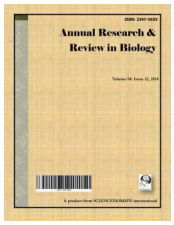

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Evaluation of Inhibition of Fungal Spore Germination by Rhizospheric Bacterial Extracts

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SMA and MMZ designed the study, wrote the protocol and interpreted the data. Authors SC, SMA, AB, MMZ and NH anchored the field study, gathered the initial data and performed preliminary data analysis. Authors SMA, NH and MMZ managed the literature searches and produced the initial draft. Authors SC and AB wrote the manuscript. While authors SMA and MMZ read and approved the publication. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This work aims to evaluate the anti-germinative activity of bacterial extracts. These bacteria were already characterized for their antagonistic capacity *in vitro* against telluric phyto-pathogens fungi: *Fusarium oxysporum* f.sp. *albedinis* (Foa), *Fusarium solani* var. *coeruleum* (Fsc) and *Phytophthora infestans* (Pi), causing Bayoud, dry rot and mildew diseases respectively.
Methodology: The OD of bacterial cultures is measured in order to determine the microbial charge producing the anti-germination substances, then centrifuged, filtered. A volume of spore suspension of determined concentration is added to a determined volume of the bacterial filtrates. After incubation, for 24 hours at room temperature the inhibition of spore germination is observed under a microscope using a Malassez cell.

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Results: The results showed that these bacterial filtrates are able of inhibiting fungal spore germination, among these filtrates XI35 ' one, gave a total inhibition (100%) on both of the tested fungi Foa and Fsc, while on Pi it only gave 16.66% of inhibition.
Conclusion: The bacterial filtrates were more active against mitosporic fungi, than oomycetes.

Keywords: Antagonistic bacteria; inhibition of germination; secondary metabolites; telluric phyto-pathogenic fungi.

1. INTRODUCTION

Plant diseases caused by soil-borne agents are economically important and, for the most part, difficult to control and / or treat. Fusarium diseases, as rots and mildew are causing considerable economic losses at the global level, affecting annual plants such as potatoes and perennials such as date palms. In these cases chemical treatment is ineffective or unnecessary.

During the twentieth century, considerable research has demonstrated the ability of microorganisms from various phylogenetic origins to inhibit different phyto-pathogenic agents [1]. These microorganisms act either by antibiosis, competition [2] or by interacting with the plant [3], leading to more complex defense mechanisms leading to biocontrol [4].

At the level of the rhizosphere and the control of soil-borne pathogens, the control is mainly via bacteria belonging to the genus *Streptomyces*, *Bacillus*, *Agrobacterium* and *Pseudomonas* and fungi of the genera *Ampelomyces*, *Candida* [5] and *Trichoderma* [5,6]. The majority of studies show that 1 to 10% of soil isolates may have some antagonist potency *in vitro*, but of these, very few have the ability to suppress phytopathogenic agents in various soils and growing conditions and yet more Small number is capable of inhibiting a broad spectrum of pathogenic species [5].

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Phythopathogenic fungi

Fungal strains were as follows: *Phytophthora infestans* (Pr Larous, LMA, Département de microbiologie, Université Sétif-1. *Fusarium oxysporum* f.sp. *albedinis* (INRAA, Alger). *Fusarium solani* var. *coeruleum* (Institut Pasteur de Paris, France).

2.1.2 Referenced bacterial strains

Pseudomonas protogene CHA0, *Pseudomonas auréofaciens* 30-84 (Pr Haas, Lausanne, Suisse) and *Pseudomonas chlororaphis* subsp. *auréofaciens* (Dr. Mezaache-aichour, LMA Département de Microbiologie, Université Sétif-1).

2.1.3 Tested strains

Bacterial strains used in the inhibition tests were previousely isolated by Belatrous [7] et Sayah [8] and Mansouri et Remadna [9]. These strains belonging to *Pseudomonads* and *Bacillus* were already characterized for their antagonistic activity *in vitro* against the cited phytopathogenic fungi [10], and_are noted: Xi29, Xi48, Xi49, Xi47, B5, B21, Xi35', Xi29', Xi37', Xi30, Xi12, Xi1.

