International Journal of Botany

ISSN: 1811-9700

science alert

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International Journal of Botany 4 (4): 369-375, 2008
ISSN 1811-9700

Biological Control Potential of *Achromobacter xylosoxydans* for Suppressing *Fusarium* wilt of Tomato

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Abstract: A bacterium identified as *Achromobacter xylosoxydans* (MM1) was isolated and evaluated for its ability to suppress *Fusarium* wilt of tomato under glasshouse conditions. The bacterial strain was applied by dipping plant roots in a cell suspension at the concentration of 1×10^{8} cfu mL^{-1}. Results of different trials showed that this bacterial strain reduced wilt incidence by about 50%. No phytotoxicity was observed on healthy plants treated with the bacterial suspension and, on the contrary, these showed a growth stimulation if compared to untreated plants. *In vitro* assays showed that *A. xylosoxydans* MM1 strain is able to produce hydroxamic siderophores and has no chitinolytic activity. Therefore a possible biocontrol mechanism could be competition for iron. This isolate should be further evaluated for potential application in biocontrol of other plant pathogens.

Key words: Antagonism, *Lycopersicon esculentum*, *Fusarium oxysporum* f. sp. *lycopersici*, increased growth response, soil rhizosphere

INTRODUCTION

Suppression of plant diseases with chemicals is being restricted as the result of increasing public concern related to the danger for human health, environment and appearing of resistance strains to usually synthetic chemicals substances. Thus, developing alternative ways to control plant diseases is of high priority. Biocontrol involves disease-suppressive microorganisms to improve plant health. Disease suppression by using biocontrol agents is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community around the plant and the physical environment (Barea *et al*., 2004). Biological control has potential for the management of many diseases.

Fungal pathogens are responsible for severe damage on many economically important plant species. *Fusarium oxysporum* Schlecht is a very dangerous plant pathogen and the *forma specialis lycopersici* occurs throughout most tomato-growing worldwide causing a vascular wilt that can severely affect the crop.

Numerous soil microorganisms have been known to be very effective against many soilborne plant pathogens, including *F. oxysporum* f. sp. *lycopersici*. They can be *Fusarium* species isolated from Fusarium wilt-suppressive soils (Monda, 2002; da Silva and Bettiol, 2005) or other biocontrol fungi, such as *Trichoderma*, *Rhizoctonia* and *Gliocladium* spp. (Larkin and Fravel, 1998; Muslim *et al*., 2003; Rose *et al*., 2003). Additionally, several least-known groups of biocontrol fungi such as *Glomus*, *Aspergillus* and *Penicillium* spp. have been used to control *Fusarium* wilt of tomato (de Cal *et al*., 1995; Larena and Melgarejo, 1996; Akköprü and Demir, 2005). Rhizobacterial strains of *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Alcaligenes* and *Bacillus* spp. also have been used to reduce wilt disease caused by *F. oxysporum* f. sp. *lycopersici* (Yuen and Schroth, 1986; Larkin and Fravel, 1998; Chin-A-Woeng *et al*., 2000; Akköprü and Demir, 2005). The use of antagonist mixtures may also provide improved disease control over the use of single organisms (Larkin and Fravel, 1998). Multiple organisms may enhance the level and consistency of control by providing multiple mechanisms of action, a more stable rhizosphere community and effectiveness over a wider range of environmental conditions. Several researchers have observed improved disease control using various combinations of multiple compatible biocontrol organisms (de Boer *et al*., 2003; Akköprü and Demir, 2005; Leeman *et al*., 2005).

