BIOCONTROL OF *PSEUDOMONAS SAVASTANOI*, CAUSATIVE AGENT OF OLIVE KNOT DISEASE: ANTAGONISTIC POTENTIAL OF NON-PATHOGENIC RHIZOSPHERE ISOLATES OF FLUORESCENT *PSEUDOMONAS*

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INTRODUCTION

Olive knot, a major bacterial disease of olive plants (*Olea europaea*), is caused by *Pseudomonas savastanoi.* There is no cost-effective way to control olive knot using available chemical bactericides (Young 2004). Furthermore, development of resistance and environmental concerns urge the necessity for alternative, more selective control methods such as biological control. A potential resource for novel compounds of interest for use in biocontrol is constituted by the diverse biomolecules that mediate antagonism among plant-associated bacteria, including peptide or proteins (bacteriocins) and secondary metabolites frequently produced by pseudomonads (Parret *et al.* 2003, 2005).

Lavermicocca *et al.* identified a bacteriocin inhibitory against *P. savastanoi* from the carob tree pathogen *P. syringae* pv. *ciccaronei.* (Lavermicocca *et al.* 1999, 2002). Notably, *P. savastanoi* was formerly regarded as a subspecies of *P. syringae* (Kennelly *et al.* 2007), indicating that this interaction represents an example of bacteriocin-mediated antagonism by a closely related bacterium. Antibacterial activity targeting *P. savastanoi* by phylogenetically more distant, non-phytopathogenic *Pseudomonas* isolates has not been reported. In the present work we explored the potential of *in vitro P. savastanoi* antagonism for a collection of such *Pseudomonas* isolates from different plant rhizospheres and geographical origins.

MATERIAL AND METHODS

Bacterial strains, cultures and growth conditions

52 rhizosphere samples of 21 wheat cultivars obtained from 10 different regions in Iran were used for isolation of 186 fluorescent *Pseudomonas* strains by plating on King's B agar (KB) and selecting for fluorescence under UV light. Using standard microbiological and biochemical tests (Palleroni 2005), the isolates were preliminary identified (data not shown). The strains were stored in the Culture Collection for Soil Microorganisms (CCSM, Soil and Water Research Institute (SWRI), Iran). 83 strains originated from rice or banana rhizosphere (Sri Lanka; Vlassak *et al.* 1992) and 54 strains were from maize or wheat rhizosphere (Belgium, France; CMPG collection). In addition, some reference strains were included (Pf-5, CHA0, KT2440, PfO-1 and SBW25).

Fourteen strains of *P. savastanoi* pv. *savastanoi* with different geographical

origins and host plants (*Fraxinus* spp., *Jasminum* sp., *Ligustrum japonicum*, *Nerium oleander*, *Olea europaea*) obtained from the BCCM/LMG Bacteria Collection (Universiteit Gent, Belgium) were used as indicators in antagonistic assays: LMG2209 (pathovar reference strain), LMG5011, LMG5154, LMG5385, LMG5386, LMG5387, LMG5388, LMG5389, LMG5484, LMG5485, LMG5486, LMG5487, LMG6768, LMG17581. All strains were routinely grown in trypticase soy broth (TSB) or agar (TSA) medium (BD Biosciences) at 30°C.

Detection of antagonistic activity

Conventional deferred antagonism assays (agar diffusion method relying on detection of halo formation in an overlay of indicator cells due to growth inhibition by chloroform-killed producer cells) were performed as previously described (Parret *et al.* 2003). The growth inhibition was quantified by measuring IZR (inhibition zone radius) from the edge of the producer colony. Alternatively, for higher throughput screening a modified direct method was devised, in which the indicator strain was streaked on a square agar plate (12 cm x 12 cm) using cotton swap tips in 8 parallel lines, and 96 producer strains spotted next to them by multi-channel pipettor. The inhibition was scored based on the size of localized growth inhibition.