2.2 Methods

2.2.1 Preparation of bacterial filtrates

The bacterial references strains as well as the tested strains were grown in 25 ml of NBY for 72 h, with constant stirring on a rotary shaker (7 G; throw 19 mm) at room temperature (20-22℃) to late exponential phase. The OD (optical density; Table 1) is measured at 625 nm in order to determine the microbial concentration producing the anti-germination substances.

The bacterial cultures are then centrifuged for 20 min at 3512 G, filtered through 0.45 µm diameter membranes (THOMAPOR®Membranfilter), and the filtrates are recovered in sterile bottles and stored at 4℃ till use.

Table 1. Optical density of the tested strains at 625 nm after 72 h of incubation

Bacterial strains	CHA0	30-84	2	Xi 29	Xi 49	Xi 29'	B 5	Xi 12	Xi 37'	B 21	Xi 47	Xi 48	X i1	Xi 35'	Xi 30
OD at 625 nm	2.23	2.17	1.3	1.97	2.1	1.91	0.52	1.96	1.3	0.59	0.56	1.92	1.2	1.8	0.87

CHA0, 30-84 and 2: Pseudomonas protogene CHA0, Pseudomonas auréofaciens 30-84 and strain 2 Pseudomonas chlororaphis subsp. aureofaciens; Xi29, Xi49, Xi29 ', B5, Xi12, Xi37', B21, Xi47, Xi48, Xi35 and Xi30: tested strains

2.2.2 Inhibition of spore germination

A volume of 1 ml of spore suspension of determined concentration (10^7 spores / ml) is placed in a series of microtubes, and a volume of 20 µl of the bacterial filtrates to be studied is added. The tubes are prepared in triplicates and incubated for 24 hours at room temperature. After incubation, the inhibition of spore germination is observed under a microscope using a Malassez cell. The number of spores germinated or not is reported.

The percentage of non-germinated spores is calculated according to the formula:

$$\%\mathrm{SNG} = \frac{SNG}{SG+SNG} x100$$

%SNG: percentage of non germinated spores.

SG: number of germinated spores.

SNG: number of non germinated spores.

2.2.3 Statistical analysis

Data were analyzed by the one way analysis of variance (ANOVA) and the test with P<0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Inhibition of *Fusarium oxysporum* f. sp. *albedinis*

The percentage of germinated spores for *Fusarium oxysporum* f. sp. *albedinis* (FOA) was zero (0%) with the extracts of isolates XI30 and B5 and maximal (100%) for the extracts of the isolates tested Xi49, Xi29 ', Xi35', Xi37 'and B21; as well as those of the reference strains 30-84, CHA0 and 2 (Fig. 1).

3.1.2 Inhibition of *Phytophthora infestans*

The percentage of germinated spores for *Phytophthora infestans* (PI) varied between 15.47% for the extract of isolate XI30 and 100% for the control. While for the referenced strains; the inhibition varied from 40% for CHA0 and 66.66% for strains 2 and 30-84 (Fig. 2).

3.1.3 Inhibition of *Fusarium solani* var. *coeruleum*

After 24 h of incubation, a significant germination of the spores of *Fusarium solani* var. *coeruleum* (FSC) was observed by the bacterial extracts.

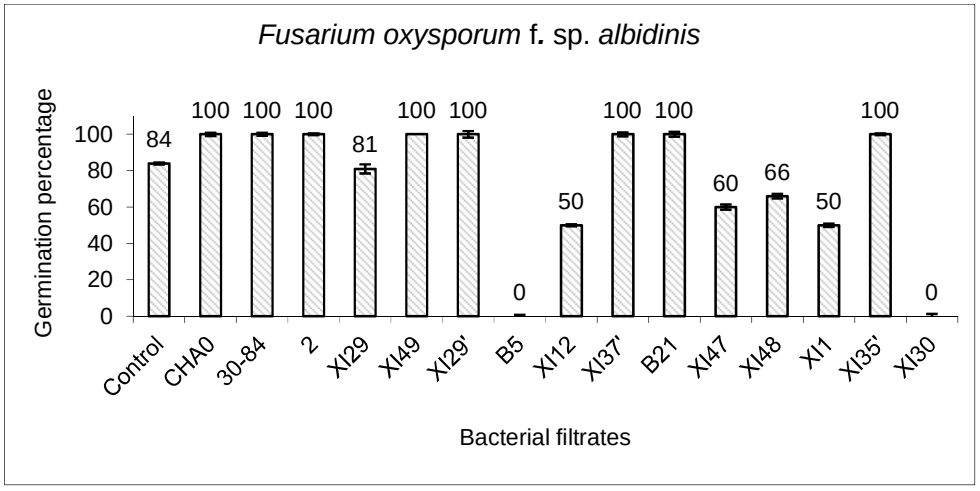

Fig. 1. Graphical representation of the percentages of the germinated spores of the *Fusarium oxysporum* f.sp. *albedinis* (Foa) treated by different bacterial filtrates