Achromobacter xylosoxydans is a gram-negative bacteria, catalase positive, frequently-retrieved in rizosphere. The use of this species in biocontrol is already known in literature (Yuen and Schroth, 1986; Benchimol *et al*., 2000; Vaidya *et al*., 2001; Yan *et al*.,

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2004). Biocontrol by *A. xylosoxydans* depends on competition for iron (Yuen and Schroth, 1986). Iron is abundant in the Earth's crust, but most of it is found in the highly insoluble form of ferric hydroxide, thus, due to the limitation of soluble iron in the rhizosphere, microbes and plants are scavenging for iron with highly sophisticated iron binding and uptake mechanisms which include siderophores production. Mutagenesis suggests that siderophores produced by bacterial biocontrol strains involved in biocontrol since siderophore-negative mutants no longer suppress disease (O'Sullivan and O'Gara, 1992; Whipps, 2001). Siderophores of *Achromobacter* sp. can act also in biocontrol as determinant of Induced Systemic Resistance (ISR) in plants (Bertrand *et al*., 2000). Another biocontrol mechanism of *A. xylosoxydans* can be chitinase production (Vaidya *et al*., 2001). It is well known that many species of bacteria, streptomycetes, actinomycetes and fungi useful for biocontrol produce chitinolytic enzymes (Herrera-Estrella and Chet, 1999). Expression and secretion of these enzymes can result in the suppression of plant pathogen activities directly. It has been recently observed that *A. xylosoxydans* also produces inhibitory substances that can control pathogenesis of *Aspergillus parasiticus* (Yan *et al*., 2004).

In this context, the objective of this research was to examine an *A. xylosoxydans* strain isolated from an Italian suppressive soil for its efficacy in controlling *Fusarium* wilt of tomato. This research was conducted as an alternative strategy to chemicals for developing an effective biological control for the management of *Fusarium* wilt disease on tomato.

MATERIALS AND METHODS

All the experiments of this study were conducted in the Centre of Competence for the Innovation in the Agro-Environmental Field (Agroinnova) in 2007.

Bacteria identification: The bacterial strain used in this work was isolated from suppressive soil in Italy and identified by 16S ribosomal DNA (rDNA) sequence analysis. Genomic DNA of the bacterium was prepared with a DNeasy tissue kit (Qiagen). A fragment of the 16S rDNA of the bacterium was sequenced. The PCR was carried out by using universal eubacterial primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991) in a 20 μL of volume containing 0.5 μM of each primer, 2.0 μL of 10X buffer (Invitrogen), 2.5 mM $MgCl_2$, 250 μM each dNTP, 1 μL of DNA preparation and 1 U of Taq polymerase (Invitrogen). PCR was performed under the following conditions: 94°C for 5 min, followed by 94°C for 1 min, 55°C for 1 min and 72°C for 1 min for 35 cycles and then 72°C for 7 min. PCR product was cloned with TOPO TA vector kit (Invitrogen) and sequenced by using the M13 reverse and forward primers with an ABI Prism 3730 XL sequencer (at the BMR Genomics srl of Padova, Italy). Phylogenetic analysis was performed with the neighbor joining method on the sequence obtained with the software MEGA4 (Tamura *et al*., 2007).

Bacteria growth: Inoculum for treatment of tomato plants was prepared from 48 h cultures grown on LB broth medium (Invitrogen) at 30°C. Bacterial suspension was concentrated by centrifugation at 8,000 rpm for 15 min and adjusted with deionized sterile water to 1×10^8 cfu mL^{-1} that corresponds an OD_{600} spectrometrically measured of 0.4.

