Evaluation of the antifungal activity of some Azospirillum strains for their possible role as biocontrol agents

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Research Article

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Abstract

Two strains of the N\textsubscript{2} fixing, plant growth promoting bacterium \textit{Azospirillum} were isolated as endophytes from roots of wheat seedlings grown in the spermosphere model that was inoculated with aliquots of two rhizosphere soil samples. \textit{Azospirillum} isolate 3Re was recovered after inoculation with \textit{Sisamum irio} rhizosphere soil and the other isolate (12Re) was recovered after inoculation with \textit{Triticum aesativum} rhizosphere soil, both were brought from Dalja village. Some physiological and biochemical characteristics of \textit{Azospirillum} strains were determined. One of the physiological studies on these \textit{Azospirillum} strains exhibited that they have a chitinolytic activity. This study aims to evaluate the antagonistic activity of these isolates against the phytopathogenic fungus \textit{Fusarium}. The investigation exhibited a positive effect for the \textit{in vitro} control of \textit{Fusarium} growth through the production of the fungal cell wall degrading enzymes (chitinase and β-1,3-glucanase) in addition to the production of volatile and non volatile antifungal compounds. The microscopic examination of the lysed fungal hyphae was recorded as a result of \textit{Azospirillum}'s fungal cell wall degrading enzymes produced. Moreover, in pot experiment \textit{Azospirillum} overcame pathogenicity of \textit{Fusarium} on wheat seedlings' growth and showed 100\% of seedlings growth compared with 30\% growth of control infested seedlings only. These results suggest that \textit{Azospirillum} has an excellent potential to be used as biocontrol agents against phytopathogenic fungi.

Introduction

Increase of the world population will require an additional agricultural production that could be based on an increase in the arable surface or on the improvement of crop productivity. The latter can be achieved in part by suitable control of losses due to biotic agents such as pests, diseases, weeds (Montesinos, 2003). The therapeutic approach of killing pest organisms with toxic chemicals has been the prevailing pest control strategy for over 50 years (Lewis et al., 1997). However, an increase in the use of chemical pesticides to support the increase in agricultural activity needed to sustain because of its hazardous effects on the environment. Increasing problems with resistance of these pests to most commonly used synthetic insecticides have spurred the search for alternative pest management strategies that would reduce reliance on synthetic insecticides (Yaman, 2003). New methods of crop protection are based on the benefits obtained from naturally occurring microbial communities which exert a biological control of pests and diseases.

Antagonistic bacteria are considered as ideal biological control agents due to their rapid growth, easy handling and aggressive colonization at rhizosphere of plant roots. These bacteria may mediate biocontrol activities by one or more types of mechanisms of diseases suppression (Weller, 1988). However, a primary mechanism of pathogen inhibition is by producing secondary metabolites e.g., antifungal metabolites and antibiotics, Fe chelating siderophores, ammonia and cyanide (hydrogen cyanide, HCN) were reported by many researchers (Lovic et al., 1993; Weller, 2007).
Many studies exploring beneficial organisms have been carried out, such as strains of *Pseudomonas* species have been used extensively for plant growth promotion and disease control because of many properties such as efficient colonization of underground plant organs, utilization of a large number of organic substrates commonly found in root exudates and production of a variety of secondary metabolites toxic to fungi and bacteria (Asha et al., 2011). *Pseudomonas fluorescens*, which was one of the examples used for the control of *Fusarium* wilt of tomato. Similarly, *Pseudomonas fluorescens* were found to be effective biocontrol agents against the *Phytophthora* disease in black pepper (Diby et al., 2005). *Bacillus* sp. producing antimicrobial compounds have been used as biocontrol agents against plant pathogenic fungi (Yilmaz et al., 2005).

