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An *in vitro* antagonistic activity evaluation of rhizobacteria against *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) isolated from the Algerian west.

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Key words: *Fusarium oxysporum* f. sp. *radicis-lycopersici*, Biological control, *Bacillus subtilis*, *Pseudomonas fluorescens, Fusarium* crown, Root rot.

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Abstract

The use of biological antagonists is a promising technique for the protection of plants without having a fatal effect on the environment. The *in vitro* confrontation tests is an obligatory step for the selection of successful antagonistic agents, what has ends in our study to the selection of two rhizobacteria: *Bacillus subtilis* and *Pseudomonas fluorescens,* isolated from the rhizosphere of tomato healthy plants and which were tested for their *in vitro* inhibitive power against five isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl). The results of the direct confrontation on different culture media demonstrated that rhizobacteria have an inhibitive effect on the pathogenic, while noticing a performance of *P. fluorescens* with an inhibition which varies between 73,77 and 80 %, comparing with 56,36 and 60,46 % for *B. subtilis*. The inhibition by volatile substances of *P. fluorescens* varies between 25 and 39,53 %, compared with 18,86 and 32,55 % by volatile substances of *B. subtilis*. Rhizobacterial cell-free supernatant showed an inhibition from the first until the seventh incubation day against the five fungal isolates, when mycelia growth reaches 5±0,2 cm in presence of *P. fluorescens* cell free supernatant, and it reaches 5,9±0,3 cm in presence of *B. subtilis* cell free supernatant compared with 8,5 cm on control plates without rhizobacteria cell free supernatant.

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Introduction

The tomato plant can be infected by different phytopathogenic fungi which affect the agriculture yield. Among these phytopathogenic fungi, we find *Fusarium oxysporum* which is the most frequent and the most important species of the fungal microflora of cultivated soils (Messiaen and Cassini, 1968). *Fusarium* crown and root rot is an important soil-borne disease, with the potential to limit productivity in glasshouse and field tomato crops. *Fusarium oxysporum* f. sp. *radicis-lycopercisi* is the forma specialis which parasites a single host who is the tomato, causing crown root which is one of the most frequent diseases and the gravest in the Algerian North (Hamini, 2010).

Various ways are used to control the tomato root rot, as the chemical, genetic, cultural, physical and biological control; the latter remains the most important intervention way (Karimi *et al.,* 2012).
Among the antagonists who reign in saturated soil by microflora equilibrated for the environment and the biotope, we meet the presence of *Pseudomonas* and *Bacillus* species that inhibit the growth of phytopathogenic fungi by direct or indirect methods (Abdulkareem *et al*., 2014).

Previous study was realised on the combination between Plant growth-promoting rhizobacteria (PGPR) and conventional pesticides to increase their efficacy and broaden the disease control spectrum (Myresiotis *et al.,* 2012).

But other researchers suggest that the use of PGPR without conventional pesticides stay the friendliest method to inhibit the growth of different spices of *Fusarium* in their natural environment without harming the environment (Nandhini *et al*., 2012; Karimi *et al*., 2012; Prashar *et al.,* 2013; Abdulkareem *et al.,* 2014).

After these results we realised a prospecting and we noticed an agricultural fields of healthy tomatoes and a good quality yield in the region of Messerghine, Oran (Algeria), which led us to set as aims the *in vitro* selection of antagonistic rhizobacteria with a successful inhibiting effect on the *Fusarium oxysporum* f. sp. *radicis-lycopersici* growth in a first part, to be used in a next study *in vivo*.

Materials and methods

Fungal isolates

Diseased tomato plants showing typical symptoms of root rot were collected from the Algerian west, from which we isolated the *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) using the method of Karkachi *et al.* (2010) with few modifications. Roots were cut in small pieces (2 cm) and placed aseptically on Potato Dextrose Agar medium (PDA). Plates were incubated at 28 °C under 12 h photoperiodic. The identification was realized on the basis of the morphological characters (Nelson *et al*., 1981; Booth 1985). The pathogenicity was detected using the method described by Hamini (2010). Five isolates were used in this study.

