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Salinity Influence upon Activity of *Trichoderma harzianum* against *Botrytis cinerea*

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ABSTRACT

Three isolates of *Botrytis cinerea* were isolated from leaves and stems of different tomato varieties taken from four areas in the North-west of Algeria where tomato is mostly grown in greenhouses and high tunnels. The purpose of this study was to determine the effect of two salts: NaCl and CaCl₂ 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 *Botrytis cinerea*, the causal agent of grey mold disease of tomato. In the absence of salt, the volatile and non-volatile secondary metabolites from *T. harzianum* showed 52, 23-79, 81% inhibition of *B. cinerea*. In the presence of salt, the inhibition percentages of the mycelial growth of *B. cinerea* by volatile metabolites were stimulated by the high concentrations of NaCl 94,70 and 90,85% for CaCl₂ compared to the control. However, non-volatile compounds from *Trichoderma*, the percentage of growth inhibition varied between 65.17% and 82, 12% for NaCl and 61.19-85.01 in the presence of CaCl₂.

Key words: Grey mould, salt stress, NaCl, CaCl₂, saline tolerance, volatile metabolites, *Lycopersicon esculentum*

INTRODUCTION

Grey mould, caused by *Botrytis cinerea* (Sclerotiniaceae family) is an important plant disease that affects a large number of plant species and is particularly important in greenhouse production of tomatoes in Mediterranean basin (O'Neill *et al.*, 1997). In greenhouse tomato, the fungus infects flowers, fruits and leaves and can grow through the petiole into the stem (Shtienberg *et al.*, 1998; Kalogiannis *et al.*, 2006).

Soil salinity is one of the major environmental factors that lead to a deterioration of agricultural land and reduction in crop productivity worldwide Keshavarzi *et al.* (2011). This problem is one of the major stresses especially in arid and semi-arid regions (Munns, 2002) and can severely limit plant growth and productivity (Allakhverdiev *et al.*, 2000; Koca *et al.*, 2007).

In Algeria, a wide range of environmental stresses (such as high and low temperature, drought, alkalinity, salinity and pathogen infection) are potentially harmful to the plants. Soil salinity and irrigation water are two of the main serious problems hindering the development of most plant species (Levignron *et al.*, 1995). Thus, the effect of these factors may result from structural and physiological changes in the plant, an increase incidence and severity of diseases caused by various species pathogen. (Bouchibi *et al.*, 1990) showed that relatively low levels of salinity (25-50 mEq

could increase the severity of phytophthora root rot of tomato with high Na:Ca ratios (10:1), *Phytophthora* (Blaker and MacDonald, 1986; MacDonald, 1982; Sanogo, 2004; Swiecki and MacDonald, 1988), *F. oxysporum* f. sp. *vasinfectum* (Turco *et al.*, 2002), *F. oxysporum* f. sp. *radicis-lycopersici* (Jones *et al.*, 1993), *Verticillium dahliae* and *Alternaria solani* (Nachmias *et al.*, 1993).

Chemical control has always been essential for the management of gray mold, caused by *Botrytis cinerea*. Continuous use of the same fungicide against the same pathogen results in the development of fungicide resistant strains of the pathogen (Kumar and Dubey, 2001; Mamgain *et al.*, 2013). Additionally chemical fungicides not only develop fungicide resistant strains but also accumulate in food and ground water as residues. In addition, it results in environmental hazards and has harmful side effects on human beings and animals. However, due to the polluting and non-biodegradable nature of such pesticides.

Biological control offers the chance to improve crop production within the existing resources, besides avoiding the problem of pesticide resistance (Khan *et al.*, 2014). However, has been considered as a more natural and environmentally acceptable alternative to existing chemical treatment methods (Baker and Paulitz, 1996; Eziashi *et al.*, 2007).