Fungal pathogen growth: The pathogen isolate Panero 6 of *F. oxysporum* f. sp. *lycopersici* was obtained from the Agroinnova Collection of the University of Torino, grown on Komada's (1975) medium agar (KM) and kept at 8°C. Fungal growth was done on Potato Dextrose Agar (PDA, Difco) plates for *in vitro* tests, while a mixture of chlamidospores and conidia obtained by growth in liquid complete medium (composition for 1 L: yeast extract 1 g; casein hydrolysate 2 g; KH_2PO_4 1,5 g; $MgSO_4$ 1 g; glucose 15 g; Trace elements solutions: 10 mL. The composition of the trace elements solutions is (g L^{-1}): LiCl 0.29 g; $CuSO_4 . 5 H_2O$ 0.05 g; $ZnSO_4$ 0.05 g; H_3BO_3 0.37 g; $Al_2(SO_4)_3$ 0.055 g; $SnCl_2 . 2 H_2O$ 0.027 g; $MnCl_2 . 4 H_2O$ 0.59 g; $NiSO_4 . 6H_2O$ 0.055 g; $Co(NO_3)_2 . 6 H_2O$ 0.055 g; Ti O_2 0.055 g; KI 0.027 g; KBr 0.027 g) and dispersed in talc powder was used as soil inoculum for *in vivo* tests. Briefly, according to Locke and Colhoun (1974), the fungal suspension was grown in shake (90 rpm) for 10 days on liquid complete medium at 25°C in the dark. Then, the culture was pelleted by centrifugation (8,000 rpm, 20 min), rinsed two times with sterile distilled water and filtered through one layer of cheese cloth to remove mycelial fragments. The conidial density was determined by direct observation on a hemocytometer (Bio Kobe) and adjusted with sterile deionized water to 1×10^7 cfu mL^{-1}. Finally, the solution was kept in a specific volume of talcum powder and when it was completely dry was used for *in vivo* assays in order to obtain a final concentration of 1×10^4 cfu g^{-1} of soil.

***In vitro* antagonism test:** The effect of the potential bacterial antagonist on mycelia growth of *F. oxysporum* f. sp. *lycopersici* was studied using PDA dual cultures assay. Three drops (0.1 mL per drops) of bacterial broth culture and a 6 mm plug from the leading edge of a 5-day-old culture of *F. oxysporum* f. sp. *lycopersici*

cultured on PDA were inoculated on opposite sides into 9-cm-diameter petri dish containing 11 mL of PDA (Suárez-Estrella *et al*., 2001). Inhibition was scored by the absence of any contact between the bacterium and *F. oxysporum* after 5-7 days of incubation at 28°C.

Glasshouse experiments: Seeds of tomato cv Cuore di Bue (Furia Sementi) chosen for its high susceptibility to *Fusarium* wilt disease were sown in an autoclaved mixture of peat:perlite:sand (70:10:20, v:v:v) in trays and maintained in a growth chamber at 25°C and 70-90% relative humidity with 16 h light for about 3 weeks. Seedlings (2-4 leaf stage) were dipped for ten minutes in a bacterial suspension at a final density of 1×10^{8} cfu mL^{-1} (OD_{600} 0.4). Treated seedlings were transplanted into two different steamed soils mixture (peat, compost broadleaf bark and clay, respectively, 60:20:20 v:v:v, treated for 30 min at 70°C): soil infested by *F. oxysporum* f. sp. *lycopersici* (prepared in a talc powder at the final rate of 10^{4} cfu g^{-1} of soil) and soil without pathogen inoculation. Control plants were inoculated with pathogenic *F. oxysporum* and left untreated. Uninoculated plants were kept as healthy controls. The four different tests used for the assay consisted of four replicates of 10 plants each. A completely randomized block design was used. Five pathogenicity assays were performed. The study was conducted in the glasshouses of Agroinnova Centre placed in Torino (Italy).

Disease evaluation: Disease progress was evaluated by monitoring the plants every week with the aid of the following arbitrary index (ADI) (0-100). ADI of 0 corresponded to healthy plant; 25, slight vascular discoloration, slight leaf chlorosis and growth reduced 25% in comparison with healthy control; 50, vascular discoloration, chlorosis and strong growth reduction; 75, extended vascular discoloration, strong leaf chlorosis and strong growth reduction; 100, dead plant. At the end of each trial, stem sections of wilted plants were harvested, surface disinfected in 0.5% household bleach (Procter and Gamble) (0.0026% sodium hypochlorite) and placed on KM to confirm the presence of the wilt pathogen.

Determination of the fresh matter weight of tomato plants: In order to evaluate a possible plant growth promotion due to the activity of the bacterial strain tested, at the end of each *in vivo* experiment (about 10 weeks after seed sowing), tomato plants were harvested and weighed.

