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Effect of Salinity on *in vitro Trichoderma harzianum* Antagonism Against *Verticillium dahliae*

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Abstract: This *in vitro* study led to test the sodium chloride tolerance of *Trichoderma harzianum* and to evaluate the impact of salinity on its antagonistic capacities, in order to use it as a biological agent controlling *Verticillium* wilt of tomato in Moroccan saline soils. *Trichoderma harzianum* tolerates the salinity for its mycelial growth but its sporulation is significantly reduced. The efficiency of the antagonistic modes of action of *Trichoderma* against *Verticillium* depends on the saline concentration used. Thus, the antagonism by competition is slightly affected by moderated salt concentrations (6 g L^{-1}). The antagonism by antibiosis decreases with the increase of salt. The antagonistic compounds produced by *Trichoderma* in presence of 8 g L^{-1} NaCl concentration still reduce the pathogen growth but have no action on the abundance of microsclerotia. The *in vitro* antagonistic properties of *T. harzianum* obtained at low salt concentrations may be applied for the biological control of *Verticillium* wilt of tomato in Moroccan saline soils where levels of salinity are equivalent to salt concentrations favorable to *Trichoderma* antagonism.

Key words: *Trichoderma, Verticillium*, antagonism, salinity, mode of action

INTRODUCTION

Verticillium Wilt diseases cause important damages on tomato crops mainly along the Moroccan Atlantic coasts where soils are subjected to the action of spindrift and irrigation waters contain a significant level of sodium chloride (0.14 - 0.49 g L^{-1})[1]. An increase in incidence of the disease has been reported in correlation with the soil salinity[2]. In addition, *Verticillium dahliae*, main causal agent of tomato wilt, tolerates high salt concentrations. Salinity seems to stimulate its growth and to increase its cellulolytic capacities[3]. Besides, the low osmotic potential of the soil can hinder the development of antagonist microorganisms of the soil, and it can consequently, favour the proliferation of the pathogens[4].

Management of *Verticillium* wilt of tomato has involved the use of chemical and genetic control. However, the effectiveness of these management practices is curtailed by *Verticillium* mode of conservation in soil as microsclerotia and the occurrence of new physiological races[5], whence the interest to apply biological control by using beneficial microorganisms antagonist to *Verticillium*.

Trichoderma harzianum is a potential antagonist largely used in biological control against soil-borne pathogens. Successful control of several phytopathogenic fungi has been reported, for example, the control of *Rhizoctonia solani*[6,7], *Botrytis*[8,9], *Pythium*[7,10], *Fusarium* spp.[11,12], *Phytophthora nicotianae*[13] and others.

The antagonistic modes of action of *Trichoderma* are well known, including competition, mycoparasitism and production of antifungal metabolites[13,14]. Moreover, *Trichoderma* improves growth response of some plants[15,16]. Furthermore, the saprophytics capacity of *Trichoderma* can be influenced by soil environmental factors as temperature, humidity and pH[17].

The purpose of this study was to report *in vitro* investigation on salt tolerance of one strain of *T. harzianum* and to study the impact of salinity on the efficacy of its antagonistic capacities against *Verticillium* in view of using this antagonist for controlling *Verticillium* wilt of tomato in Moroccan saline soils.

MATERIALS AND METHODS

The pathogen: One isolate of *Verticillium dahliae* (P80, race 2) was selected for its highest aggressiveness towards tomato, var Marmande[3]. The fungus was grown in Petri dish in Potato Dextrose Agar (PDA, Difco)

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amended with NaCl with different concentrations varying from 0 to 8 g L^{-1} or from 0 to 16 g L^{-1} according to the experiment.

The antagonist: The isolate of *Trichoderma harzianum*, obtained from the Laboratory of Phytopathology (University Pierre and Marie Curie, Paris), was grown in PDA and into liquid Malt medium (2%). The two media were added with the same concentrations of NaCl previously described. The growth of the antagonist was estimated by the dry weight of mycelium; after filtration on muslin of *Trichoderma* liquid cultures two weeks old, the collected mycelium was washed twice then put to dry at 80°C during a night. The level of sporulation was determined on the filtrate after counting in haemocytometer . All cultures are incubated in darkness at 25°C±1.

Measure of the *Trichoderma* antagonism

Dual plating essays: *Verticillium* isolate was paired with the isolate of *Trichoderma* in the same petri dish at a distance of 4 cm. After three days, *Trichoderma* colonization capacity was determined by the ratio C as follows[18]:

$$C=DT/dx100$$

DT is the distance in mm covered by *Trichoderma* front growth on the axis connecting the cuttings of the two fungi and d is the distance separating the two.