To study the influence of siderophore production or repression on antagonistic interactions, strains were cultured in iron-limiting medium (KB) or in KB supplemented with iron (0.1 mM $FeCl_3.6H_2O$).

Sensitivity of antagonistic substances to proteolytic enzymes and heating were tested according to Parret *et al.* (2003).

RESULTS AND DISCUSSION

Direct versus deferred antagonism

To facilitate faster screening of large numbers of bacteria for antagonistic activity we examined a setup assaying for direct interaction (avoiding prior killing of producer bacteria and subsequent overlay with indicator bacteria). Using this assay, 15 antagonistic strains of the Sri Lanka and Belgium-France collections were initially identified, of which 10 strains were confirmed active by the deferred method.

It is of interest to further examine the antagonisms identified only in the direct method. Such growth-inhibitory effect may be caused by competition for nutrients. On the other hand, one might envisage that interaction between two bacterial strains may trigger production of some antagonistic compound, subsequently affecting growth of the opponent bacterium. Such inducible production of an antibacterial metabolite would not be detected by the standard assay method in which no viable producer cells are confronted with potential target cells.

Antagonism of *P. savastanoi* by fluorescent *Pseudomonas* spp.

Several strains studied in the present work, inhibited growth of *P. savastanoi* (Figure 1). All active strains displayed a different antagonism spectrum for the *P. savastanoi* target strain tested. The IZR on TSA varied from 0.5-20 mm and both clear and turbid halo formation was observed, presumably resulting from growth inhibition and growth retardation, respectively.

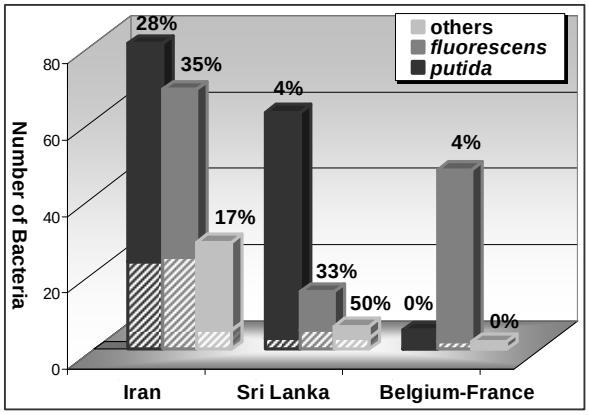

Figure 1. Frequency of *P. savastanoi* inhibitory activity detected among fluorescent *Pseudomonas* strains from different geographical origins. The dashed bars represent the percentage of antagonistic strains for each subset of strains.

The Iranian collection displayed the highest proportion of inhibitory strains (29 %), while 13% and 3% of the strains from Sri Lanka and Belgium-France, respectively, showed antagonistic activity. Antagonist strains belonged mainly to the *P. fluorescens* species (Figure 1).

The strains RW10S1, RW10P3a and RW10P3b (from rice, Sri Lanka) and SWRI196 (from wheat, Iran) showed the biggest halo size for all indicator strains. Inhibition of some other *Pseudomonas* species was previously observed for strain RW10S1 and the active substance is currently being characterized (Estrada de los Santos *et al.* 2006, Li *et al.* 2008). The antagonistic spectrum towards different indicators of RW10P3a and RW10P3b is quite similar to that of RW10S1. As these strains were isolated from the same biological sample (hence the code RW10), it cannot be excluded that the same (or a closely related) strain may have been isolated. As the corresponding biosynthetic gene cluster of strain RW10S1 has been identified recently in this laboratory, it will be possible to verify the presence of these genes in addition to an assessment of strain relatedness (by BOX-PCR fingerprinting).

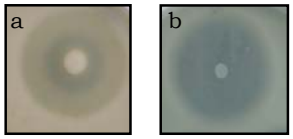

Figure 2. Comparison of *P. savastanoi* growth inhibition halo generated by SWRI196 (a) and RW10S1 (b).

