Biological control of pathogenic and secondary (non-pathogenic) fungi associated with Barley (Hordeum vulgare) seeds

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ABSTRACT: Plant pathogenic fungi are a major problem in agriculture with effects on yield and quality of agricultural product. In this study, antagonistic effects of five fungi biocontrol agent Aspergillus niger, Aspergillus ostianus, Aspergillus versicolor, Penicillium sp., Trichoderma harzianum and one species of bacterium Pseudomonas aeruginosa isolated from the seeds of barley were evaluated against plant pathogenic fungi Rhizoctonia solani and secondary (non-pathogenic) fungi Aspergillus flavus. The ability of isolated microorganisms in antagonizing or inhibiting the growth of phytopathogenic fungi was tested by measuring the growth inhibition percentage over control. P. aeruginosa obtained higher inhibition with R. solani and A. flavus (88%, 76%) respectively, but A. ostianus obtained the lower inhibition with R. solani and A. flavus (59%, 53%) respectively. The results also showed that significant differences were recorded in mycelial growth of pathogens in presence of biocontrol agent when compared with control. Also, the results showed that non-significant differences were recorded between R. solani and A. flavus when treated with different antagonisms.

Key words: Bacteria, phytopathogenic, antagonism, competition, pollution, fungicides.

INTRODUCTION

Fungal plant pathogens are the most important factors that cause serious losses to agricultural products annually. Fungicides are commonly used to control the diseases in plants. Modern agriculture heavily depends on the application of agrochemicals for fertilizing soils and diseases control. However, the over use of chemical compounds poses potential risk to human health and the environment and can also lead to resistance in causal agents (Akintonkun and Taiwo, 2016). It is always better to adopt biological method as an alternative disease control method in order to reduce the hazards, which is also ecology conscious and eco-friendly. Antifungal compounds produced by microorganisms may be used as biocontrol agents. Some soil borne fungi, bacteria and actinomycetes have been identified and used as antagonistic microbes. A number of bacterial species have been tested as biocontrol agents. Antifungal metabolites produced by bacteria like Pseudomonas spp., Bacillus spp. (Moita et al., 2005; Siddiqui et al., 2005; Nourozian et al., 2006; ChristyJeyaseelan et al., 2012). For instance Pseudomonas fluorescens used against Rhizoctonia solani and Pythium damping off of cotton and Bacillus used for seeds treatment (Agrios, 2005). The mechanisms underlying these bacterial antagonisms for plant pathogens involve antibiotics, competition for nutrients or space, enhancement for root and plant development, induction of plant resistance and or inactivation of the pathogen's enzymes (Harman, 2000). Cereals can be very susceptible to toxigenic fungal growth in the field, during storage or during processing. Their presence in stored products can significantly decrease quality and economic value of the harvested grain (La Penna et al., 2004). The purpose of this paper was to study the activity of A. niger, A. ostianus, A. versicolor, Penicillium sp, T. harzianum and P. aeuginosa isolated from barley seeds against R. solani.
and A. flavus.

MATERIAL AND METHODS

Isolation and Identification of Fungal isolates

The fungi strains used in this study were isolated from barley seeds. barley seeds were collected from local market in Al-Nasiriya city (South of Iraq). Potato Dextrose Agar (PDA) supplemented with 250 mg/L Chloramphenicol to suppress bacterial growth was used to isolate fungi from the seeds. Plates and media were incubated at 25°C in the dark. Single colonies were picked from the plates under a dissecting microscope and transferred to PDA medium to allow fungal development. Stock cultures were maintained on the potato dextrose agar slant, subcultured periodically until pure cultures were gotten and stored at 4°C. Fungal isolates were examined under light microscope and morphologically identified using Pictorial atlas of soil and seed fungi provided by Watanabe (2000).

Isolation and Identification of Bacterial isolates

The bacterium used in this study was isolated from barley seeds. Nutrient agar supplemented with 30 µg/L Nystatin to suppress fungal growth was used to isolated bacteria from the seeds. Plates and media were incubated at 37°C in the dark. Stock cultures were maintained on nutrient agar, subculture periodically until pure cultures were gotten and then stored at 4°C. Bacterial isolates were examined under light microscope and identified using morphological characters, gram stain, biochemical tests and API20E (Holt et al., 1994) and taxonomical keys provided in the bacteriological keys (Reddy, 2010; Bergy's and Holt, 1994).

Antagonistic assay (dual culture method)

The isolated bacterium was streaked as a thick from edge to center of PDA plates. Then a 4 mm diameter disc of pathogenic fungus R. solani was cut from an actively growing culture by a sterile cork borer and placed onto the center of PDA plates. The petri dishes were incubated at 25°C in incubator in dark till the complete growth was observed in control plates. The same procedure also was carried out for A. flavus. The antagonistic effect of five fungi A. niger, A. ostianus, A. versicolor, Penicillum sp and T. harzianum with R. solani and A. flavus separately were recorded and the percentage growth inhibition compared with control were calculated according to the formula given by Vincent (1927). The experiments were carried out in three replicates.

\[
I = \frac{R_1 - R_2}{R_1} \times 100
\]

Where: \( I \) = percentage inhibition of mycelia growth, \( R_1 \) = Mycelial growth in control and \( R_2 \) = Mycelial growth in treatment.