CHA0, 30-84 and 2: Pseudomonas protogene CHA0, Pseudomonas auréofaciens 30-84 and strain 2 Pseudomonas chlororaphis subsp. aureofaciens filtrates. Filtrates of the tested strains: Xi29, Xi49, Xi29 ', B5, Xi12, Xi37', B21, Xi47, Xi48, Xi35 ', Xi30. The first column corresponds to the untreated control

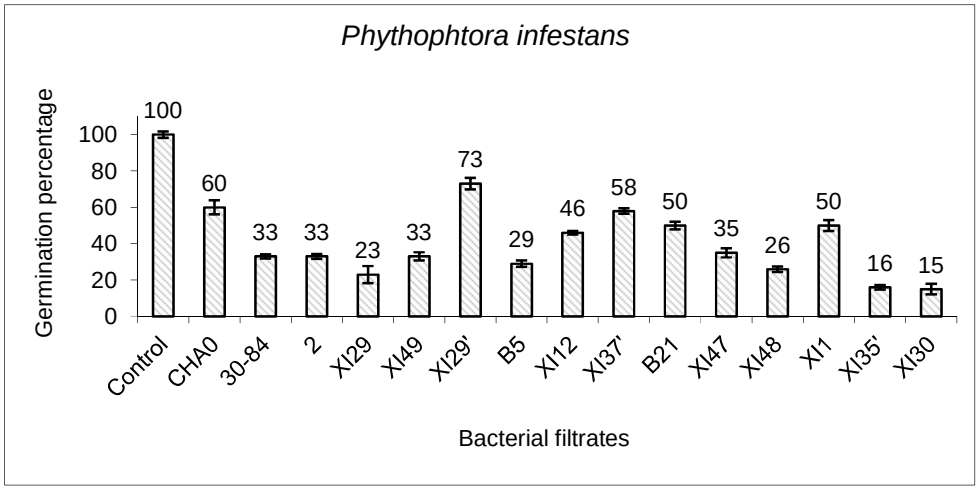

Fig. 2. Graphical representation of the percentages of the germinated spores of the *Phytophtora infestans* (Pi) processed by different bacterial filtrates

C, 3 and 2: Pseudomonas protogene CHA0, Pseudomonas auréofaciens 30-84 and strain 2 Pseudomonas chlororaphis subsp. aureofaciens filtrates. Filtrates of the tested strains..., B .....: Xi29, Xi49, Xi29 ', B5, Xi12, Xi37', B21, Xi47, Xi48, Xi35 ', Xi30. The first column corresponds to the untreated control

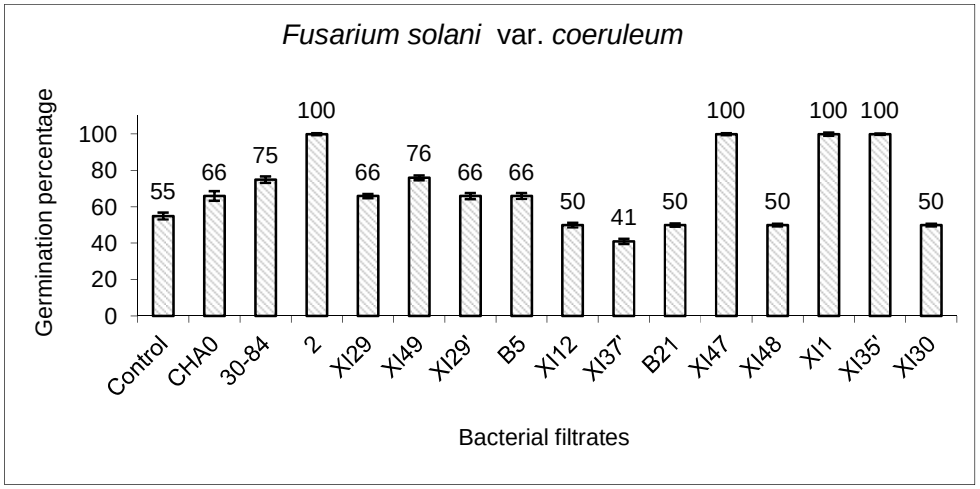

Fig. 3. Graphical representation of the percentages of the germinated spores of the *Fusarium solani* var. *coeruleum* (Fsc)Pi treated by different bacterial filtrates