Evaluation of siderophore production: Siderophore production by *A. xylosoxydans* MM1 was detected using the Chrome Azurol Sulphate (CAS) assay modified by Milagres *et al*. (1999). This assay was chosen due the toxicity of CAS Blue-agar on the growth of this bacterial strain. Petri dishes were prepared with Malt Extract Agar (MEA) and after becoming solid the medium was cut into halves, one of which was replaced by CAS-blue agar. The halves containing MEA medium were inoculated with the bacterial strain. The inoculum was placed as far as possible from the borderline between the two media. The plates were incubated at 30°C for about three weeks in the dark. As negative control we used MEA supplemented with 10^{-4} M $FeCl_3$.

Hydroxamate nature of siderophore was detected by Csaky (1948) test and catecholate nature of siderophores was detected by Arnow (1937) test.

Detection of chitinolytic activity on plates: To test for chitinolytic activity on plates, bacterial cells were streaked on minimal medium solidified with 1.5% agar and supplemented with colloidal chitin (0.2%) prepared by the method of Rodriguez-Kabana *et al*. (1983) by partial hydrolysis with 10 N HCl for 1.5 h at room temperature. The colloidal chitin was then washed several times with distilled water to adjust the pH. The plates were incubated at 30°C for 72-96 h.

Statistical analysis: The data from all the experiments were analysed by analysis of variance (ANOVA) and statistical significance between treatments was evaluated with the Tukey's Honestly Significantly Different test at 5% significance level. Statistical analysis was carried out with SPSS software (SPSS for Windows 5.01).

RESULTS

Bacteria identification: The nucleotide sequence analysis of the 16S rDNA amplified by PCR and cloned in TOPO TA vector was performed with the software BLASTN available online from the National Center for Biotechnology Information (NCBI). This analysis showed that bacterial strain tested belongs to *Achromobacter xylosoxydans* species (E value = 0). A phylogenetic tree obtained with the software MEGA4 using the 16S rDNA sequence of our bacterium is showed in Fig. 1.

***In vitro* antagonism test:** The hypothetical antagonistic bacterium *A. xylosoxydans* MM1 did not inhibit growth of *F. oxysporum* f. sp. *lycopersici* on PDA plates and the fungus overgrown bacterial streaked cells (data not shown).

***In vivo* evaluation for *Fusarium* wilt suppression in the glasshouse:** *A. xylosoxydans* MM1 significantly reduced *Fusarium* wilt of tomato. Efficacy in reduction of disease incidence ranged from 23.8 to 72.1% (mean value of 51.1%). Moreover pathogen was not isolated from stem

