INTRODUCING AN ENDOPHYTE FOR CONTROLLING TOMATO EARLY BLIGHT DISEASE

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ABSTRACT

Diseased tomato samples showed typical symptoms of the early blight disease were collected from four Egyptian governorates, i.e. Sharkia, Kalubia, Gharbia and Ismailia. All Alternaria solani isolates affect Peto 86 tomato cultivar. Ismailia was the most aggressive isolate. Three Bacillus subtilis isolates (were isolated from planosphere of tomato leaves) and two Trichoderma viride isolates (were isolated from rhizosphere) inhibited A. solani growth with different levels. The most aggressive B. subtilis isolate was chosen as a tool to induce disease resistance for tomato seedlings. The application of Bacillus subtilis on tomato seeds achieved a profuse proliferation of endophytically colonized seedlings, tomato plants were then challenged with A. solani suspension. The av. height of endophytically colonized plants increased from 38 cm for the check plants to 57 cm for the treated ones. After 45 days keeping under greenhouse conditions, the endophytically colonized and challenged tomato plants exhibited a significant reduction in early blight severity (7% compared with 19% for check plants). The endophytic bacteria was recovered from treated tomato seedlings, indicating that the endophytic B. subtilis has the potential to move systemically throughout the plant tissues.

Keywords: Bacillus subtilis, endophyte, tomato early blight, Alternaria solani, disease resistance inducing.

INTRODUCTION

Tomato (Lycopersicon esculentum) is one of the most important vegetable crops in Egypt, and in other countries. The plants are subjected to attack by several diseases that cause great losses for yield production. Early blight caused by Alternaria solani (Ell. & Mart.) is one of the most serious diseases of all tomato diseases in Egypt (El-Saman, 1986).

Recent researches reported that endophytes (microorganisms inhabit plants without causing visible disease symptoms) may exudate chemicals inhibit the growth of causal organisms, including harmful insects and mammalian herbivores (Carroll, 1998 and Azevedo, 2000), produce some plant-growth-promoters (Varma et al., 1999 and Adeline et al., 2008) and induce plant resistance against different pathogens (Redman et al., 1999, Narisawa et al., 2000, Arnold et al., 2003 and Henson et al., 2007). There is always a degree of antagonistic balance between endophytes and their plant hosts (Schulz, Barbara and Christina Boyle, 2005) except when the host is under stress conditions, so, endophytes appear to be associated with initial degradation of the plant tissues followed by senescence (Amico, Margherita et al., 2007).
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Many successful trials were carried out on tomato plants including artificially introducing antagonistic endophytes to induce plant disease resistance, i.e. *Acremonium* sp. against *Fusarium* wilt of tomato (Brgmann and Schonbeck, 1992 and Grunewald-Stoecker et al., 1998), a nonpathogenic strain of *Colletotrichum* sp. against tomato anthracnose (Redman et al., 2002), *Bacillus* against tomato wilt (Algam, Soad et al., 2005), Actinomycetes against *Ralstonia solanacearum* (Tan et al., 2006) and nonpathogenic strain of *Fusarium solani* against tomato soil pathogens (Kavroulakis et al., 2007). Grunewald-Stoecker and Alten (2003) stated that the injected endophyte must be permanently present within plant tissues before pathogenic infection, to maintain successful systemic disease protection.

In the present study we isolated some organisms from leaves and root tissues of tomato plants to identify the possibility for being biocontrol agents against *Alternaria solani* and to induce disease resistance against tomato early blight disease, as one of the most effective methods in the integrated best management program.

**MATERIALS AND METHODS**

Diseased tomato samples showed the typical symptoms of the early blight disease were collected from four Egyptian governorates, i.e. Sharkia, Kalubia, Gharbia and Ismailia. Two fields from each governorate were chosen. The samples were transmitted to the laboratory for isolation and identification. Isolation, purification using single spore technique, and identification was carried out by Ellis, 1971.

**Pathogenicity test:**

Pathogenicity test was carried out in the greenhouse using the identified isolates as *Alternaria solani*. The inoculum was prepared by growing the pathogenic fungus on Czapek's liquid medium for 10 days. Tomato seeds of the susceptible cultivar Peto 86 were obtained from the Agric. Res. Center, Giza. Seeds were planted in a shallow for 1.5 month, then seedlings were transplanted into green house. Inoculation was carried out by spraying the inoculum of *A. solani* isolets (5x10³ conidia/ml) on plant leaves. Plants were sprayed with distilled water were used as check. Disease assessment was carried out 10 days after inoculation according to Christ (1991) as follows:

- 0.1= a few circular lesions (1 -2 mm).
- 0.5= up to 5 % of the leaf area spotted.
- 0.75= above 5 %, but below 10 % of leaf area spotted.
- 1= above 10 %, but below 25 % of leaf area spotted.
- 2= above 25 %, but below 50 % of leaf area spotted.
- 3= above 50 %, but below 75 % of leaf area spotted.
- 4= above 75% to 100 % of leaf area spotted.