C, 3 and 2: Pseudomonas protogene CHA0, Pseudomonas auréofaciens 30-84 and strain 2 Pseudomonas chlororaphis subsp. aureofaciens filtrates. Filtrates of the tested strains..., B .....: Xi29, Xi49, Xi29 ', B5, Xi12, Xi37', B21, Xi47, Xi48, Xi35 ', Xi30. The first column corresponds to the untreated control

Indeed, this germination varied between 41.9% for the extract of the isolate XI37 ', and 100% for the extracts of the isolates XI1, XI35' and Xi47. Whereas for the referenced strains CHA0, 30-84 and Strain 2, a percentage of 66.66, 75 and 100% respectively was observed (Fig. 3 above).

Depending on fungi and bacterial isolates, significant differences were recorded.

3.2 Discussion

Several antagonists certainly exist in nature and exert a more or less effective biological control on the pathogens of plants, the result is an increase in the inhibitory activities of the antagonists against the pathogens, the potential of a possible control of the diseases with this method is currently limited because, unlike laboratories and *in vitro*; the results in the field

are not usually of a particular success. The major problems are due to the fact that introduced microorganisms generally fail in their competition with the existing micro flora [11]. Such microorganisms are called plant growth-promoting rhizobacteria (PGPR), which use several mechanisms for biological control. However, antibiotic synthesis seems to be the main mechanism used against crop pathogens by different genera such as *Pseudomonas*, *Bacillus*, *Azospirillum*, *Rhizobium* and *Serratia* [12].

Among the isolates tested which showed activity on fungi, the filtrates of the isolates XI29 and XI49 (Gram +ve bacilli), B5 (*Bacillus megatherium*) and XI29' (*Pseudomona*s sp.), inhibited the spore's germination of the studied fungi. XI 29, XI49 and B5 were the most active on Fsc and Pi with respective percentages ranging from 24.04 to 33.33% and 66.66 to 77%. However, the inhibition of the Foa was maximum for the filtrate of isolate B5 and zero for others as XI37', XI1….

While the filtrates of isolates XI1 (*Bacillus licheniformis*), XI35 'and XI47 (*Bacillus subtilis*) were completely inactive on Fsc (100% of germination). The filtrates of isolates XI12, XI37' and B21 (Gram + ve bacilli) showed an effect varying from 59.9 to 50%. Indeed, Chan et al. [13] showed that the filtrate of *Bacillus subtilis* D1 / 2 was found to be effective in preventing the spread of *Fusarium graminearum*. This antifungal activity was attributed to the extracellular lipopeptides identified as fengycins [13].

XI37' (*Bacillus cereus*) showed an average activity of 58.33% on Pi, an oomycete. Silo-Suh et al. [14] demonstrated that the filtrate of the *Bacillus cereus* culture contained two antibiotics that suppress alfalfa disease caused by another oomycete *Phytophthora medicaginis*. The purified zwittermicin A reduces reversibly the elongation of the germinal tubes derived from cysts of *P. medicaginis*, and antibiotic B is responsible for the deformations of the germinal tubes.

On the other hand, the partial inhibition of germination of the control Foa and Fsc in our study could be the result of self-inhibition of the germination frequently observed when the concentrations of spores are high thus imposing a state of dormancy, whereas the frequency of germination increases as the concentration decreases. This phenomenon was observed for the first time by Edgerton (1910); who noted that when more than 12 to 15 conidia of *Colletotrichum lindemuthianum* are included in 1 cubic millimeter, the percentage of germination of spores is reduced. In some cases this inhibition is due to the effects of specific chemical inhibitors, but in other cases it may simply be due to an inadequate concentration of oxygen or nutrients [15].