There are a variety of fungal species and isolates that have been reported as biocontrol agents, although *Trichoderma* species clearly dominate, perhaps due to their ease of growth and wide host range (Whipps and Lumsden, 2001). *Trichoderma* as a potent fungal biocontrol agent against a wide range of plant pathogens has attracted considerable scientific attention (Hermosa *et al.*, 2012; Joshi *et al.*, 2010; Moran-Diez *et al.*, 2012). The effectiveness of this antagonist species depends on the suppress plant diseases by direct antibiosis or mycoparasitism as well as indirect IR (Lorito *et al.*, 2010; Shores *et al.*, 2010). The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, competition and secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Harman *et al.*, 2004). For the most effective control of disease, it seems necessary to examine the impact of salinity on its antagonistic capacities, in order to use it as a biological agent controlling *B. cinerea* of tomato.

MATERIALS AND METHODS

Fungal isolates: *Botrytis cinerea* isolates were obtained from decayed tomato (*Lycopersicon esculentum*) in North-western Algeria. The leaf fragments were placed on filter paper moistened with sterile water in a Petri dish. *B. cinerea* was cultured on Potato Dextrose Agar (PDA) incubated at 25°C.

Antagonist: The isolate of *Trichoderma harzianum*, obtained from the laboratory of plant protection (University of Mostaganem, Algeria) was grown in PDA. The media were added with the same concentration of NaCl (50, 100, 150, 300 mEq).

Colony growth inhibition assay with *Trichoderma harzianum* in dual culture method: Interactions between antagonistic of *T. harzianum* against *Botrytis cinerea* were determined by the method described by Dennis and Webster (1971). A 4 mm diameter mycelial disc from the margin of the *Trichoderma* one week old culture and the pathogen *Botrytis cinerea* were placed on opposite of the plate at equal distance from the periphery and in control plates only *Botrytis cinerea* was placed. The plates were incubated at 25±1°C, observed after 4 days and the colony interactions were measured as percentage of inhibition of radial growth of *Botrytis cinerea* by following equation:

$$L = \frac{(D1 - D2)}{D1} \times 100$$

where, L is the percentage inhibition of radial mycelial growth, D1 is radial growth of the pathogen in the control, D2 is radial growth of the pathogen in the presence of *T. viride* (Edgington *et al.*, 1971).

Evaluation of volatile metabolites: The effect of the volatile metabolites released by *T. harzianum* on the mycelial growth of the pathogens was evaluated by the inverted plate technique as described by Dennis and Webster (1971). The 5 mm mycelial discs of *T. harzianum* obtained from the margin of young cultures were placed centrally on the PDA glass dish and incubated in 25±1°C for 72 h. In the control plates, sterile PDA media discs 5 mm in diameter were placed on the plates as mentioned above. At the end of the incubation period, the top of each petri dish was replaced with the bottom of the petri dish inoculated with pathogen and sealed together with adhesive tape. A completely randomized experimental design was used with three replicates. Radial growth of the pathogens was recorded on the 5th day of incubation and L was calculated, as described above.

The influence of NaCl and CaCl₂ on the diameter growth was determined by growing the isolates in a PDA medium at 4 NaCl and CaCl₂ levels (50, 100, 150, 300 mEq), control medium was not amended with salts.

Statistical analysis: All statistical analyses were analyzed by the software of statistics (STATBOX 6.0.4 grammersoft). The data was analyzed by two-way factorial. Comparison of means and interactions was performed by Duncan's multiple range tests. Statistical significance was assessed at the level of p = 0.05 or p = 0.01.

RESULTS

Effect of non-volatile metabolites from *T. harzianum* on the radial growth of *B. cinerea*:

In the absence of salt (Table 1), isolates of *B. cinerea* do not present the same profile of growth inhibition, the optimum percent for growth inhibition of this fungus was from 63.76-68.15 of F27 and TR46, respectively. In the dual culture experiment, *T. harzianum* had a marked significant inhibitory effect on the growth of isolates (Table 1). Growth inhibition decreased as follows: TR46 (68.15%)>B27 (66.8 %) >F27 (63.76%). Data in Table 1 indicate that, application of sodium salt caused a significant increase the growth inhibition at various concentrations tested compared with control except TR46 (p>0.05), growth inhibition was decreased for sodium concentrations of 50 and 100 ppm can be obtained from this isolate relative to the control.