Antagonistic bacteria

One isolate of *Bacillus subtilis* and another of *Pseudomonas fluorescens* were obtained from the collection of the applied microbiology laboratory, Faculty of Nature Sciences and Life, University of Oran, Algeria. They were isolated from rhizosphere samples of healthy western Algerian tomato plants on selected nutrient agar medium (NA) and purified using the method described by Prashar *et al.* (2013).

Direct confrontation test

The method used is the one described by Zebboudj *et al.* (2014) with few modifications. This test was realised to compare the inhibition growth effect of antagonistic bacteria in different culture mediums. *Bacillus subtilis* and *Pseudomonas fluorescens* isolates were streaked on PDA, King B and nutrient agar medium in two streak parallels of 2 cm, and the centre of the same plates containing antagonistic bacteria was inoculated with 5 mm fungal agar disc 3 days pre-cultivated on PDA medium. Control plates containing fungus without antagonistic bacteria. Petri dishes were incubated at 30°C for 5 days. Mycelia growth inhibition was determined using the method

described by Jafar-Pour *et al.* (2008). The experiments were performed in triplicate.

Volatile substances activity

The method used is the one described by Nandhini *et al.* (2012) with few modifications. In this technique, plates containing PDA medium sowed by each bacterial isolate were incubated 24 h at 28°C, then in sterile conditions, the lid was replaced by the base of another plate containing PDA medium inoculated by a 5 mm fungus plug (Forl). Bases of plates were sealed with parafilm and incubated 5 days at 30°C : Fungus upper and bacterial isolate lower. In control plates, bacterial isolates were replaced with sterilised distilled water. The experiments were performed in triplicate and mycelia growth inhibition was also determined using the method described by Jafar-Pour *et al.* (2008).

Culture filtrate assay

Bacterial cell-free supernatant activity was determined by method used by Laref and Guessas (2013) with few modifications. *Bacillus subtilis* and *Pseudomonas fluorescens* were inoculated in nutrient and King B broth respectively. After 24 h of incubation at 30°C, cultures were centrifuged at 8000 g for 10 min, and supernatant was filtered by Millipore filter (0,22 µm). 100 µl of *B. subtilis* and *P. fluorescens* cell-free supernatant was spotted onto every one of the four wells in nutrient agar and King B medium respectively. Centre of plates containing bacterial cell-free supernatant was inoculated with 5 mm plug 3 days pre-cultivated on PDA medium. Control plates containing fungus without antagonistic bacterial cell-free supernatant. Petri dishes were incubated at 30°C, and mycelia growth was measured every 24h during 7 days. The inhibition zones were microscopically examined. Growth inhibition tests were done in triplicate.

Statistical analyze

The statistical study was realized by "Microsoft Office Excel 2007".

Results

Direct confrontation test

The results illustrated on Fig. 1 present a comparative analysis of Forl growth inhibition by antagonistic bacteria on PDA medium, and it shows that each bacteria used have an inhibitive activity against the five fungal isolates. We also see that *P. fluorescens* has a more important antagonistic activity with an inhibition which varies between 61,36 and 65,11%, compared with 56,36 and 60,46% by *B. subtilis*.

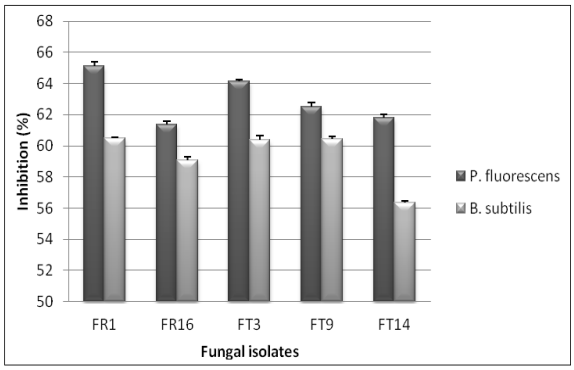

Fig. 1. Comparative analysis of Forl growth inhibition by *P. fluorescens* and *B. subtilis* on PDA medium.

The results illustrated on Fig. 2 present a comparative analysis of growth inhibition by *P. fluorescens* on PDA and King B medium, and it show that this antagonistic bacterium has a more important inhibitive activity on King B medium with an inhibition which varies between 73,77 and 80%, compared with 61,36 and 65,11% on PDA medium.