Mean disease severity for a plant was calculated as the mean of disease severity values of all leaves of that plant.
Isolation and identification of antagonistic organisms:

_Bacillus subtilis_ was isolated from planosphere of tomato leaves and identified according to Harrigan and McCance (1976), while, _Trichoderma viride_ was isolated from rhizosphere of tomato roots and identified according to Rifai (1969).

The antagonistic tests:

The antagonistic effect of both organisms above was studied against _A. solani_ in vitro. Regarding _Bacillus_ isolates, The method described by Abo El-Naga, Heidi, 2006 was applied as follows: Plates of PDA medium was inoculated with two equal discs of _A. solani_. The antagonistic bacterium (_B. subtilis_) was streaked at the centre of each plate by a loop loaded with 48 hr old bacterial culture grown at 27°C on nutrient broth. Four plates were used for each combination. Antagonistic effect was determined by measuring zones between the antagonistic bacteria and the tested fungus. Petri dishes with _A. solani_ discs only were served as control check.

However, mycelial discs (5 mm in diameter) were cut from 7-day-old PDA cultures of _A. solani_ and _T. viride_, two discs (one of each fungus) were transmitted into each experimental dish (containing PDA media), and facing each other with 40 mm distance between them (one disc in each side). Four plates per each combination were incubated at 25±2°C. Dishes containing PDA medium and discs of _A. solani_ only were used as control check. Inhibition zone diameters (in mm) were determined after 7 days of incubation period (Sabet and Khan, 1969).

Endophytically colonization of tomato seeds:

Pots (50 cm in diameter) were filled with sterilized sandy-clay soil. At the same day of planting, surface sterilized tomato seeds of the susceptible cultivar, Peto 86, were soaked five hours before planting in Petri-dish containing a 3-days-old culture of _Bacillus subtilis_ (10^8-10^9 cfu/ml) and was grown in liquid tryptic soybean medium (Anonymous, 2003). One liter of the medium contains: 17g Tryptone, 3g Soytone (enzymatic digest of soybean meal), 2.5g Dextrose, 5g Sodium Chloride and 2.5g K_2HPO_4. Ten treated seeds were planted in each pot to be artificially inoculated with _Alternaria solani_. Surface sterilized un-soaked seeds were served as a control check. Five pots were used for each treatment, Five pots were cultivated with treated seeds also prepared to study the bacterial effect on tomato growth. Tomato seedlings were sprayed by spore suspension of _A. solani_ (5x10^3 conidia/ml) after five rows of true leave stage.

Assessment of disease severity of tomato early blight was estimated after 7 days of inoculation as described by (El-Saman, 1986). The recovery of _Bacillus subtilis_ was carried out. All the seedlings were left under greenhouse conditions. Plant height was recorded at 30 and 45 days after sowing. The experiment was implemented for the tow successive seasons (2007 and 2008).

Statistical analysis:

The obtained results were statistically analyzed according to Gomez and Gomez, 1984.
RESULTES AND DISCUSSION

Isolotion and identification of the causal organism:
Tomato plants showing leathery, dark brown, necrotic lesions on the older leaves, which develop concentric black rings due to the conidia formation, were taken to isolate the causal pathogen from the affected leaves. The obtained cultures were identified according to Ellis (1971) as *Alternaria solani* fungus.

Pathogenicity test:
Data present in table (1) indicate that all the tested isolates affected tomato plants. Ismailia isolate was the most aggressive one flowed by Kalubia isolate, while Sharkia and Gharbia isolates were less effective, this may be due to the environmental conditions in Ismailia and Kalubia that was more favorable to the prevalent isolate of *A. solani*, and to the development of early blight disease. Similar result was obtained in the investigation of (Rashed, 1999) on the same tomato cultivar.

Table (1) : Effect of different isolates of *Alternaria solani* on early blight disease severity of tomato plants cv. Peto 86.

<table>
<thead>
<tr>
<th>Governorate of isolate</th>
<th>Location</th>
<th>Disease severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gharbia</td>
<td>Kafr El-Zayat</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Mahala</td>
<td>7.0</td>
</tr>
<tr>
<td>Ismailia</td>
<td>Ismailia</td>
<td>23.2</td>
</tr>
<tr>
<td></td>
<td>Kasaseen</td>
<td>20.4</td>
</tr>
<tr>
<td>Kalubia</td>
<td>Banha</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>Kanater El-Khyria</td>
<td>14.3</td>
</tr>
<tr>
<td>Sharkia</td>
<td>Zagazig</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>Hehia</td>
<td>9.5</td>
</tr>
<tr>
<td>LSD at 5 %</td>
<td></td>
<td>2.9</td>
</tr>
</tbody>
</table>

Isolation and identification of antagonistic organisms:
Two *Trichoderma viride* isolates were isolated from rhizosphere of tomato roots and three *Bacillus subtilis* isolates were isolated from planosphere of tomato leaves. The antagonistic tests which carried out in vitro using discs on PDA media indicated that all the tested isolates of *B. subtilis* or *T. viride* inhibited the mycelial growth of *Alternaria solani* but the bacterium was more antagonistic than Trichoderma.