Calcium chloride stimulates mycelial growth of *T. harzianum*. These observations indicate that the growth inhibition of the F27 and B27 isolates might increase (85.01, 70.17%), even at high salinity (150 ppm). However, higher concentrations of calcium chloride (300 ppm) caused F27 and B27 to reduce the percentage of inhibition growth by 81.63 and 65.84%, respectively. Calcium chloride was reducing growth inhibition of the TR46 in the dual culture for all concentrations. The interaction between salt and concentration was significant (p<0.001).

Effect of volatile metabolites from *T. harzianum* on the radial growth of *B. cinere*: In the absence of salt, TR46 and F27 was most resistant and revealed lowest percent inhibition of mycelial growth as 52,23% and 53,13, respectively in combination with *T. harzianum*.

Table 1: Growth inhibition of three isolates by *Trichoderma harzianum* in dual culture after incubation at 25°C for 3 days

Botrytis cinerea isolates			
Treatments	F27	B27	TR46
NaCl			
0	63.76+4.34 ^c	66.80+4.32 ^{bc}	68.15+3.01 ^{abc}
50	74.39+0.83 ^b	72.56+1.44 ^{bc}	65.17+0.86 ^{bc}
100	71.01+5.22 ^b	66.80+3.81 ^{bc}	67.16+1.49 ^{abc}
150	82.12+2.21 ^a	71.12+2.9 ^{bc}	71.63+1.49 ^{ab}
300	80.18+3.01 ^a	78.83+0.83 ^a	73.13+0 ^a
CaCl₂			
50	76.81+2.90 ^a	73.06+0.83 ^b	67.16+2.58 ^{abc}
100	80.67+0.83 ^{bc}	73.54+0.83 ^b	67.16+2.58 ^{abc}
150	85.01+0.83 ^b	70.17+0.83 ^{bc}	65.66+2.98 ^{abc}
300	81.63+2.21 ^{bc}	65.84+0.83 ^c	61.19+5.38 ^c
Two-way factor analysis of variance			
Salt	***	NS	***
concentration	***	***	NS
Salt*concentration	NS	***	***

*, **, ***Significant effects at 0.05, 0.01 and 0.001, respectively, NS: Not significant

Table 2: *In vitro* growth inhibition of three isolates of *Botrytis cinerea* by volatile compounds of *T. harzianum* in PDA medium after incubation at 25°C for 3 days

Botrytis cinerea isolates			
Treatments	F27	B27	TR46
NaCl			
0	53,13+0.83 ^c	79,81+2,88 ^c	52,23+2,98 ^c
50	61,83+3.01 ^b	80,30+0,80 ^c	48,75+0,86 ^c
100	77,29+0.83 ^a	88,93+0,83 ^b	65,66+5,37 ^b
150	71,97+1.67 ^a	90,37+1,66 ^b	68,65+6,50 ^b
300	73,91+2.9 ^a	94,70+0,83 ^a	78,6+3,75 ^a
CaCl₂			
50	76,80+5.79 ^a	90,37+0,83 ^b	65,66+3,94 ^b
100	77,77+7.14 ^a	90,85+0,83 ^b	67,65+6,03 ^b
150	81,15+3.83 ^a	92,78+0,0 ^{ab}	62,68+6,50 ^b
300	81,64+4.65 ^a	90,25+0,83 ^b	63,18+3,75 ^b
Two-way factor analysis of variance			
Salt	***	***	***
Concentration	***	***	NS
Salt*concentration	*	***	***

****, **, ***Significant effects at 0.05, 0.01, 0.001, respectively, NS: Not significant

After 3 days of incubation, it is observed that volatile compounds from *Trichoderma harzianum* exhibited maximum growth inhibition by adding 300 ppm of NaCl to the culture medium when compare to the others salts (Fig. 1). The highest growth inhibition, 94,70, 78,6 and 73,91% was obtained in the high concentrations from isolates B27, TR46 and F27, respectively. The interaction between salt and concentration was significant for all salts isolates ($p < 0.05$) (Table 2). In the presence of low salt concentrations (50 and 100 ppm), the percentage of inhibition growth due to the interaction between fungi and *T. harzianum* was still very important with CaCl₂ more than NaCl. However, the antifungal activity of volatile metabolites produced by *T. harzianum* showed 90.037% maximum inhibition of mycelia growth with B27 in presence of higher concentrations of calcium chloride (300 ppm). On the other hand the effect of CaCl₂ on *T. harzianum* volatile metabolites action on the growth of B27 isolat presented in table 2 showed that the volatile metabolic substances of antagonistic present the same profile of the percentage inhibition of mycelia growth in the different concentrations tested.