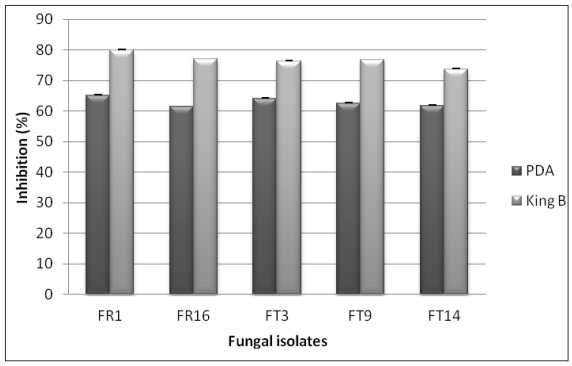

Fig. 2. Comparative analysis of Forl growth inhibition by *P. fluorescens* on PDA and King B medium.

The results illustrated on Fig. 3 present a comparative analysis of growth inhibition by *B. subtilis* on PDA and nutrient agar medium, and it show that this antagonistic bacterium has a more important

inhibitive activity on PDA medium with an inhibition which varies between 56,36 and 60,46%, compared with 23,91 and 52,63% on nutrient agar medium.

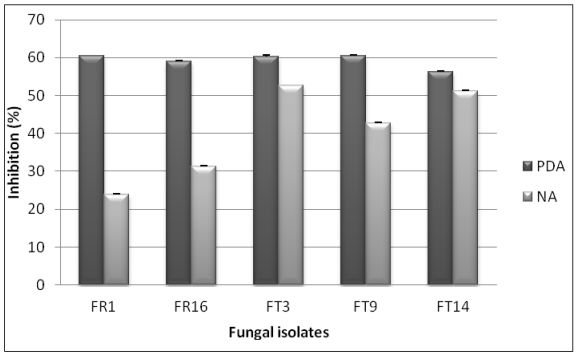

Fig. 3. Comparative analysis of Forl growth inhibition by *B. subtilis* on PDA and nutrient agar medium.

The results illustrated on (Fig. 4 and Fig. 5) show that radial growths of fungal isolates in dual culture with antagonistic bacteria are lower than those in control-plates on different culture mediums.

Volatile substances activity

The results illustrated on Fig. 6 show the Forl growth inhibition by volatile substances of *P. fluorescens* and *B. subtilis* on PDA medium after incubation 5 days at 30°C, and we can see that volatile substances of *P. fluorescens* have an effect with an inhibition which varies between 25 and 39,53%, compared with 18,86 and 32,55% by volatile substances of *B. subtilis*.

Fig. 4. Antagonistic activity of *P. fluorescens* in co-culture with Forl on King B medium after incubation at 30°C for 5 days. (a) Test plate; (b) Control plate.