Inhibition of germination of Foa spores was complete with the filtrates of isolates B5 and XI30. The bacteria associated with fungi have a considerable influence on their pathogenesis; their associations with *Fusarium oxysporum* appear to be important in their ability to adopt an invasive state / or pathogenic growth. Indeed, it has been demonstrated that volatile compounds produced by *Bacillus amyloliquefaciens* NJN-6 prevent the growth and germination of spores of *Fusarium oxysporum* f. sp. *cubense* [16]. On the other hand, a negative effect on fungal pathogenesis was observed with the inhibition of germination of phytopathogenic spores *Botrytis cinerea* by an antagonistic bacterial community on Chrysanthemum leaves [17].

Pseudomonas has multiple biocontrol mechanisms, the production of antibiotics seems to be the main mechanism of action against harvesting microorganisms, by the secretion of Pyrrolnitrin, Aerugine, Phenazines and Pholoroglucinols such as 2,4-DAPG (diacetyl-Phloroglucinol). Among these metabolites 2,4-DAPG is active against *Fusarium oxysporum* f.sp. *pisi*; in addition to other antifungal metabolites such as proteases, lipases and chitinases [12]. Some of the isolates tested (Xi35', Xi48 and Xi49) produce metabolites with the same biochemical characteristics of DAPG [18].

The filtrates of the reference strains tested gave an inhibition of spore germination but with different percentages depending on the fungus studied. While they showed important inhibition of PI. Inhibition of Fsc and Foa by the filtrates of these reference strains is of lesser effect except for strain 2 which gave important inhibition of PI. In addition to this strain 2, the isolates studied produce siderophores [19]. A direct relationship was established *in vitro* between the synthesis of siderophores in *Pseudomonas fluorescens* and their ability to inhibit the germination of *F. oxysporum* chlamydospores [20]. Indeed, Mezaache-Aichour et al. [21], demonstrated that this bacterium (strain 2) inhibits in dual culture

the mycelial growth of Foa, *Fusarium oxysporum* f. sp. *lycopersici*, *F. solani*, *Rhizoctonia solani* and the oomycete *Pythium ultimum*.

On the other hand, the filtrate of the isolate XI29' showed an inhibition of the spore germination of the Fsc of 33.33%. In contrast, the filtrate of isolates XI48 and XI30 (Gram-ve bacilli) showed a greater effect with 50% inhibition. Concerning the Foa, the filtrate of the isolate XI30 showed an inhibition of 100%, while that of XI29 showed no action on the germination of the spores of this fungus. Lim et al. [22] demonstrated that *Pseudomonas stutzeri* Ypl-1 filtrate containing lytic enzymes such as chitinase and laminarinase inhibited mycelial growth rather than spore germination, but also caused lysis of mycelia and germinal tubes in *F. solani*.

However for Pi, the respective inhibitions were 73.33 and 15.47% with XI30 and XI29'. These isolates produce similar molecules to phloroglucinol and its derivatives [18]. This polycetide, produced by biocontrol bacteria such as *P. fluorescens*, prevents zoosporogenesis and alters the motility of the zoospores of *Plasmopara viticola* and *Aphanomyces cochlioides*. Generally, zoospores are first immobilized after a short exposure to DAPG, then they undergo lysis [23]. The low sensitivity of Pi spores to the extract of isolate XI29' may be due to the low concentration of active molecules. Tofazzel and Tiedemann, [23] demonstrated that when treated with a small fraction of DAPG, instead of being lysed, zoospores of *A. cochlioides* germinate and form around the cystospores multiple germinal tubes with several branches.

Finally, in this study, the most effective isolates on the inhibition of germination of fungal spores are: isolates B5, XI29, XI30, XI35', XI37' and XI49 in addition to the reference strains tested.

4. CONCLUSION

The protection of crops against phythopathogens is a major issue in agriculture. Chemical control is widely used, but it is not always effective and can lead to contamination of food and the environment. The ability of these bacteria to produce antagonist substances that inhibit the germination of spores of phythopathogenic fungi as different as the isolated Foa and Fsc suggests the possibility of using these microorganisms in the control of fungal diseases of the date palm and Potato. This is by delaying infections, or contributing to weakening the pathogen and predisposing it to other chemical or biological substances.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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