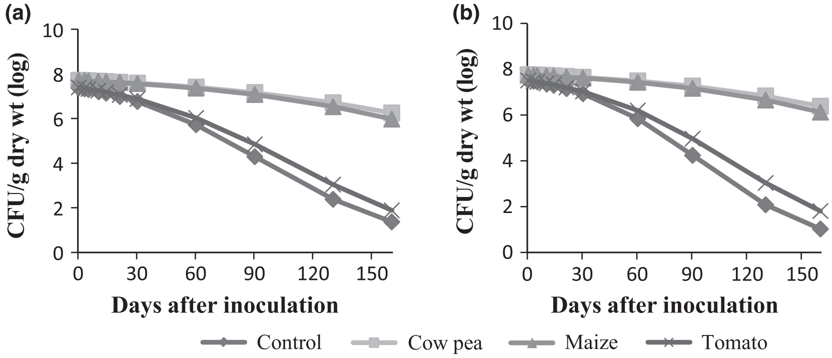

Fig. 1 Decline in the density of *Stenotrophomonas maltophilia* (cfu/g dry soil) in different crops cultivated in clay (a) and sandy soil (b) under greenhouse conditions. *S. maltophilia* was inoculated just prior the cultivation of the desired crops at a concentration of 10^8 cfu/ml. Data were recorded from zero up to 160 days (90 days for tested rotated crops plus 70 days for the potato maturation stage) after inoculation. The lines are the predicted values from the logistic decline model: $C_t = a_m/(1 + \exp(-d^*(t-c)))$. Where C_t, log of transformed number of bacteria; a_m, initial count of the pathogen (asymptote); *d*, decline rate (per days); *t*, time (days); and *c*, 50% reduction time in days based on (Franz et al. 2005). R^2 for the fit of the data to the logistic model with asymptote were around 0.95 for all tested treatments.

maize (from 7.9 to 1.88 and 1.83 cfu/g) for both crops, respectively, 70 days after soil inoculation. On the other hand, the decline of the pathogen was lowest in non-cultivated soil (control) and those cultivated with tomato (from 7.9 to 7.39 and 7.86 cfu/g), respectively, and giving similar results for clay and sandy soil (Fig. 2). The observed values fit well with the logistic decay model with asymptote, with R^2 values around 0.98 for clay soil and 0.94 for sandy soil and control, respectively. When comparing *R. solanacearum* survival in different crops, a significant difference in pathogen survival in different cultivated soils (MANOVA: Wilk's lambda (1.5×10^3) for crop, (5.32) for soil: $P < 0.001$), where ANOVA for (*c*), $F = 1.75 \times 10^3$, $P < 0.001$ and for (*d*), $F = 58.6$, $P < 0.001$, was observed. The experiment was conducted twice and showed similar results.

Survival of *R. solanacearum* in three different crop root exudates cultivated in two types of soils inoculated with *S. maltophilia* (PD4560)

Decline in log-transformed cfu count of *R. solanacearum* was the highest in root exudates of cowpea and maize cultivated in both soil types (from 6.1 to 1.5 and 2.38 cfu/ml), respectively, forty days after inoculation. On the other hand, the decline of pathogen was low in water (control) and root exudates of tomato (from 6.1 to 4.8 and 3.97 cfu/ml, respectively) (Fig. 3). Soil type did not affect the survival of *R.*