Some chitinolytic bacteria have been shown to be potential agents for biological control of the plant diseases caused by various phytopathogenic fungi and insect pests. *Azospirillum* species are gram negative aerobic heterotrophs that fix N₂ under microaerobic conditions. They grow extensively in the rhizosphere of gramineous plants (Roper and Ladha, 1995). They can also penetrate the root to grow endophytically in intercellular crevices (Okon and Kapulink, 1986). *Azospirillum* is a plant growth promoter bacterium (PGPBs), and it is not known as typical biocontrol. Chitinase production by some *Azospirillum* strains could have a promising future for application of Azospirilla as biological fungicides and biological insecticides.

*Fusarium* is a well distributed large genus of filamentous saprophytic fungus affecting plant, animal and human health as they enter the food chain (Agrios 1988; Smith et al. 1988), also they produce toxins, fumonisins and trichotheccenes. *F. oxysporum* has a variety of hosts that include sugarcane, garden beans, cowpeas, potatoes, banana, water melon, prickly pear, tomato, cucumber, pepper, muskmelon, tobacco, cucurbits, sweet potatoes, asparagus, vanilla, strawberry and cotton (Naik et al. 2010; Nikam et al. 2011).

Hence, the objectives of this study were to isolate, identify and test some physiological characteristic of two locally isolated chitinolytic *Azospirillum* isolates, moreover, evaluate their antagonistic potential including the production of antagonistic metabolites and cell wall degrading enzymes against *Fusarium* as one of phytopathogenic fungi.

**Materials And Methods**

**Microbial organisms**

*Fusarium* spp. were isolated from the botanical garden of Botany and Microbiology Department, Faculty of Science, Minia University, El-Minia, Egypt. *Fusarium* isolates were identified by Mycology Unit of Assiut University, and one of them is *F. oxysporum* and the other is *Fusarium* sp. fungal isolates were maintained on potato dextrose agar (PDA) at 4°C. All microorganisms were included in the culture collection of Microbiology Lab., Faculty of Science, Minia University, El-Minia, Egypt.
Azospirillum strains used in this study were isolated as endophytes from roots of wheat seedlings grown in the spermosphere model (Bauzon et al., 1982) that was inoculated with aliquots of two rhizosphere soil samples from Dalja village, Dairmawas city, El-Minia, Egypt. Azospirillum isolate 3Re was recovered after inoculation with Sisamum irio rhizosphere soil and the other isolate (12Re) was recovered after inoculation with Triticum aestivum rhizosphere soil.

Isolation of Azospirillum from endorhizosphere of wheat seedlings

The inoculated wheat roots were surface-sterilized by soaking them in series of ethanol (70%/2min) followed by Sodium hypochlorite (2%/3min) and finally ethanol (70%/30s) and consecutively rinsed with distilled sterile water. Sterilized roots were aseptically spread on nutrient agar and incubated at 35°C for 24–48 h to verify their surface sterility. Wheat roots were aseptically segmented into small pieces, suspended in 0.05 M PBS buffer and ground with a sterile mortar, 1ml of suspension was inoculated onto test tubes that contained 5ml of semisolid malate DN medium (Döbereiner, 1988), and incubated at 30°C for 48 h.

After the incubation period positive cultures showed a white, dense, undulating, diffuse pellicle was observed 1 to 4 mm below the surface (Fig. 1A). Loopfuls of subsurface pellicles were diluted in sterile 3ml of NaCl 0.8%. Dilutions were streaked on plates of Congo red (RC) medium (Caceres, 1982) and incubated at 30°C. Colonies that have a Light-pink color after 48 h that became scarlet After 72 h due to the absorption of Congo red indicating the presence of Azospirillum (Fig. 1B). Isolates must be re-streaked on plates of RC medium to check the purity of the isolates. Single colonies were picked for further study. Pure Azospirillum cultures were maintained on NA slants.