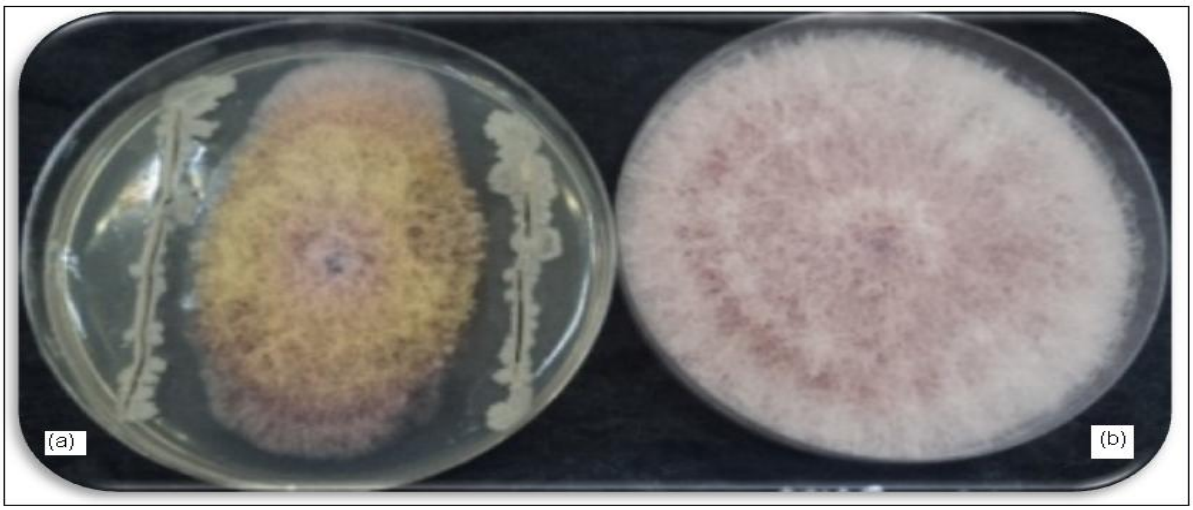

Fig. 5. Antagonistic activity of *B. subtilis* in co-culture with Forl on PDA medium after incubation at 30°C for 5 days. (a) Test plate; (b) Control plate.

Culture filtrate assay

This test was realized for analyzed the effect of bacterial cell free supernatant on Forl mycelia growth. As showed on (Fig. 7, Fig. 8 and Fig. 9), there is a growth inhibition by bacterial cell-free supernatant from the first until the seventh incubation day against the five fungal isolates used, when mycelia growth reaches 5±0,2 cm in presence of *P. fluorescens* cell free supernatant, and it reaches 5,9±0,3 cm in presence of *B. subtilis* cell free supernatant compared with 8,5 cm on control plates without bacterial cell free supernatant.

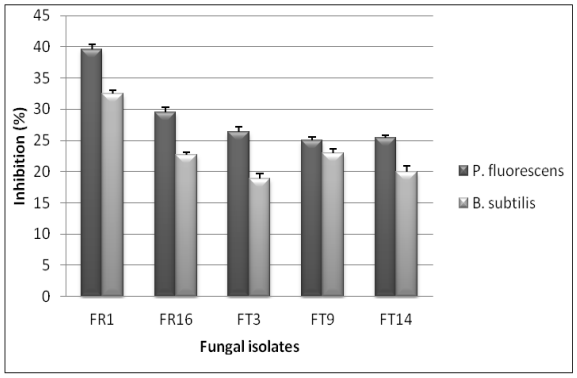

Fig. 6. Growth inhibition of Forl by volatile substances of antagonistic bacteria.

Microscopic observations in inhibition zones illustrated on Fig. 10 show a sterile mycelium what means that there is an inhibition of conidia germination.

Discussion

The crown and root rot caused by *Fusarium oxysporum f.sp radicis lycopersici* is a very important disease which made the object of several recent studies with the aim of finding methods to control this disease. The researchers suggest that the use of biological antagonistic agents is the friendly method to inhibit the growth of the pathogen in there natural environment without harming the environment (Nandhini *et al.*, 2012 ; Karimi *et al.*, 2012 ; Prashar *et al.,* 2013 ; Abdulkareem *et al.,* 2014).

The tests of biological control with these bacterial strains showed that it was possible to limit the growth of Forl. Nevertheless, the use of these biological agents allows stabilizing the disease in an acceptable threshold. Other works allowed demonstrating the importance of inhibitive action exercised by strains of *Pseudomonas* spp. on the *Fusarium oxysporum* f. sp. *ciceri*, the agent of chickpea fusariose (Inam-ul-Haq *et al.*, 2003).

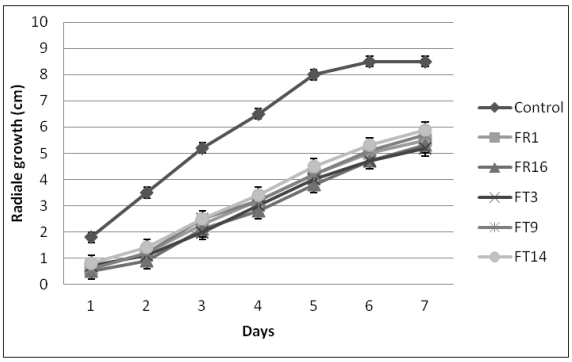

Fig. 7. *B. subtilis* cell-free suparnatant activity on different fungal isolates incubated 7 days at 30°C on nutrient agar medium.