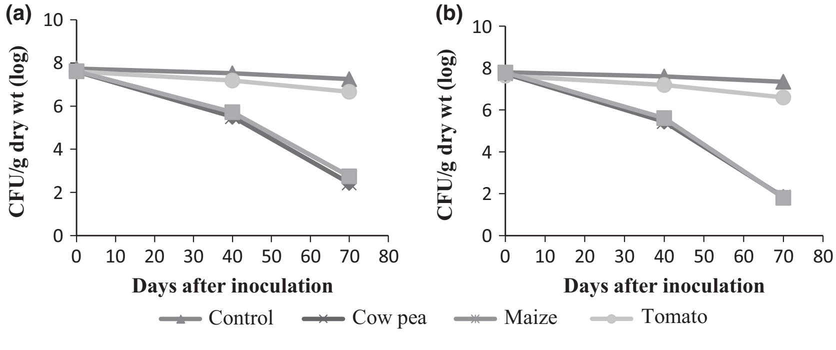

Fig. 2 Decline in *Ralstonia solanacearum* density (cfu/g dry soil) in different crops cultivated in clay (a) and sandy soil (b) pots under greenhouse conditions. Note: *R. solanacearum* was inoculated after 90 days from the application of the biocontrol *S. maltophilia* at a concentration of 10^8 cfu/ml. Data were recorded from zero up to 70 days (potato maturation stage) after inoculation. The lines are the predicted values from the logistic decline model. The lines stand for the predicted values from the logistic decline model: $C_t = a_m/(1 + \exp(-d^*(t-c)))$. Where C_t, log of transformed number of bacteria; a_m, initial count of the pathogen (asymptote); *d*, decline rate (per days); *t*, time (days); and *c*, 50% reduction time in days based on (Franz et al. 2005). R^2 for the fit of the data to the logistic model with asymptote were around 0.95 for all tested treatments.

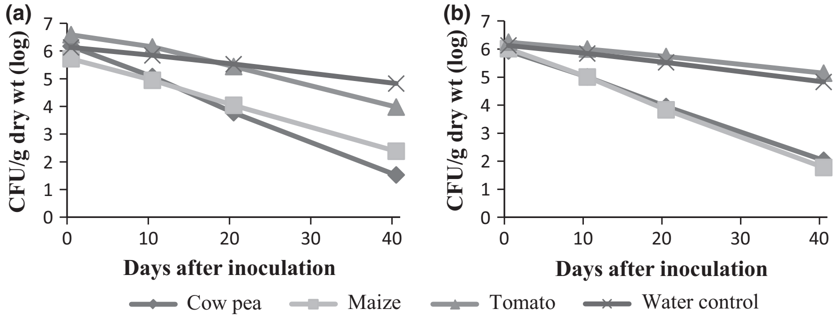

Fig. 3 *In vitro* decline in density (cfu/ml) of *Ralstonia solanacearum* in different plant root exudates cultivated in clay (a) and sandy soil (b). Note: the experiment was terminated forty days after the inoculation of root exudates by *R. solanacearum* at a concentration of 10^6 cfu/ml, incubated at 28°C. The lines stand for the predicted values from the logistic decline model: $C_t = a_m/(1 + \exp(-d^*(t-c)))$. Where C_t, log of transformed number of bacteria; a_m, initial count of the pathogen (asymptote); *d*, decline rate (per days); *t*, time (days); and *c*, 50% reduction time in days based on (Franz et al. 2005). R^2 for the fit of the data to the logistic model with asymptote were around 0.95 for all tested treatments.

solanacearum in root exudates of different crops cultivated in clay or sandy soil. The goodness of the data fitting to the model (R^2) was around 0.99 for the different treatments. Survival of *R. solanacearum* in different crops was significant in both soils as mentioned above (MANOVA: Wilk's lambda (76.7) for crop, $P < 0.001$) where ANOVA for (*c*), $F = 93.7$, $P < 0.001$ and for (*d*), $F = 9$, $P < 0.001$. The correlation test between survival of *R. solanacearum* that expressed by *c* and *d* values in different crop root exudates and log-transformed cfu count of *S. maltophilia* persisting in root exudates showed that there is a significant negative regression between survival of both organisms where $r = -0.88$ for *c*, $P < 0.001$; $r = -0.51$ for *d*, $P < 0.009$). By other words, when the population counts of *S. maltophilia* increase, the population counts of *R. solancearum* decrease. The significant regression indicates a clear relation (interaction) between the survival of both organisms, where decline in log-transformed cfu count of *R. solanacearum* was highest in soils cultivated with cowpea and maize crop (from 7.9 to 1.88 and 1.83 cfu/g) for both crops, respectively, after 70 days of soil inoculation. On the other hand, *S. maltophilia* survived well in soil cultivated with cowpea and maize with a slight decline after 160 days of soil inoculation (from 7.92 to 5.66 and 5.68 cfu/g) for both crops, respectively.