Preparation of filter of antagonistic fungi and bacteria

50 mL of Potato Dextrose Broth (PDB) was transferred to 250 mL conical flasks and sterilized with 121°C 15 lbat 15 mins by using an autoclave. This was followed by the addition of a 4 mm diameter disc of antagonistic fungi from 7 days old culture separately. Also, the procedure was carried out with antagonistic bacteria of 2 day old culture, but control treatment flasks were contained (PDB) without any microorganisms. All flasks were placed in an incubator in the dark at 25°C for two weeks. At the end of incubation, the extracts were filtered through sterilized Buchner funnel under aseptic conditions and the filtrate was further filter through 0.45 µm sterilized Millipore filter paper.

Effect of antagonistic filter of fungi and bacteria on a diameter of mycelia growth of R. solani and A. flavus

The filtrate of antagonistic fungi and bacteria of about 10% was added to PDA medium. After mixing the filtrate and medium, the mixture was poured in sterilized petri dishes, while the control treatment only 10% from PDB was added to PDA. Then a 4 mm diameter of each mycelia disc was cut using a sterile cork borer and placed in the center of the PDA plates separately under aseptic conditions. Mycelia disc from R. solani and A. flavus on PDA medium without antagonistic fungi and bacteria were used as control. The culture were incubated in incubator in the dark for 7 days and diameter of the fungal mycelia growth was measured (Matar et al., 2009). The experiments were carried out in three replicates.

Statistical analysis

The results of the antagonistic activity were subjected to statistical analysis using analysis of variance ANOVA test with the aid of software SPSS windows version 10.0.

RESULTS AND DISCUSSION

Effect of antagonistic organisms on mycelia growth of pathogenic and secondary fungi

The antagonistic effect of fungi and bacteria isolates was carried out by using five isolates including A. niger, A. ostianus, A. versicolor, Penicillum sp and T. harzianum and one isolate of P. aeruginosa. They were all tested against R. solani and A. flavus. After a week of incubation, the growth of targeted fungal pathogens towards and
Table 1. Effect of antagonistic organisms on mycelia growth of pathogenic and secondary fungi after 7 days incubation.

<table>
<thead>
<tr>
<th>Biocontrol agent</th>
<th>Pathogens</th>
<th>Mycelia growth in control (cm)</th>
<th>Mycelia growth of pathogen in presence of biocontrol (cm)</th>
<th>% inhibition of mycelia growth over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>R. solani</td>
<td>8.5</td>
<td>2.5*</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>A. flavus</td>
<td>8.5</td>
<td>3.0*</td>
<td>65</td>
</tr>
<tr>
<td>A. ostianus</td>
<td>R. solani</td>
<td>8.5</td>
<td>3.5*</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>A. flavus</td>
<td>8.5</td>
<td>4.0*</td>
<td>53</td>
</tr>
<tr>
<td>A. versicolor</td>
<td>R. solani</td>
<td>8.5</td>
<td>3.0*</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>A. flavus</td>
<td>8.5</td>
<td>2.5*</td>
<td>71</td>
</tr>
<tr>
<td>Penicillium sp</td>
<td>R. solani</td>
<td>8.5</td>
<td>2.0*</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>A. flavus</td>
<td>8.5</td>
<td>3.0*</td>
<td>65</td>
</tr>
<tr>
<td>Trichoderma harzianum</td>
<td>R. solani</td>
<td>8.5</td>
<td>3.5*</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>A. flavus</td>
<td>8.5</td>
<td>2.0*</td>
<td>76</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>R. solani</td>
<td>8.5</td>
<td>1.0*</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>A. flavus</td>
<td>8.5</td>
<td>2.0*</td>
<td>76</td>
</tr>
</tbody>
</table>

*Significance P< 0.05.

away from the antagonistic fungi isolates were recorded. The results showed that significant differences were recorded in mycelial growth of pathogens in presence of biocontrol agent when compared with control (Table 1).