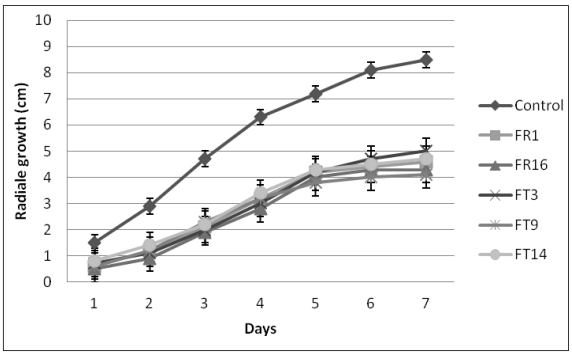

Fig. 8. *P. fluorescens* cell-free suparnatant activity on different fungal isolates incubated 7 days at 30°C on King B medium.

Baysal *et al.* (2008) identified a new *Bacillus subtillus* strain (EU07) and was selected as the best antagonist and evaluated against FORL *in vitro* studies. Strain EU07 was able to reduce disease incidence by 75%, when applied as an inoculants.

Uppal *et al.* (2008) noted a significant reduction of the verticilliose incidence on the potato culture by strains of *Pseudomonas* spp. As we can note several works which demonstrated a very important inhibitive effect of *Pseudomonas* spp. against *Fusarium oxysporum* f. sp. *lycopersici* (Kumar *et al.,* 2002 ; Karkachi *et al.,* 2010 ; Ardebili *et al.,* 2011).

Ahmed Idris *et al.* (2007) and Zhang *et al.* (2008) indicated that there are enzymes secreted by Bacillus spp. which have a role in the hyphale lyses of certain phytopathogenic fungi such as *Fusarium oxysporum, Rhizoctonia solani, Pythium ultimum* and *Alternaria solani*. Tan *et al.* (2013) showed the role of a novel antifungal proteine wich has stronger antagonism against *Fusarium oxysporum* f. sp. *cubense Corynespora cassiicola*, *Alternaria solani, Botrytis cinerea* and *Colletotrichum gloeosporioide*. In the majority of the works relative to the use of *Pseudomonas* spp. and *Bacillus* spp., the pre-selection of strains is based largely on the *in vitro* antagonism activity. However, the correlation between the potentialities shown *in vitro* and the levels of the bio control actions or bio stimulation of the vegetable growth, is not always obvious (Benchabane *et al.*, 2000).

This study was realized as part of an *in vitro* biological control test using *Bacillus subtilis* and *Pseudomonas fluorescens* against the pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the agent responsible for the *Fusarium* crown and root rot, and it having to notice that the tomato cultivated in the region of Messerghine situated in Oran (Algerian west), where from we isolated the rhizobacteria.

Direct confrontation between Forl and rhizobacteria demonstrated that *P. fluorescens* and *B. subtilis* have an inhibitive effect on five fungal isolates, as he has us allowed to make a comparative study between the inhibitive effect of both rhizobacteria, as well as, the inhibitive effect of each on different cultural media. The results showed that *P. fluorescens* has a more important inhibitive effect than that of *B. subtilis* on the PDA medium and that the antagonistic effect of the latter is more important on the PDA medium than on NA medium, contrary to *P. fluorescens* that were more successful on King B medium, what concords with the results of Sundaramoorthy and Balabaskar, (2013).