Bacterial wilt (brown rot) incidence after different crops cultivated in two types of soil

The data in Table 1 show 100% reduction in disease severity and AUDPC for sandy soil pots treated with *S. maltophilia* previously cultivated with maize as compared to control pots. A similar trend was observed for pots previously cultivated with cowpea (96.7% reduction in disease severity and 99% reduction in AUDPC) for sandy soils. Clay soil pots previously cultivated with maize or cowpea showed similar reductions in disease severity and AUDPC were obtained after the same crops for, 98% reduction in disease severity and 77% reduction in AUDPC were observed, as compared to control pots. On the other hand, there was only 15% reduction in AUDPC for pots with clay soil and 24% for pots with sandy soil previously cultivated with tomato as compared to control pots. (All soils pre-inoculated with *S. maltophilia*). The log-transformed cfu counts of *R. solanacearum* were detected in soil, rhizosphere and plant tissues of potato plants. In soils which were previously inoculated with *S. maltophilia* and cultivated with cowpea, maize, tomato crop or left non-cropped, the highest final level of the pathogen was detected in previously tomato cropped (6.7 cfu/g for both clay and sandy soils, giving a reduction of nearly 10-fold compared to non-cropped soils (7.4 cfu/g for clay soil and 7.3 cfu/g for sandy soil). The final level of the pathogen (log transformed) in soil previously cultivated with maize was 1.8 cfu/g for clay soil and 2.8 cfu/g for sandy soil, giving a reduction of nearly 10^5- and 10^4-fold for clay and sandy soil, respectively. While for cowpea, the detected level of the pathogen was 1.8 cfu/g for clay soil and 2.5 cfu/g for sandy soil giving a reduction of nearly 10^5- and 10^4-fold for clay and sandy soil, respectively. On the other hand, the log-transformed cfu counts of *S. maltophilia* that were finally detected in soil, rhizosphere and plant tissues of the potato plant previously cultivated with cowpea and maize were more (around 5.5 cfu/g soil for both crops) with similar results for sandy and clay soils compared to around 2.6 cfu/g in tomato soils and 2.2 cfu/g for non-cropped soils for both sandy

Table 1 Disease incidence[a] of *Ralstonia solanacearum* potato wilt disease on potato cv. Lady Rosetta cultivated after different crops (cowpea, maize and tomato) in two types of soil under green house conditions