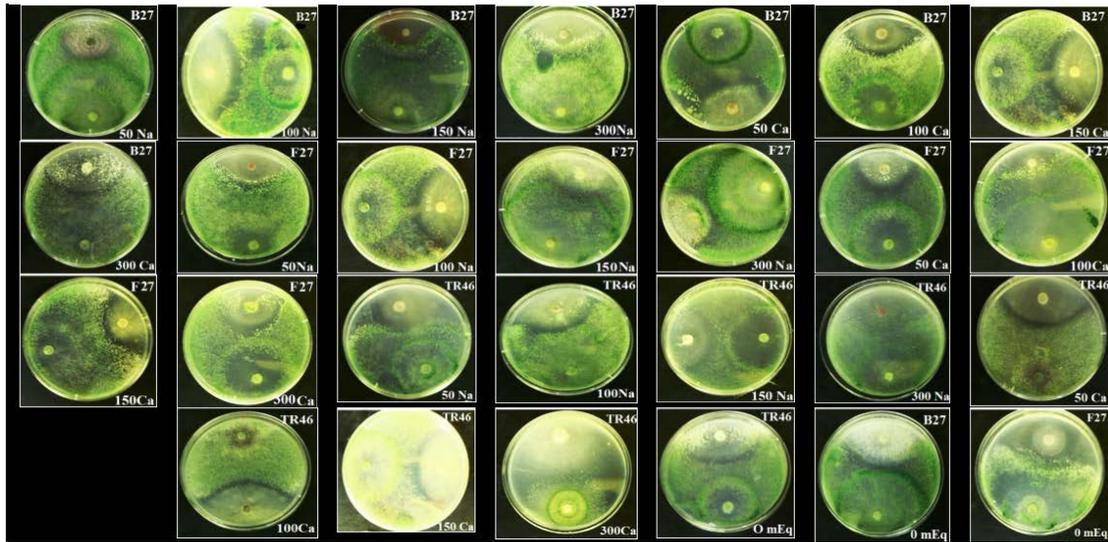


Fig. 1: Antagonistic effect of the isolates on *Botrytis cinerea* in dual culture assay at 25°C on potato dextrose agar media

DISCUSSION

In the present study, we tested effect of salinity on *in vitro* *Trichoderma harzianum* antagonism against *B. cinerea* for their production of volatile and non-volatile compounds.

In the absence of salt, The direct confrontation of *T. harzianum* against the different isolates of *B. cinerea* *in vitro* on PDA medium showed that *T. harzianum* inhibited the growth of pathogenic fungi at varying degrees.

The non-volatile secondary metabolites from *T. harzianum* were found more effective in suppressing the mycelial growth of all isolate when compared to volatile compounds except B27 isolate, growth inhibition was recorded by volatile compounds up to 79,81%.

The inhibition in radial growth of two interacting organisms in dual culture has been attributed to secretion of extracellular hydrolytic enzymes (Schirmbock *et al.*, 1994) by the production of antibiotics Howell (1998) or as well as some cell walls degrading enzymes such as chitinases, glucanases that break down polysaccharides, chitins and β -glucanase, thereby destroying cell wall integrity (Elad, 2000). *Trichoderma* species are known to produce a number of antibiotics such as Trichodernin, Trichodermol, Harzianum A and Harzianolide (Dennis and Webster, 1971; Kucuk and Kivanc, 2004).