Some physiological and nutritional features of Azospirillum isolates

Some physiological and biochemical characteristics of Azospirillum isolates were determined. Nitrogenase activity was tested by using the ARA, as described by Eckert et al., (2001). Cellulase, pectinase, starch, chitinase, gelatinase, indole production were qualitatively assayed as described in (Mahmoud, 2010). Modified mineral DN agar medium (Döbereiner J, 1988) made with 0.4% colloidal chitin instead of malic acid and supplemented with ammonium chloride (2.0 gm/l) as the sole nitrogen source was used to test the qualitative chitinase production by Azospirillum isolates. Also antibiotic sensitivity by disk diffusion method and nitrate reduction test was determined.

Preparation of Azospirillum's crude extract for testing fungal growth inhibition

Azospirillum isolates were cultured in 50 ml of nutrient broth (NB) medium in 250-ml Erlenmeyer flasks and incubated at 30°C in a rotary shaker at 120 rpm for 24 h. Cells were harvested by centrifugation at 3500 g for 10 min and washed once in sterile PBS buffer (0.1 M potassium phosphate buffer, pH 6.8,
supplemented with 0.15 M NaCl). Washed bacterial cells were re-suspended in PBS buffer at a concentration of approximately $10^{10}$ CFU ml$^{-1}$ and were immediately used as bacterial inoculum. Culture filtrate was sterilized by membrane filter 0.22 µm and stored in refrigerator till need.

**Preparation of colloidal chitin (Hsu and Lockwood, 1975)**

Forty grams of bleached chitin, ground dry in an electric blender, was dissolved in 400 ml of concentrated HCl by stirring for 30 to 50 min. The chitin was precipitated as a colloidal suspension by adding it slowly to 2 liters of cold water at 5 to 10°C. The suspension was collected by filtration on a coarse filter paper and then washed by suspending it in about 5 liters of distilled water and refiltering. The washing was repeated at least three times or until the pH of the suspension was about 3.5. At least 85% of the chitin was recovered. Water content of the chitin was determined by drying a sample at 65°C for overnight. Chitin agar medium was usually prepared using 4 gm of the dry preparation per liter, or a sufficient volume of colloidal chitin suspension to give 4 gm of chitin. After autoclaving plates were poured, the plates were dried for overnight, spot-inoculated with actively growing cultures and incubated for 4 days at 28°C for the qualitative estimation of chitinase production. Chitinase activity of *Azospirillum* isolates growing on the colloidal chitin agars was monitored as clear zones surrounding the colonies.

**In vitro detection of antagonistic activity:**

**Inhibition assay by bacterial isolate**

For in vitro inhibition assays, one disk (9 mm diam.) of each tested fungal isolate (from a PDA plate) was added to the center and two disks (4 mm diam.) of *Azospirillum* 3Re or 12Re (from a NA plate) were added to the periphery of each fresh PDA plate. These PDA plates were incubated at 28°C for 5 days prior to measuring the zone of inhibition of hyphal growth. For this, the distance between the bacterial colony and the edge of the fungal pathogen mycelium was determined. The experiment was performed three times with three replicate PDA plates.

**Fungal growth inhibition by the crude extract of *Azospirillum***

The ability of the bacterial isolate to produced antifungal compounds was tested against *Fusarium* spp. by using a bioassay described by Broglie et al. (1991) with some modification. An agar disk (5 mm in diameter) of *Fusarium* which was derived from the fungus in an actively growing state previously cultured on PDA was placed in the center of a Petri dish containing PDA. The plates were incubated at 28°C for two days. Wells were subsequently punched into the agar at a distance of 25 mm from the center of the plate. 100 µl of *Azospirillum*s culture filtrate were placed into the wells, and control wells contain sterile dH$_2$O. The plates were incubated for 4 days at 28°C and then photographed.

**Production of diffusible antifungal substances**
In this experiment, diffusible antifungal substance(s) was produced in sandwich agar plate, which was prepared according to the procedure as described by Dikin et al. (2002), the experiment was performed three times with five replicate. Results were expressed as mean of % inhibition of Fusarium growth in presence or absence of bacterial isolate.