						Pathogen[b]						Biocontrol[c]					
		Disease* severity		AUDPC*,[6]		Log_{10} cfu* *R. s*[1] soil		Log_{10} cfu *R. s* Rhizosphere*		Log_{10} cfu *R. s* crown[3]*		Log_{10} cfu *S. mph*[2] soil*		Log_{10} cfu *S. mph* Rhizosphere*		$\log_{10}$ cfu *S. mph* in crown *	
Crop/ cultivar	Soil type	Mean rank	Mean ± SE	Mean rank	Mean ± SE	Mean rank	Mean ± SE	Mean rank	Mean ± SE	Mean rank	Mean ± SE	Mean rank	Mean ± SE	Mean rank	Mean ± SE	Mean rank	Mean ± SE
Cowpea*	Clay	4.6	2.5 ± 2.5	4.6	20 ± 20	5	1.8 ± 0.34	5.1	1.54 ± 0.06	4.3	0.14 ± 0.13	14.2	5.68 ± 0.009	14.5	6.55 ± 0.01	14.5	3.23 ± 0.02
Maize*	Clay	4.3	2 ± 2	4.3	19.5 ± 19.5	4	1.8 ± 0.29	3.8	1.46 ± 0.09	4.6	0.17 ± 0.17	10.7	5.58 ± 0.036	10.5	6.32 ± 0.04	10.5	3.04 ± 0.03
Tomato*	Clay	12.5	100 ± 0	11	719.1 ± 45.1	10.5	6.7 ± 0.02	11.5	7.18 ± 0.08	14	5.34 ± 0.01	5.5	2.67 ± 0.084	6.5	2.45 ± 0.02	5.2	1.57 ± 0.3
Control*	Clay	12.5	100 ± 0	14	841.5 ± 32.4	14.5	7.4 ± 0.01	13.5	7.31 ± 0.07	11	5.28 ± 0.02	3.5	2.22 ± 0.32	2.5	1.36 ± 0.04	3.7	1.42 ± 0.2
Maize*	Sandy	4	0 ± 0	4	0 ± 0	5.2	2.8 ± 0.4	5.5	3.01 ± 0.48	4.2	0.82 ± 0.24	12.2	5.39 ± 0.045	10.5	6.15 ± 0.04	10.5	3.08 ± 0.04
Cowpea*	Sandy	5	3.3 ± 3.3	5	9.1 ± 9.1	3.7	2.5 ± 0.32	3.5	2.22 ± 0.31	4.7	0.66 ± 0.05	12.7	5.45 ± 0.04	14.5	6.42 ± 0.01	14.5	3.24 ± 0.01
Tomato*	Sandy	12.5	100 ± 0	11	674.4 ± 17.1	10.5	6.7 ± 0.01	12.2	7.1 ± 0.078	13.8	5.27 ± 0.02	5.5	2.66 ± 0.083	6.5	2.35 ± 0.01	5.7	2.2 ± 0.25
Control*	Sandy	12.5	100 ± 0	15	883.1 ± 29.3	14.5	7.3 ± 0.01	12.7	7.17 ± 0.1	11.1	5.2 ± 0.022	3.5	2.29 ± 0.31	2.5	1.62 ± 0.21	3.2	1.85 ± 0.26

1, *R. solanacarum*; 2, *S. maltophilia*; 3, Rhizosphere; 4, Plant tissues of potato plants; 5, Standard error; 6, Area under disease progress curve.

*Significance: $P < 0.001$.

[a] Disease incidence for bacterial wilt of potato caused by *R. solanacearum* was recorded daily after each crop rotation under greenhouse conditions in presence of *S. maltophilia* biocontrol agent; Disease severity and AUDPC was recorded according to scale adopted by Kempe and Sequeira (1983).

[b] *R. solanacearum* log count in soil, rhizosphere and plant tissues of potato plants after 70 days from soil inoculation with a concentration 10^8 cfu/ml.

[c] *S. maltophilia* log count in soil, rhizosphere and plant tissues of potato plants after 160 days from soil inoculation with the same previous concentration.

and clay soil (Fig. 3). Soil type did not have an effect on the count of organisms, neither pathogen nor antagonist.

The data in Table 1 show that counts of the pathogen in rhizosphere of potato plants were higher than the count in both soil and crown area, respectively, in case of tomato (around 7.1 cfu/g in rhizosphere soil compared to 6.7 cfu/g in soil. The reverse rhizosphere effect was observed for cowpea and maize (around 1.5 cfu/g in rhizosphere soil compared to 1.8 cfu/g in soil). The statistical analysis (nonparametric analysis) showed a significant difference in disease incidence after maize and cowpea on one hand and tomato and control on the other when previously cultivated in both soils (clay and sandy) for all parameters (disease severity, AUDC, log *R. solanacearum* and *S. maltophilia* count in soil, rhizosphere and plant tissues where $P < 0.01$ for all parameters), but there is no significant difference according to soil type. The mean rank of the two types of soil previously cultivated with tomato and non-cultivated soil was higher in disease severity (more than three times), AUDPC and log count of *R. solanacearum* in soil, rhizosphere and crown area of potato plants than those previously cultivated with cowpea and maize, respectively. 'The mean rank, however, was higher (three times and four times) in the case of *S. maltophilia* log cfu count in soil, rhizosphere and plant tissues previously cultivated with cowpea and maize, respectively, compared to tomato and non-cultivated soil'.