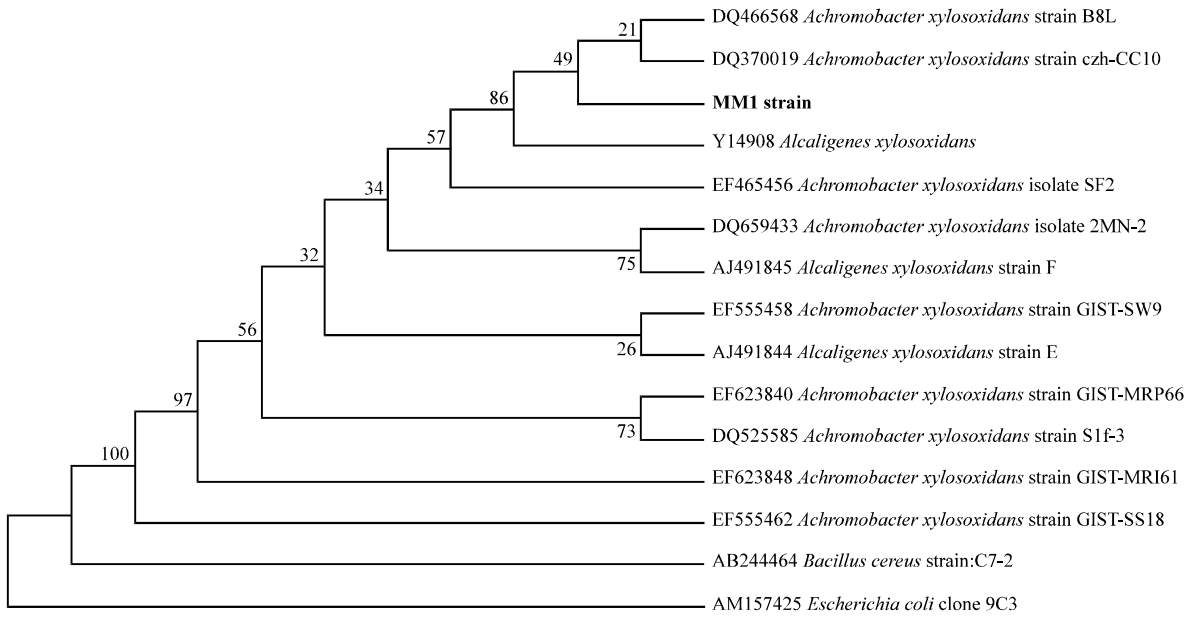

Fig. 1: Phylogenetic tree performed with the neighbor joining method with the software MEGA4 on the 16S rDNA sequence obtained from the bacterial strain analyzed in this study

Table 1: Control efficacy of *A. xylosoxydans* MM1 for control *F. oxysporum* f. sp. *lycopersici* in tomato. Since repeated tests yielded similar results, representative mean values are presented

	Treatments	Disease severity (%)[A]	Efficacy (%)[B]
With pathogen inoculation in the soil	Positive control[C]	67.2b	-
	A. xylosoxydans MM1[D]	37.0ab	51.1
Without pathogen inoculation in the soil	*A. xylosoxydans* MM1[E]	1.2a	-
	Healthy test (negative control)[F]	0.0a	-

[A]Mean values followed by the same letter(s) are not significantly different according to Tukey's Honestly Significantly Different test at 5% significance level. [B]Control efficacy in wilt severity compared with the pathogen alone (positive control), [C]Healthy plants transplanted into soil infested by *F. oxysporum* f. sp. *lycopersici* (pathogen alone), [D]Treated plants with *A. xylosoxydans* MM1 transplanted into soil infested by *F. oxysporum* f. sp. *lycopersici*, [E]Treated plants with *A. xylosoxydans* MM1 transplanted into soil without pathogen inoculation, [F]Healthy plants transplanted into soil without pathogen inoculation

Table 2: Fresh weights of tomato plants affected by *Fusarium oxysporum* f. sp. *lycopersici* and *A. xylosoxydans* MM1 treatments. Since repeated tests yielded similar results, representative mean values are presented

	Treatments	Fresh weight (g plant^{-1})[A]	Increased growth (%)
With pathogen inoculation in the soil	Positive control[B]	8.6b	-
	A. xylosoxydans MM1[C]	19.1a	55.0[D]
Without pathogen inoculation in the soil	*A. xylosoxydans* MM1[E]	18.4a	14.4[F]
	Healthy test (negative control)[G]	15.8ab	-

[A]Mean values followed by the same letter(s) are not significantly different according to Tukey's Honestly Significantly Different test at 5% significance level, [B]Healthy plants transplanted into soil infested by *F. oxysporum* f. sp. *lycopersici* (pathogen alone), [C]Treated plants with *A. xylosoxydans* MM1 transplanted into soil infested by *F. oxysporum* f. sp. *lycopersici*, [D]Percentage of increased tomato growth in *A. xylosoxydans* MM1 treated test in presence of the pathogen in the soil compared with the positive control (pathogen alone), [E]Treated plants with *A. xylosoxydans* MM1 transplanted into soil without pathogen inoculation, [F]Percentage of increased tomato growth in *A. xylosoxydans* MM1 treated test in absence of the pathogen in the soil compared with the negative control (healthy test), [G]Healthy plants transplanted into soil without pathogen inoculation

sections of asymptomatic plants protected with the biocontrol treatment, indicating that the disease assay was effective in identifying all diseased plants and that the asymptomatic plants were generally free of disease and did not represent attenuation of symptom development. Finally *A. xylosoxydans* MM1 demonstrated any significative phytotoxicity on tomato plants (Table 1).