Ajith and Lakshmidevi (2010) examined the potential of *Trichoderma harzianum* which suppress the mycelia growth of *Colletotrichum capsici* causing anthracnose on bell peppers. Doi and Mori (1994) reported volatile compounds produced from *Trichoderma* species were able to arrest and inhibit the hyphal growth of various plant pathogenic fungi. Amin *et al.* (2010) reported the effectiveness of volatiles produced by *T. harzianum* (Th-1) causing 53.63% inhibition of *Colletotrichum capsici*.

In the case of the evaluation the effect of NaCl and CaCl₂ on the *Trichoderma harzianum* against *B. cinerea*, salinity is most favorable to the increase growth of *Trichoderma harzianum*, especially at high concentrations. *T. harzianum* was able to tolerate the presence of salt in the medium culture even at high concentrations.

The analysis shows that CaCl_2 stimulate the development of the *Trichoderma harzianum* as compared to sodium chloride.

Our data indicate that volatile compounds from *Trichoderma* were found more effective in suppressing the mycelial growth of all fungal pathogens when compared to non-volatile metabolites.

In indirect confrontation, the effect of salinity on *T. harzianum* volatile compounds action is perceived in salt concentrations demonstrate that *T. harzianum* was able to colonize the space and to inhibit the mycelial growth of *Botrytis cinerea*. It was found that NaCl inhibited the growth of pathogen (94,70%) more than CaCl_2 (92,78%).

In the direct confrontation, the growth of *Botrytis cinerea* was intensely inhibited by non-volatile metabolites was stimulated by salt and moderately inhibited the pathogen growth. The percentage of growth inhibition varied between 65.17 and 82, 12% for NaCl and 61.19-85.01 in the presence of CaCl_2 .

The growth and multiplication of *T. harzianum* were stimulated by the addition of salt to the growth media, it showed also some good antagonist abilities at the high concentrations of salinity demonstrated by confrontation techniques. The strain activity of *T. harzianum* was able to tolerate the presence of salt in the medium culture even at high concentrations. Benyahya (1998) showed that increasing the salinity of the medium promotes the *in vitro* mycelia growth of *Phytophthora citrophthora* and *P. parasitica* agents of root rot of citrus with an optimum between -1.44 and -3.11 bars. The mycelium growth of *T. harzianum* developed in saline media between 2 and 6 g L⁻¹ of NaCl doses not differ from the control cultures without salt. Nevertheless, the capacity C declines significantly starting from 6 g L⁻¹ of NaCl but C value remain high (80%), showing an important capacity of the antagonist to cover the colony of the pathogen in presence of salt (Regragui and Lahlou, 2005).

Growth inhibition of the pathogens by the *Trichoderma* metabolites has been reported by several workers., such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Curvularia lunata*, *Bipolaris sorokiniana* and *Colletotrichum lagenarium*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides* (Yang *et al.*, 2006; Zivkovic *et al.*, 2010). The antagonistic activity of *Trichoderma* depends on multiple synergistic mechanisms (Nallathambi *et al.*, 2009; Howell, 2003). The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, competition and secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Harman *et al.*, 2004). Also it is found that there is large variety of volatile secondary metabolites produced by *Trichoderma* such as ethylene, hydrogen cyanide, aldehydes and ketones which play an important role in controlling the plant pathogens (Vey *et al.*, 2001). Previous studies have demonstrated that before mycelia of fungi interact, *Trichoderma* sp. produces low quantities of extracellular exochitinases (Kullnig *et al.*, 2000; Brunner *et al.*, 2003). The diffusion of these enzymes dissolves cell fragments of host cells. These cell fragments in turn induce the production of further enzymes and trigger a cascade of physiological changes, stimulating rapid and directed growth of *Trichoderma* sp. (Zeilinger *et al.*, 1999).

Although, *Trichoderma* sp. species is widely used to control many plant pathogens but not much work has been done to give the effect of salinity on the antagonist effect of *Trichoderma* sp. Knowledge of soil salinity and its potential effects on the antagonist against diseases is essential for disease management strategies. Therefore, it is important that biocontrol potential of *Trichoderma harzianum* under salinity conditions should be further evaluated *in vivo* against *Botrytis cinerea* causing grey mould disease.

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