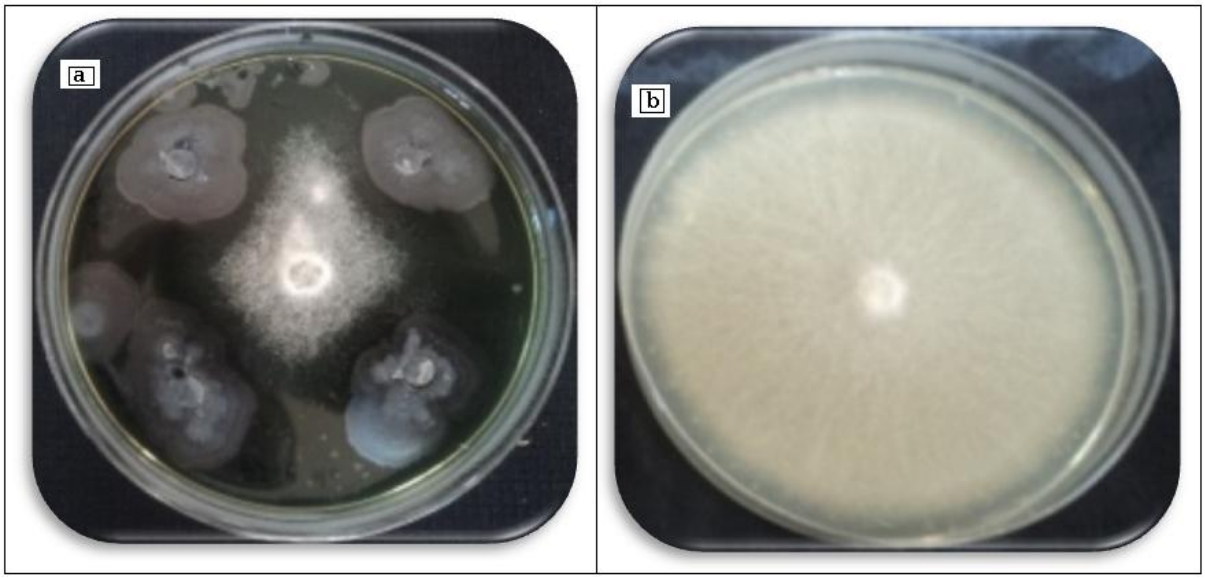

Fig. 9. Growth inhibition of Forl by *P. fluorescens* cell-free supernatant on King B medium after incubation at 30°C for 7 days. (a) Test plate; (b) Control plate.

The evaluation of the volatile substances production has allows to highlight the important inhibitive effect of *P. fluorescens* that was known by its volatile substances production (Altinok *et al.*, 2013), as well as, the effect of *B. subtilis* who is used as agent of bio control seen its production of a wide range of metabolites with a biological effect, which allows it to stay alive in its environment (Sansinenea and Ortiz, 2011).

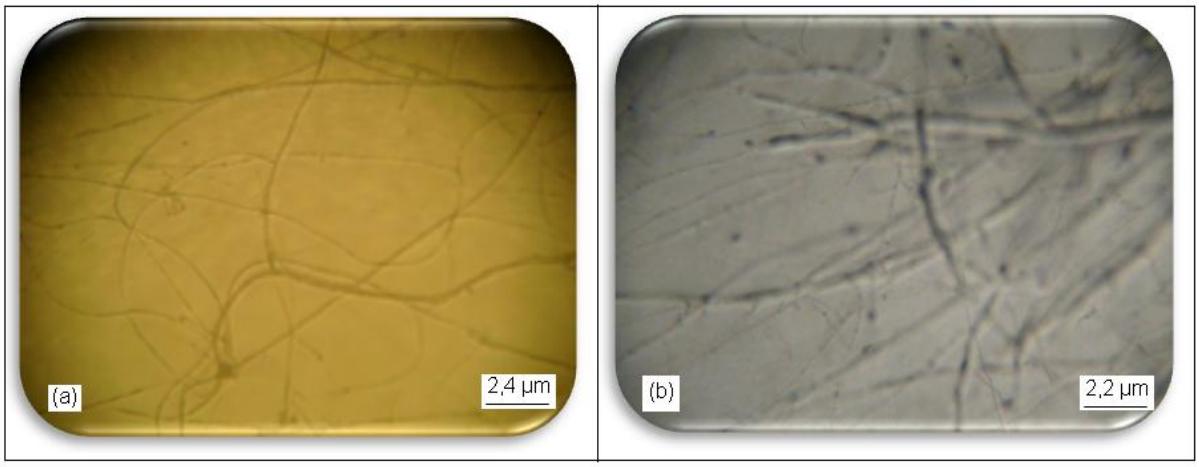

Fig. 10. Microscopic observations showing inhibition of conidia germination by bacterial cell free supernatant. (a) In presence of *Pseudomonas fluorescens* cell free supernatant; (b) In presence of *Bacillus subtilis* cell free supernatant.