Fresh matter weight of tomato plants: Impact of *A. xylosoxydans* activity on tomato growth promotion was determined by comparison of the fresh matter weight of the treated and untreated plants in the presence of the pathogen in the soil. Also treated plants in soil without *F. oxysporum* f. sp. *lycopersici* were analyzed in comparison with healthy plants. Efficacy of the *A. xylosoxydans* treatment on *Fusarium* wilt control of tomato was confirmed by the significant differences among the fresh matter weight of the treated and untreated plants grown in the presence of the pathogen (Table 2). When *A. xylosoxydans* was used in the absence

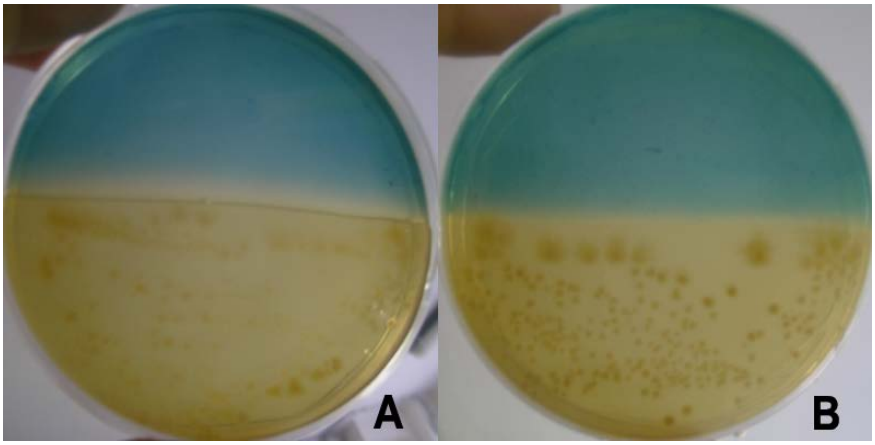

Fig. 2: Siderophore production *in vitro* by *A. xylosoxydans* MM1 in MEA medium (A) and in MEA medium supplemented with 10^{-4} M $FeCl_3$ (B). When iron is absent in the medium, the bacterial strain is stimulated to produce siderophores: see the colour change of CAS-blue agar

of the pathogen to test any hypothetical effect on tomato plants, an unexpected biofertilizer activity was discovered. *A. xylosoxydans* MM1 stimulated about 14% the tomato growth of treated healthy plant if compared with untreated healthy ones.

***In vitro* siderophore production:** *A. xylosoxydans* MM1 showed siderophore production (Fig. 2). When MEA, a medium poor of iron, was supplemented with 10^{-4} M $FeCl_3$, any change in the colour of CAS-blue agar edge occurred. In contrast when *A. xylosoxydans* is grown on MEA it is stimulated to produce siderophores. To investigate siderophore chemical nature, the tests of Csaky and Arnow were performed. Only Csaky's test gave positive result, suggesting that siderophore molecules produced by *A. xylosoxydans* MM1 belong to hydroxamic class.

Chitinolytic activity *in vitro*: *A. xylosoxydans* MM1 did not hydrolyze colloidal chitin after 72-96 h of growth on minimal medium agar supplemented with colloidal chitin as the sole carbon source. This result suggested that chitinolytic enzymes are not secreted by this strain *in vitro*.

DISCUSSION

A. xylosoxydans was isolated for the first time from human ear discharge by Yabuuchi and Yano (1981). Many beneficial functions of this species have been reported in experimental assays, including control of some plant pathogens (Yuen and Schroth, 1986; Benchimol *et al*., 2000; Vaidya *et al*., 2001), stimulation of ionic transport to promote plant growth (Bertrand *et al*., 2000) and inhibition of aflatoxin production in *Aspergillus* sp. (Yan *et al*., 2004).