Antibiosis essays

Antagonistic activity of volatile compounds: *T. harzianum* was cultured in petri dishes in PDA amended with NaCl. After five days, the lid of each dish was replaced by a bottom containing PDA medium inoculated with a cutting from young *Verticillium* culture. The two dishes were taped together with parafilm to constitute a tight proof. The pathogen is thus exposed to the gas emitted by *Trichoderma*. The antagonistic action is estimated, after five days incubation, by the inhibition of the pathogen growth (I) calculated using the formula:

I % = dc – di / dc x 100
dc: Diameter of the control colony
di : Diameter of the colony exposed to *Trichoderma*

Antagonistic activity of non volatile compounds: *Trichoderma* cultures were grown in PDA medium added with salt which was covered with a sterile cellophane sheet 24 h before. After seven days of incubation, the cellophane, carrying *Trichoderma*, was removed and a cutting from *Verticillium* culture was deposited The antagonistic activity was evaluated according to two parameters; pathogen growth and microsclerotia abundance. The latter is recorded by measuring the diameter of the pigmented area after four weeks.

The data, average of six replicates are analyzed for statistical significance according to Student test.

RESULTS

Effect of salinity on *Trichoderma harzianum* development

Morphology of the culture: Seven-days-old cultures, developed without salt, show a hyaline mycelium more or less frothy with green sporogenous area situated on the periphery of the Petri dish. In saline media, the sporogenous area becomes less dense in correlation with increasing salt concentrations. At 8 g L^{-1} of NaCl, the green coloration completely disappears. The medium is invaded only with whitish mycelia.

Mycelial growth: The mycelial growth of *Trichoderma* developed in saline media between 2 and 6 gL^{-1} of NaCl does not differ from the control cultures without salt (Table 1). A slight but significant decrease of mycelium dry weight is recorded at 8 g L^{-1}.

Sporulation: The results (Table 1) show that spores density gradually decreases with the increase of salinity. Sporulation is very weak at 8 g L^{-1} of NaCl; it's 0.7x 10^6 spores mL^{-1} which corresponds to an inhibition of 95% as compared to control cultures.

Effect of salinity on antagonistic activities against *Verticillium dahliae*

Competition: The capacity of colonization C is the result of competition between *Trichoderma* and *Verticillium* for space occupation. After three days of incubation, *Trichoderma* shows a high capacity to colonize the space whether in absence or presence of salt (Table 2). Nevertheless, the capacity C declines significantly starting from 6 g L^{-1} of NaCl but C values remain high, (80%), showing an important capacity of the antagonist to cover the colony of the pathogen in presence of salt.

The speed of growth of *Trichoderma* exceeding that of *Verticillium* results in the inhibition of the pathogen growth (Table 3). In presence of low salt concentrations, the percentages of inhibition growth due to the physical presence of *Trichoderma*, are similar to those of the control dual cultures without salt (65.7%). At 8 g L^{-1} of NaCl, the percentage of inhibition growth decreases to 52.45%.

Table 1: Effect of salinity of culture medium on mycelial growth and sporulation of *T. harzianum* after 15 days of incubation

NaCl (g L^{-1})	0	2	4	6	8
Mycelium dry weight (mg)	194.6a	195.5a	194.5a	177.5a	159.8b
Density of spores (x 10^6spores.ml^{-1})	14.1a	9.05b	5.06c	1.22d	0.76e

Numbers in the same line followed by the same letter are not significantly different (p=0.05) according to Student test

Table 2: Effect of salinity on the capacity of colonization of *T. harzianum* confronted with *Verticillium dahliae* in dual culture

NaCl (g L^{-1})	0	2	4	6	8
Capacity of colonization C(%)	90.62a	92.06a	90.62a	84.37b	80.04c

Numbers followed by the same letter are not significantly different (p=0.05) according to Student test

Table 3: Effect of salinity on *T. harzianum* antagonistic activity on *Verticillium dahliae* growth in dual cultures

NaCl (g L^{-1})	0	2	4	6	8
Inhibition (%)*	67.57 a**	65.20 a	62.71 ab	59.00 b	52.45 c

*Inhibition of mycelial growth of *Verticillium* confronted with *Trichoderma* in the same plate after 7 days of incubation

**Numbers followed by the same letter are not significantly different (p=0.05) according to Student test

Table 4: Effect of salinity on antagonistic action of volatile and non volatile metabolites produced by *T. harzianum* on *Verticillium dahliae* growth

	Inhibition of mycelial growth of *Verticillium dahliae* (%)				
NaCl (g L^{-1})	0	2	4	6	8
Volatile metabolites	38.33a	37.66a	37.93a	41.60a	25.00b
Non volatile metabolites	61.98a	55.82b	49.70c	45.20d	37.32e

Numbers in the same line followed by the same letter are not significantly different (p=0.05) according to Student test

Table 5: Effect of salinity on the antagonistic action of non volatile metabolites produced by *T. harzianum* on microsclerotia abundance of *Verticillium dahliae*

	Diameter of pigmented area on colony of *Verticillium dahliae* (mm)				
NaCl (g L^{-1})	0	4	8	12	16
Control colonies	52.80a	57.80b	42.16c	19.66d	13.83e
Colonies exposed to *Trichoderma*	20.33d	30.00f	30.66f	27.78f	31.5f

Numbers in the same line followed by the same letter are not significantly different (p=0.05) according to Student test

Antagonism by antibiosis: This form of antagonism concerns the capacity of *Trichoderma* to produce volatile and non volatile compounds diffused in culture medium.