The use of rhizobacteria culture filtrate showed that there is a significant reduction of Forl mycelia growth and it corresponds to the results of (Ardebili *et al.,* 2011) which demonstrated that *P. fluorescens* can inhibited the growth of Forl by the secretion of enzymes, as well as in the results of (Abdulkareem *et al.,* 2014) which showed that *B. subtilis* reduced fundamentally the growth of the pathogenic by the secretion of inhibitive diffuse substances.

Microscopic observations in inhibition zones show a sterile mycelium what means that there is an inhibition of conidia germination. The same observations were reported by Ström (2005); Muhialdin and Hassan (2011); Laref and Guessas (2013) and Zebboudj *et al.* (2014).

Conclusion

The current study suggested that the application of *B. subtilis*, *P. fluorescens* and rhizobacteria cell-free supernatant can be used to inhibit the growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Further studies *in vivo* should be carried in a second part to determine the potential of there rhizobacteria.

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References

Abdulkareem M, Aboud HM, Saood HM, Shibly MK. 2014. Antagonistic activity of some plant growth rhizobacteria to *Fusarium graminearum*. International Journal of Phytopathology **3,** 49-54.

Ahmed Idris H, Labuschagnea N, Korstena L. 2007. Screening rhizobacteria for biological control of *Fusarium* root and crown rot of sorghum in Ethiopia. Biological control **40**, 97-106. http://dx.doi.org/10.1016/j.biocontrol.2006.07.017

Altinok HH, Dikilitas M, Yildiz HN. 2013. Potential of *pseudomonas* and *bacillus* isolates as biocontrol agents against *Fusarium* wilt of eggplant. Biotechnology and biotechnological equipment **27**, 3952-3958. http://dx.doi.org/10.5504/BBEQ.2013.0047

Ardebili ZO, Ardebili NO, Mahdi Hamdi SM. 2011. Physiological effects of *Pseudomonas fluorescens* CHA0 on tomato (*Lycopersicon esculentum* Mill.) plants and its possible impact on *Fusarium oxysporum* f. sp. *lycopersici*. Australian Journal of Crop Science **5**, 1631-16-38.

Baysal Ö, Çalışkan M, Yeşilova Ö. 2008. An inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp. *radicis-*

lycopersici. Physiological and Molecular Plant Pathology 73, 25-32.
http://dx.doi.org/10.1016/j.pmpp.2008.11.002

Benchabane M, Bakour R, Toua D, Boutekrabt A. 2000. Mise en évidence de l'effet antagoniste de *Pseudomonas fluorescens* vis-à-vis de la fusariose vasculaire de la tomate. EPPO Bulletin 30, 243-246.
http://dx.doi.org/10.1111/j.13652338.2000.tb00888.x

Booth C. 1985. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, 3ème Edition.

Hamini N. 2010. Diversité génétique des populations de *Fusarium* pathogène de la tomate sur le pourtour méditerranéen. PhD thesis, University of Oran 1, Algeria.

Inam-ul-Haq M, Javed M, Ahmad R, Rehman A. 2003. Evaluation of different strains of *Pseudomonas fluorescens* for the biocontrol of *Fusarium* wilt of chick pea. Pakistan Journal of Plant Pathology **2,** 65–74.
http://dx.doi.org/10.3923/ppj.2003.65.74

Karimi K, Amini J, Harighi B, Bahramnejad B. 2012. Evaluation of biocontrol potential of *Pseudomonas* and *Bacillus* spp. against *Fusarium* wilt of chickpea. Australian Journal of Crop Science **6,** 695-703.

Karkachi N, Gharbi S, Kihal M, Henni JE. 2010. Biological control of *Fusarium oxysporum* f. sp. *lycopersici* isolated from Algerian tomato by *Pseudomonas fluorescens, Bacillus subtilis, Serratia marcesens* and *Trichoderma harzianum*. Research Journal of Agronomy **4,** 31-34.
http://dx.doi.org/10.3923/rjagr.2010.31.34

Kumar N, Thirumalai V, Gunasekaran P. 2002. Genotyping of antifungal compounds producing plant growth promoting rhizobacteria *Pseudomonas flurescens*. Current Science **82,** 1463-1466.