Table (2) : Effect of the antagonistic organisms on *A. solani* growth after 7 days of incubation.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Location</th>
<th>Av. of inhibition zone after 7 days (in mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Ismailia</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>Kasaseen</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>Banha</td>
<td>19.0</td>
</tr>
<tr>
<td><em>Trichoderma viride</em></td>
<td>Kasaseen</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>Mahala</td>
<td>8.5</td>
</tr>
<tr>
<td>Check (<em>A. solani</em> alone )</td>
<td>Ismailia</td>
<td>0.0</td>
</tr>
<tr>
<td>LSD at 5 %</td>
<td></td>
<td>5.5</td>
</tr>
</tbody>
</table>
The three *Bacillus subtilis* isolates exhibited inhibition effect with different degrees against *Alternaria solani* growth on PDA medium. The most aggressive *B. subtilis* isolate (Ismailia) was chosen as a tool to induce disease resistance in tissues of tomato seedlings. This effect agreed with the effect of using *B. pumilus* to inhibit mycelial growth of many other fungi (Bollone and Pelaso, 2003 and Zhang et al., 2007).

**Endophytically colonization of tomato seedlings:**

The application of *Bacillus subtilis* on tomato seeds achieved many effects:

- No significant damage was noticed on endophytically colonized seedlings. This is in agreement with the meaning of the term "endophyte" of Carroll, 1998.
- There was a profuse proliferation of endophytically colonized plants. The height of endophytically colonized plants increased from 38 cm for the check plants to 57 cm for the treated plants. So, there was a remarkable enhancement to the growth of the endophytically colonized tomato plants. This result is in harmony with the findings of Verma, 1999 and Redman et al., 2002.
- Seven days after infection with *Alternaria solani*, the endophytically colonized and challenged tomato seedlings exhibited a significant reduction in early blight disease under greenhouse conditions.

**Table (3): Blighted tomato seedlings % and plant height/seedling (in cm.) after *Bacillus* application inside plant tissues, seasons 2007 and 2008.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Blighted seedlings %</th>
<th>Plant height / seedling (in cm.) after</th>
<th>Blighted seedlings %</th>
<th>Plant height / seedling (in cm.) after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 days</td>
<td>45 days</td>
<td>30 days</td>
<td>45 days</td>
</tr>
<tr>
<td>Protected seedlings</td>
<td>7.0</td>
<td>35.0</td>
<td>57.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Control I*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control II**</td>
<td>2.6</td>
<td>30.0</td>
<td>54.0</td>
<td>2.2</td>
</tr>
<tr>
<td>LSD at 5 %</td>
<td>1.5</td>
<td>5.3</td>
<td>4.2</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* Control I = Free endophyte and challenged seedlings.
** Control II = Endophytically colonized and not challenged seedlings.

This result agreed with those reported by Georgy, 1977, Podile, 1993 and Abo El-Naga, Heidi, 2006. They used *Bacillus subtilis* to induce resistance against the pathogens of onion white-rot, peanut root-rot and sugar beet damping-off, respectively.

To discover the mechanisms behind inducing systemic effects of delayed and reduced tomato early blight symptoms and reduce pathogen spread by using *Bacillus* spp., Katz and Demain, 1977 stated that *Bacillus* species produce 167 biological compounds active against pathogenic bacteria, fungi, protozoa and viruses. Several peptide antifungal compounds synthesized by *Bacillus* spp. are active against filamentous fungi and yeast such as Mycocerein, Pumiciadin, Rhizoctcin, Fungicin M₈ and Hcxxcine (Wakayama et al., 1984, Karuse et al., 1990, Kugler et al., 1990, Lebbadi et
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al. 1994, Kudryashava et al., 2005). Also, Fiddaman and Rossak, 1993 reported that *Bacillus subtilis* produces some antifungal volatiles.

The endophytic bacteria was recovered from the imerged treated seeds after tow weeks from application, indicating that the endophytic *B. subtilis* has the potential to move throughout the plant tissues . This result is in agreement with the findings of Grunewaldt-Stoecker et al., 1998 who stated that endophytic organisms are easily detected inside the target plant tissues in the second week after injection.

REFERENCES


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