While the large halo formed by RW10S1 is clear (on average IZR>20mm), that of SWRI196 is smaller and exhibits a zonal aspect (partly clear and turbid; IZR>10mm) (Figure 2). It cannot be excluded that this may be due to formation of multiple inhibitory substances.

As expected, siderophore production is widespread among the fluorescent *Pseudomonas* collections studied here (Vlassak *et al.* 1992, Rasouli Sadaghiani *et al.* 2006). As nutrient limitation by sequestering iron may

contribute to growth inhibition, the effect of inducing or repressing siderophore production on antagonistic activity, was investigated. Almost all strains displayed growth inhibitory activity in iron-poor KB medium which disappeared in KB supplemented with iron, indicating siderophore-mediated growth inhibition of *P. savastanoi*. Six strains (including SWRI196) retained their antagonistic ability in the presence of iron, pointing to a siderophore-independent activity.

Antagonistic activity among *P. savastanoi* strains

Bacteriocins typically mediate antagonism against closely related strains. No such activity could be detected when confronting the 14 *P. savastanoi* strains as producer-indicator pairs. However, we noticed that *Pseudomonas* sp. LMG5390 (a strain isolated from *Nerium oleander* stem gall in New Zealand and originally described as '*P. syringae* pv. *savastanoi*') exhibits antagonistic activity against several *P. savastanoi* strains. In TSA medium, growth of five strains was affected (LMG5487, LMG5154, LMG5485, LMG5486 and LMG6768). In KB medium activity against almost all indicator strains was observed but antagonism was suppressed in the presence of iron, except for three strains (LMG5487, LMG5485 and LMG6768).

Preliminary characterization of antagonistic activities

Three promising strains, RW10S1, SWRI196 and LMG5390, were selected for further characterization. The active compounds produced by strains RW10S1 and SWRI196 were not inactivated by protease or heat treatment. LMG5390 activity against LMG6768 was protease sensitive but heat resistant, suggesting that a heat-resistant protein or peptide may be involved. Strains RW10S1 and SWRI196 have a relatively broad anti-*Pseudomonas* activity. A narrow inhibitory capacity is exhibited by strain LMG5390 (Table 1).

Table 1: Spectrum of antagonistic activity exhibited by selected strains against other *Pseudomonas*.

Indicators	RW10S1	SWRI196	LMG5390	Indicators	RW10S1	SWRI196	LMG5390
P. aeruginosa PAO1	(+++)	(+++)	-	*P. stutzeri* LMG1228	+++	++	-
PA14	+++	(+++)	-	LMG2333	+++	+++	-
				P. syringea pv.			
P. agarici LMG2112	+++	+++	-	*syringae* LMG1247	(+++)	(+++)	-
P. diminuta LMG2088	(++)	(+++)	-	*glycinea* LMG5066	+++	(+++)	-
				P. fluorescens			
P. mendocina				Pf-5	-	(+++)	-
LMG1223	(+++)	(+++)	(+++)	SBW25	-	(+++)	(++)
P. putida KT2440	+++	-	-	WCS365	+++	(+++)	(+++)
OE 47.1	+++	+++	-	BW11S1	(+++)	-	-
P. tolaasii LMG2342	-	+++	-	*P. virdiflava* LMG2352	+++	+++	-

-: no inhibition, ++: 0.5<IZR<4mm, +++: IZR>4mm, (): turbid halo

CONCLUSION

Although a related phytopathogenic *Pseudomonas* strain has been reported to inhibit *P. savastanoi* by producing a bacteriocin, this report is the first study exploring the potential of non-pathogenic *Pseudomonas* rhizobacteria as antagonists of *P. savastanoi*. In addition to ongoing work with strain RW10S1, the active compounds of two other promising strains, SWRI196 and LMG5390, will be studied further with respect to chemical structure and biosynthetic genes. Such molecular knowledge is instrumental to assess olive knot biocontrol potential.

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