Laref N, Guesses B. 2013. Antifungal activity of newly isolates of lactic acid bacteria. Innovative Romanian Food Biotechnology **13,** 80-88.

Messiaen CM, Cassini R. 1968. Recherches sur les Fusarioses. IV- La systématique des *Fusarium*. Annales des Epiphytes**19**, 387-454.

Muhialdin B J, Hassan Z. 2011. Screening of lactic acid bacteria for antifungal activity against *Aspergillus oryzae*. American Journal of Applied Sciences **8,** 447-451.
http://dx.doi.org/10.3844/ajassp.2011.447.451

Myresiotis CK, Karaoglanidis GS, Vryzas Z, Papadopoulou-Mourkidou E. 2012. Evaluation of plant-growth-promoting rhizobacteria, acibenzolar-S-methyl and hymexazol for integrated control of *Fusarium* crown and root rot on tomato. Pest Management Science **68**, 404-411.
http://dx.doi.org/10.1002/ps.2277

Nandhini S, Sendhilvel V, Babu S. 2012. Endophytic bacteria from tomato and their efficacy against *Fusarium oxysporum* f. sp. *lycopersici*, the wilt pathogen. Journal of Biopesticides **5**, 178-185.

Nelson PE, Tousson TA, Cook RJ. 1981. *Fusarium*: Diseases, Biology and Taxonomy. The Pennsylvana state University Press. University Parck and London.

Prashar P, Kapoor N, Sachdeva S. 2013. Isolation and characterization of *Bacillus* sp with *in-vitro* antagonistic activity against *Fusarium oxysporum* from rhizosphere of tomato. Journal of Agricultural Science and Technology **15**, 1501-1512.

Jafar-Pour S, Hajieghrari B, Salehi A. 2008. A *in vitro* study on the fungicidal effects of Percidin 535® (Per Acitic Acid 15%) against phytopathogenic fungi. Biotechnology **7**, 830-832.

Sansinenea E, Ortiz A. 2011. Secondary

metabolites of soil *Bacillus* spp. Biotechnology Letters **33**, 1523-1538.
http://dx.doi.org/10.1007/s10529-011-0617-5

Ström K. 2005. Fungal inhibitory lactic acid bacteria - Characterization and application of *Lactobacillus plantarum* MiLAB 393. Doctor's dissertation. ISSN 1652-6880, ISBN 91-576-7036-6

Sundaramoorthy S, Balabaskar P. 2013. Evaluation of Combined Efficacy of *Pseudomonas fluorescens* and *Bacillus subtilis* in Managing Tomato Wilt Caused by *Fusarium oxysporum* f. sp. *lycopersici* (Fol). Plant Pathology Journal **12**, 154-161.
http://dx.doi.org/10.3923/ppj.2013.154.161

Tan Z, Lin B, Zhang R. 2013. A novel antifungal protein of *Bacillus subtilis* B25. SpringerPlus **2**, 543.
http://dx.doi.org/10.1186/2193-1801-2-543

Uppal AK, Hadrami AE, Adam LR, Tenuta M, Daayf F. 2008. Biological control of potato *Verticillium* wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. Biological Control **44**, 90–100.
http://dx.doi.org/10.1016/j.biocontrol.2007.10.020

Zebboudj N, Yezli W, Hamini-Kadar N, Kihal M, Henni JE. 2014. Antifungal activity of Lactic Acid Bacteria against *Fusarium oxysporum* f. sp. *albedinis* isolated from diseased date palm in south Algeria. International Journal of Biosciences **5**, 99-106.
http://dx.doi.org/10.12692/ijb/5.9.99-106

Zhang SS, Raza W, Yang XM, Hu J, Huang QW, Xu YC, Liu XH, Ran W, Shen QR. 2008. Control of *Fusarium* wilt disease of cucumber plants with the application of a bioorganic fertilizer. Biol Fertil Soils **44**, 1073-1080.