Table 2 Percentage (%) of amino acids[a] detected in different root exudates of cowpea, maize and tomato crops tested in this study

	Root exudates of crops/Type of soil					
	Cowpea		Maize		Tomato	
Amino acids[a]	Clay	Sandy	Sandy	Clay	Clay	Sandy
Asparagine	22.55	20.75	16.45	14.88	5.87	7.42
Glutamine	17.61	15.73	20.28	19.45	6.60	7.74
Methionine	8.38	9.78	8.5	6.2	0	0
Arginine	8.32	12.32	9.74	5.75	27.30	26.62
Lysine	7.68	0	1.09	2.03	21.23	24.91

[a]Determination of the amino acids concentration based on the relative concentration with the amino acids standards listed above, using amino acid analyser (AAA400; INGOS Ltd.) in the tested root exudates of crops cultivated individually in clay and sandy soil separately. Sampling of root exudates was taken 90 days after inoculating each of the tested soil with *S. maltophilia* at a concentration of 10^8 cfu/ml under greenhouse conditions. Numbers are means of two replicated measurements performed in two repeated experiments.

The amino acid analysis for different root exudates of different crops (Table 2) showed that asparagine amounts were high in cowpea root exudates (around 21%) followed by maize (around 16%) and tomato (around 6%), respectively, in both types of soil. Glutamine amino acid was higher in maize root exudates (around 20%) than in cowpea (around 16%) and tomato root exudates (around 7%), respectively, in both soils. Methionine amino acid was found only in cowpea (around 9%) and maize root exudates (around 7%) and was not found on the tomato root exudates. Arginine (around 27%) was found higher in tomato root exudates than those of cowpea (around 11%) and maize (around 7%), respectively. Lysine was found in highest percentages in tomato root exudates (around 22%), while low in cowpea and maize (around 3%). The correlation test between *c* and *d* for both *R. solanacearum* and *S. maltophilia* in one side and amino acids found in different crop root exudates in other side revealed a significant positive correlation between the per cent of asparagine and glutamine amino acids and *c*, *d* values for *S. maltophilia*, where $r = +0.95$, $P = 0. 004$, $r = +0.93$, $P = 0. 006$ for *c* and *d*, respectively. On the other hand, negative correlation was observed between asparagine and glutamine and survival of *R. solanacearum*, as expressed by *c* and *d*, and $r = -0.93$), $P = 0. 007$, $r = -0.85$), $P = 0.032$ for *c* and *d*, respectively. In case of methionine, there was a positive correlation with (*c*, d) value for *S. maltophilia* where $r = +0.98$, $P < 0.001$, $r = +0.97$, $P < 0.001$ for *c* and *d*, respectively, and negative correlation with *c*, *d* value for *R. solanacearum*r $= -0.99$, $P < 0.001$ and ($r = -0.93$, $P < 0.006$ for *c* and *d*, respectively. In case of arginine, there was a negative correlation with (*c*, *d*) value for *S. maltophilia* where $r = -0.95$, $P = 0.004$ and $r = -0.95$, $P = 0.003$ and positive correlation with *c*, *d* value for *R. solanacearum*; $r = +0.99$, $P = 0.009$ $r = +0.93$, $P = 0.009$ for *c* and *d*, respectively. From all above, we can conclude that high percentages of asparagine and glutamine amino acids are associated with high survival of the antagonist and hence low survival of the pathogen. On the other hand, high percentage of arginine and lysine are associated with low survival of the antagonist and high survival of the pathogen.