In this study, the isolate MM1 of *A. xylosoxydans* collected from a *Fusarium* suppressive soil in Italy was found to be effective in the control of *Fusarium* wilt of tomato. This strain provided from 23.8 to 72.1% reduction of disease relative to pathogen-treated under severe disease pressure which resulted in wilt incidence of 51.4 to 81.3%. The range shift of disease reduction was probably due to the seasonal effects or, in other words, to the more or less conductive conditions in which experiments were performed. This level of control is comparable with those reported in literature using rhizobacterial strains in controlling *Fusarium* wilt diseases (Larkin and Fravel, 1998; Chin-A-Woeng *et al*., 2000; Monda, 2002; de Boer *et al*., 2003; Bora *et al*., 2004; Akköprü and Demir, 2005).

Vaidya *et al*. (2001) reported that an *A. xylosoxydans* strain produces chitinolytic enzymes that could be useful in biocontrol. However, in our testes, this activity seems absent. This may be due to differences in the genome of the bacterial strain or in the genomic expression profile or in the assay method used.

So, the inhibitory in vivo effect of *A. xylosoxydans* MM1 against *F. oxysporum* f. sp. *lycopersici* could be related to production of siderophores that limit the availability of iron. In our experiments, in fact, this peculiarity was demonstrated, even if only *in vitro* and not yet in soil. Yuen and Schroth (1986) demonstrated that the strain MFA1 of *Alcaligenes* sp. (the old name of the genus *Achromobacter*) was able to control Fusarium wilt of carnation by siderophore production. Probably this

could occur also in our case in which we found the production of hydroxamate type of siderophores. This activity is considered advantageous for its exploitation in biocontrol because these compounds are comparatively stable, have high iron chelating ability and can impart suppressiveness to soil (Mazzola, 2002).

It could be speculated that our strain can also act by Induction of Systemic Resistance (ISR) in tomato due the fact that *A. xylosoxydans* MM1 did not inhibit the *in vitro* growth of *F. oxysporum* f. sp. *lycopersici*, therefore antagonism could be mediated through the host plant. Moreover it is already demonstrated that *Achromobacter* sp. can induce ISR in planta by improvement of NO_3^- uptake rate (Bertrand *et al*., 2000) and in our case we also observed a tomato growth stimulation in plants inoculated with the bacterium. It was no checked if the hypothetical elicitation of ISR occur in tomato but the application of this bacterial strain to the roots of tomato not only inhibited and delayed the establishment of *F. oxysporum* f. sp. *lycopersici* in root tissues, but also increased tomato growth. *A. xylosoxydans* MM1 stimulated about 14% the tomato growth of treated healthy plant if compared to untreated healthy ones.

Many rhizobacteria have been described and tested as biofertilizers (Kennedy *et al*., 2004; Zahir *et al*., 2004) and encourage to improve selection procedures and production of quality inocula for practical application. Plant Growth Promoting Rhizobacteria (PGPR) are involved in many important ecosystem processes such as biological control of plant pathogens, nutrient cycling and seedling growth (de Boer *et al*., 2003; Barea *et al*., 2004; Zahir *et al*., 2004) and probably also in our case we could have a PGPR strain.

Work is in progress with this strain to determine the specific mechanisms, interaction, condition and requirements responsible for effective biological control such as production of volatile organic compounds and non-volatile metabolites. It was just identified a possible new bacterial antagonistic strain against *Fusarium* wilt of tomato and this is only the first step toward the development of a biocontrol organism. It is necessary also to implement at a practical level the knowledge of this strain such as in field application and ecological interaction. Through an understanding of much more information we can establish the limitation as well the potential for biocontrol and develop strategies for its implementation and management.

ACKNOWLEDGMENT

Authors want to thank Dr. Jeanne Griffith (University of Torino, Italy) for improving the English of the original manuscript. This research was supported by the European grant of the CROP BIOTERROR project n° 6403 and by the Italian Ministry for the Environment, Land and Sea.

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