Action of volatile compounds: The growth of the *Verticillium* subjected to volatile compounds liberated by *Trichoderma* was estimated after five days incubation. This time constraint is due to the fact that the antagonist mycelia quickly invaded the lid where *Verticillium* was cultured. In non saline media, the effect of volatile compounds reduced the pathogen growth by 40% as compared to non exposed control cultures (Table 4). The same inhibition is recorded for the low salt concentrations varying from 2 to 6 g L^{-1} of NaCl. At 8 g L^{-1}, the inhibition growth action decreases significantly. The inhibition percentage is of 24.4%.

Action of non volatile compounds

Action on mycelial growth: The pathogen cultures conducted on saline media, where *Trichoderma* had previously been cultured, show a considerable reduction of their growth as compared to their respective controls of similar salinity i.e. developed without *Trichoderma* compounds (Table 4) In absence of salt, the inhibition of the pathogen growth is above 62%. In presence of salt, the antagonistic action decreased as salinity increased. However, the inhibition growth was still very significant at 6 g L^{-1} (37%) and steadily persists in high salt concentrations up 16 g L^{-1}.

Action on sclerogenesis: Microsclerotia appear from the second week of incubation in control cultures without *Trichoderma* compounds in all salt concentrations of the experiment. Yet, microsclerotia was not observed on *Verticillium* cultures developed in the presence of the *Trichoderma* compounds. After four weeks, sclerogenesis is emphasized in all control cultures and lately appeared on the other cultures with less importance. In saltness media, the diameter of pigmented area is significantly weaker than that in the non exposed control cultures to *Trichoderma*. In presence of salt, the antagonistic action on sclerogenesis decreases and completely disappears with NaCl concentrations superior to 8 g L^{-1}. Even, the latter concentrations cause a significant increase in the abundance of microsclerotia as compared to control cultures of the same salinity developed in media lacking *Trichoderma* antagonistic compounds (Table 5).

DISCUSSION

The results presented in this study show that the sodium chloride presence in the media modifies the morphological aspect of *T. harzianum* and affects its antagonistic capacities against *Verticillium* tomato isolate.

In non-saline media, the antagonistic activity of the strain of *T. harzianum* used inhibited the pathogen mycelial growth and reduced its sclerogenesis intensity. Present results are in line with others studies which have demonstrated the antagonism of *T. harzianum* against vascular fungi namely *Verticillium dahliae*[19] and *Fusarium oxysporum*[20]. In fact, this antagonist is able to produce volatile compounds having either a fungistatic

effect such as the acetaldehyde and/or fungicide one as the alkyl pyrones[21]. Moreover, this antagonist releases some antibiotics and exude cellulase or chitinase, enzyme that break down the cell walls of pathogens[22,14].

Salinity proved to have different impacts on *T. harzianum* mode of action. Hence, the antagonism by space colonization is less affected by salt. The effect of salinity on *Trichoderma harzianum* volatile compounds action is perceived in salt concentrations beyond 6 g L^{-1} where the antagonistic effect on the pathogen's growth decreases. A threshold of salinity seems to be necessary to disturb the release of volatile compounds and/or to decrease their efficiency on *Verticillium* growth. As to the antifungal non-volatile compounds emitted by *Trichoderma*, salt amendment leads to a progressive drop of their antagonistic action on the pathogen; their effect on the inhibition of pathogen growth decreases and stabilized from 8 g L^{-1} on and was still observed for high salt concentrations, while the antagonistic action inhibiting sclerogenesis tended to disappear. We suppose then that salinity slows down the release of antifungal metabolites, however, the quantities produced prove to be sufficient to insure a noticeable inhibition of the pathogen growth. The inhibition of *Verticillium* sclerogenesis seems to exact larger quantities of antifungal metabolites to maintain a good antagonistic level. This accounts for the absence of the antagonistic action on microsclerotia under high salt concentrations.

Soil salinity may constitute an environmental factor which limits *Trichoderma* capacities. This should be taken into account in biological control programs against plant pathogenic fungi. The results of the *in vitro Trichoderma* antagonism recorded with low salt concentrations constitute a first phase in biological control program which aims in the long run, to control in natural conditions, *Verticillium* wilt of tomato using *Trichoderma* introduced in soil. We also note that the level of salinity of Moroccan soils are closer to the salt concentrations favorable to *Trichoderma* antagonism. Research on new strains of *Trichoderma* more tolerant to salt will help select more performing strains to be used as biological agents in regions where salinity of soils constitutes a factor aggravating fungal diseases such as tracheomycosis. It is necessary to study the adaptation of these antagonist strains to saline soil conditions and their interactions with others soil microorganisms before introducing them in different environments.

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