Discussion

Safe use of the *S. maltophilia* (PD4560) as a biocontrol agent

Some strains of *S. maltophilia* are known opportunistic human pathogens (Berg et al. 2005), and others are saprophytes and non-pathogenic to humans (Wolf et al. 2002). Our strain (PD 4560) was confirmed to be non-human pathogenic as it was able to grow at temperature 28 and 4°C and was unable to grow at 40°C. Moreover, our strain was able to use xylose as a carbon source and has the same dominating fatty acid groups resembling the non-pathogenic strain *S. rhizophila* (unpublished data, present authors, Wolf et al. 2002).

Survival of *S. maltophilia* in three different crops

The aim of this study was to study the survival of the biocontrol agent in rhizosphere of three different crops in rotation with potato and correlate this survival to specific amino acids in plant root exudates. The relationship between survival of the biocontrol agent and disease suppression was also investigated. Increasing the sustainability of the biocontrol agent by the selection of suitable crops that enhance the survival of *S. maltophilia* in the soil is expected to result in better potato wilt suppression. Decline in log-transformed cfu count of *S. maltophilia* was slower in soil cultivated with cowpea and maize with a slight decline 160 days after soil inoculation for both crops, respectively (Fig. 1) as compared to those cultivated with tomato and non-cultivated soil (control), respectively. The longest (160 days) survival in soil cultivated with cowpea and maize is explained by the presence of certain amino acids in the different crop root exudates studied. Analysis of the amino acids revealed high percentages of asparagine and glutamine in cowpea and maize as compared to tomatoes root exudates, while methionine was found only in cowpea and maize root exudates. Kraffczyck et al. (1984) reported that amino acids in general were found as minor components of the latter crop. All of these amino acids in root exudates act as a nutrient source (Dakora and Phillips, 2002) which may enhance the survival of *S. maltophilia*. Glutamine was thought to play an important role in nitrogen fixation by bacteria and act as nitrogen donor in various biosynthetic reactions such as synthesis of nucleic acid and protein synthesis as determined for some proteobacteria (Streicher et al. 1974). Methionine as a sulphur-containing amino acid is suggested to support the long survival of *S. maltophilia* in rhizosphere of cowpea and maize. Methionine is needed for the methylation of a membrane protein that is involved in chemotaxis (Kort et al. 1975). Martin et al. (1975) imply that methionine or some of its derivatives are involved in transducing chemical stimuli to bacterial responses. Debette and Blondeau (1980) reported that *S. maltophilia* is a common micro-organism in the rhizosphere of cruciferous plants. *S. maltophilia* has also been found in association with maize and beets, and this association is promoted by the high content of sulphur-containing amino acids in root exudates, which are growth factors for *S. maltophilia*. Many strains of *Stenotrophomonas* require the addition of growth factors (methionine or cysteine plus glycine) for growth on chemically defined media. Stimulation of growth by the sulphur-containing amino acids was present in 100% of *S. maltophilia* strains studied by Van Den Mooter and Swings (1990). Harsh-Pal et al. (2004) reported that root exudates have a key role in communication between plants and microbes in the rhizosphere. Previous work by Elhalag (2008) showed that the use of root exudates of certain plants such as the Chinese chive (*Allium tubersum*) and the sweet basil (*Ocimum basilicum*) plants was effective in inhibition of *R. solanacearum* in *in vitro* trials (Elhalag 2008).

Bacterial wilt (brown rot) incidence after different crops cultivated in two types of soil