The percentage inhibition of mycelia growth compared with control was tabulated. All antagonistic fungal isolates and P. aeruginosa significantly inhibited the mycelia growth of pathogens. The highest inhibition of mycelia growth was recorded for P. aeruginosa against R. solani (Table 1). These results were in agreement with the findings of (Shaikh Farah and Sahera, 2016). They showed that both bacterial isolates of P. aeruginosa and Bacillus subtilis could inhibit the mycelia growth of R. solani and Fusarium oxysporum (Tharmila et al., 2013). Also, Tharmila et al. (2013) showed that Pseudomonas maltophilia had the highest antifungal activity against all tested fungi on PDA medium. This antagonistic ability was due to the production and secretion of antifungal compounds that was able to reduce the growth of fungi. Also, P. aeruginosa can produces antibiotics such as HCN, phycocyanin, pyrocnitri and pseudomonic acid. The results (Table 2) showed that the filtrate of T. harzianum decreased the growth of R. solanion solid medium (PDA) to 4.03 cm and the inhibition percent reached to 59% (Table 1). The results also showed that non-significant differences were recorded between R. solani and A. flavus when treatment with different antagonisms (Table 2). These results were in line with the findings of (Harman et al., 1989) which showed that the only T. harzianum isolate tested was able to exhibit the wide spectrum of inhibition. However T. harzianum and T. koningii have also been applied in soil, and on cowpea leaves, as a biocontrol agent against Rhizoctonia solani on cotton in a greenhouse environment (Lartey et al., 1994; Latunde, 1991), and against wood degrading fungi (Canessa and Morrell, 1996). Both T. viride and T. harzianum are recognized biopesticides mainly against Rhizoctonia, Sclerotinia and Botrytis (Lewis and Papavizas, 1991). Under in vitro studies R. solani, Pythium ultimum and Chalara alegans were strongly inhibited by T. viride, T. harzianum, T. pseudokoningii and T. koningii. These results indicate that the biocontrol efficacy of Trichoderma asseems to perform not only in the medium, but also in the field level (Marchetti et al., 1992). Mycoparasitism involving lytic enzymes have been already described as the mechanism of action of Trichoderma isolates in the biological control of commercial important plant pathogens (Bruce et al., 1995). Trichoderma employs a variety of antagonistic mechanisms for combating other fungi. The simplest one is probably competition for non - structurally - bound nutrients, however, volatiles and soluble antifungal metabolites are also involved (Horvath et al., 1995). These metabolites are composed of harzanic acid, alamthincins, tricholin, peptobales, antibiotics, 6-phenyl-alpha-pyron, massoilactone, viridian, gilovirinid, glisoprenins, heptelicid acid, and other suppressive compounds (Vey et al., 2001; Kucuk and Kivanc, 2004). Also, T. virens strain, that produced high amount of gilovirinid antibiotics, protected cotton seedlings from seedling blight caused by P. ultium (Chetet et al., 1997). The most effective T. harzianum isolates against take-all agent produced pyron antibiotic (Monte, 2001). In the same time, general PDB was a more suitable medium for the production of antibiotic compounds (Santamarina et al., 2002).
Table 2. Effect of filter of antagonistic organisms on mycelia growth of *Rhizoctonia solani* and *A. niger* on solid medium.

<table>
<thead>
<tr>
<th>Biocontrol agent</th>
<th><em>R. solani</em></th>
<th><em>A. flavus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td>3.50*</td>
<td>4.2</td>
</tr>
<tr>
<td><em>A. ostianus</em></td>
<td>3.5 **</td>
<td>3.24</td>
</tr>
<tr>
<td><em>A. versicolor</em></td>
<td>4.17 **</td>
<td>5.5</td>
</tr>
<tr>
<td><em>Penicillium sp</em></td>
<td>2.5 **</td>
<td>2.9</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>4.03 **</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>3.17 **</td>
<td>1.2</td>
</tr>
<tr>
<td>Control</td>
<td>8.5</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*(cm), ** Non significance P< 0.05.

**Effect of filter of antagonistic organisms on mycelia growth of *R. solani* and *A. flavus***

Table 2 showed that the filtrate of *Penicillium sp* decreased the mycelia growth of *R. solani* and *A. flavus* on PDA plates. This result was due to the release of antibiotic by fungus. These results were in agreement with the findings of Jijakli and Lepoivre (1999) which showed that *Penicillium guillermondi* was able to degrade fungal cell walls by producing enzyme β- 1,3 glucanase.

It also shown from Table 2 that *A. niger*, *A. ostianus* and *A. versicolor* decreased the mycelia growth of *R. solani* and *A. flavus* on solid medium (PDA). This was due to the ability of these fungi to have higher growth and to compete with other fungi on PDA. These results were similar to the findings of Ikotum and Agboola (1992) which showed that *A. niger* had ability of high growth and compete with *Curvularia lunata*. However these ability might also be due to the possibility that these fungi produced toxin materials, and these toxins were able to inhibit pathogenic fungi. Also, the present study was similar to the findings of Egorov (1985) which showed that the genus of *Aspergillus* produces many antibiotics such as fumagillin and griseofulavin. These antibiotics have a wide spectrum of action against microorganisms.

**Conclusion**

All fungi isolated in this study had antagonistic ability against pathogenic fungal strains and secondary fungi. *P. aeruginosa* showed the highest inhibitory effect on mycelia growth and with an inhibition percentage up to 88%. According to our results, it is concluded that *P. aeruginosa* is the best biocontrol agent against fungal pathogens associated with barley seeds. This is due to it possibility to reduce and control the environmental pollution by reduction of chemical treatments. It should persuade the farmers to choose biological control.

**REFERENCES**


Antagonistic and inhibitory effect of Bacillus subtilis against certain plant pathogenic fungi. International Biotechnology, 8, 53-61.