The biocontrol agent *S. maltophilia* was previous found to be effective in controlling potato wilt disease in *in vitro* bioassays and in greenhouse experiments. A reduction of 36.4% in percentage of infected plants was observed in antagonist-amended soil compared to non-amended non-sterilized soil (Messiha et al. 2007). Our present study shows that the decline in the log-transformed cfu count of the *R. solanacearum* was highest in clay and sandy soil cultivated with potato and pre-cultivated with cowpea and maize crop than the soil which was non-cultivated (control) by any crop apart from the clay and sandy soil which were cultivated with tomato plants. In this regard, it is known that both tomato and potato are highly susceptible hosts for the *R. solanacearum* (He et al. 1983). *R. solanacearum* can easily attack and colonize the roots of these hosts via chemotactic attraction of the root exudates of these hosts. Root exudates may thus act as messengers in communicating and initiating the biological and physical interactions between the roots and certain soil organisms (Walker et al. 2003). Peters and Verma (1990) found that certain bacterial catabolic genes encode chemoattractants; examples are *β*-ketoadipate that enables the microbe to use monocyclic compounds and naphthalene that enables the use of dicyclic compounds in order to facilitate the carbon and nitrogen acquisition with regard to specific interactions with plant roots. Bacilio-jimenez (2003) studied host interaction systems between the rice endophytes *Corynebacterium flavescens* and *Bacillus pumilus* (Gram positive bacteria). They concluded that rice root exudates contain amino acids and carbohydrates which are an important source of nutrients for micro-organisms and also participate in the early colonization and the initiation of induced chemotactic responses. The fore-mentioned work has stimulated us to analyse the amino acids that were present in the root exudates of the different crops studies.

Arginine was the most abundant amino acid in the tomato root exudates. Arginine is thought to be an important source of nitrogen for bacteria by the hydrolytic cleavage of arginine to ornithine and urea by arginase. The twin-arginine translocation (Tat) pathway is a prokaryotic transport system that enables the transport of folded proteins across the cytoplasmic membrane (Cline et al. 1992). However, in recent years, it has become clear that the Tat pathway has a broader role and it is also an important virulence enhancing factor in different bacterial pathogens (Cline et al. 1992; Bogsch et al. 1998; Weiner et al. 1998; Berks et al. 2003 and Palmer et al. 2005). This high level of arginine in tomato root exudates may explain the high disease incidence in potato plants planted just after tomato as well as high population of *R. solanacearum* which was associated with low population of the biocontrol *S. maltophilia*.

On the other hand, the high level of methionine in maize and cowpea root exudates may explain the low disease incidence in potato plants planted just after maize and cowpea as well as low population of *R. solanacearum* which was associated with a high population of the biocontrol agent, *S. maltophilia* (Table 1). Sulphur-containing amino acids present in root exudates of maize and cowpea may support the growth of *S. maltophilia* and its survival and hence suppress the potato bacterial wilt disease, regardless of the soil type. The disease incidence as expressed by disease severity and the area under disease progress curve (AUDPC) of potato plants was decreased by 97% after maize and cowpea as compared to the non-cultivated clay and sandy soil that served as control (Table 1). This would be of value when a biocontrol management is recommended to be performed in clay or sandy soil in our Egyptian ecosystem. In turn, this would reflect on expanding the potato-cultivated areas in Egypt for a sustainable food production. One of the underlying mechanisms of the biocontrol agent *S. maltophilia* utilized in this study is possibly largely or entirely based on the production of the alkaline serine protease.

Expression of *prtP* gene by *S. maltophilia* isolate was investigated by Elhalag (2014). This gene encodes the precursor of the serine proteases (Vos et al. 1989). The results of the antagonistic bioassay of *S. maltophilia* using this enzyme in an *in vitro* plate bioassay against *R. solanacearum* revealed total inhibition of *R. solanacearum* growth. Therefore, another bioprotection mechanism apart from the previously mentioned survival mechanism would be through the production of the above-mentioned alkaline serine protease enzyme that hydrolyses the cell wall of *R. solanacearum* pathogen.

Conclusions and Future Perspectives

We conclude from the data of this work that *S. maltophilia* is an efficient biocontrol agent against the potato *R. solanacearum* wilt disease in Egyptian clay and sandy soils. *S. maltophilia* persists and survives for the long period (up to 160 days) in soil previously cultivated with leguminous and gramineous plants such as cowpea and maize. The two crops can be recommended as natural reservoir for *S. maltophilia* in clay and sandy soil to control *R. solanacearum* race 3 biovar 2. Further studies would be warranted to investigate the natural shift of microbial diversity after different crops before cultivating potato, that is, to explore whether there are other microbes which would further contribute in combination with *S. maltophilia* in suppressing bacterial wilt disease.

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