**Production of volatile compounds**

The ability of the bacterial isolate to produce volatile compounds was evaluated by taking 10-mm disk of two days old pure culture of Azospirillum and placed at the centre of one half Petri dish containing NB agar medium, and a 10-mm disk of four days old pure culture of F. oxysporum or Fusarium sp. was placed at the centre of another Petri dish containing PDA. Both half plates were placed face to face preventing any contact between the pathogen and the bacterial cells and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at 28°C for 5 days and the growth of the pathogen was measured and compared to controls developed in the absence of Azospirillum. Each experiment was run in triplicate and repeated at least three times. Results were expressed as means of % inhibition of growth of Fusarium spp. in the presence and absence of Azospirillum.

**Enzyme production**

Chitinase and β-1,3-glucanase production by Azospirillum spp. in presence of fresh mycelium of Fusarium oxysporum

The capability of Azospirillum isolates to produce chitinase and β-1,3-glucanase in DN mineral salt medium amended with ammonium chloride as the utilisable nitrogen source and 5% w/v fresh mycelia of F. oxysporum as sole of carbon source were investigated.

First, Carbon starved inocula of the used Azospirillum isolates were inoculated into a 20 ml of DN medium in 100ml Erlenmeyer flasks. The concentration of the utilisable carbon, malic acid, in the DN medium was decreased to 1/25 of its original concentration (0.2gm/l instead of 5gm/l); cultures were incubated at 28°C in a rotary shaker at 150 rpm for 16 h. After incubation, the Azospirillum cultures were starving from shortage of an utilisable carbon and dispiritly need it, and then aseptically washed. Fusarium mycelium grown as a still culture in DN medium containing sucrose 0.20 gm/l and 2.0 gm/l ammonium chloride was added to the conical flasks of the starving Azospirillum. Incubation continued at the same shaking conditions for 36 hours. Control was prepared by aseptically adding washed Fusarium mycelium to conical flasks of sterile Mineral–carbon free DN medium without Azospirillum or any utilisable carbon.

Samples of culture filtrate as well as samples of fungal mycelia were taken each 4 hours till 36 hours of incubation. Enzyme activity was tested in culture filtrate and fungal mycelia were examined under light microscope.

**Enzyme activity assay**
Chitinase activity was assayed using the colorimetric method described by Molano et al. (1977). The reducing sugars produced were determined by the method of Miller (1959) using dinitrosalicylic acid (DNS) reagent. The amount of reducing sugars released was calculated using a standard curve recorded for N-acetyl-glucosamine (GlcNAc).

β-1,3-glucanase was assayed based on the release of reducing sugars from pure laminarin (Sigma) as a substrate. The activity was routinely assayed by incubating 500 µL of 0.5% (w/v) laminarin in 50mM acetate buffer (pH 4.8) with 200 µL of crude enzyme preparation at 45°C for 30 min, and the reducing sugars produced were determined by the method of Miller (1959) using DNS reagent. Total protein concentration was assayed by the method of Bradford (Bradford, 1976) with bovine serum albumin as the protein standard.

A pot experiment indicating the biocontrol of Fusarium by the locally isolated Azospirilla:

*F. oxysporum* was grown on PDA agar slants at 25°C for 10 days. Spore suspension of *Fusarium* from three agar slants in sterile dH2O that had an optical density of 0.2 at 660 nm was mixed with 700 gram of dry fine wood reich after wetting with sterile water. *Fusarium* inoculated wood reich was divided into six pots and were used as soil replacement.

The inoculants of *Azospirillum* for treating wheat seeds were prepared by suspending cells from agar plates in a sterile 0.2% NaCl solution at concentration of $10^5$ CFU ml$^{-1}$ by measuring optical density (OD) at 660 nm. For *A. brasilense*, at this wavelength, an OD of 1.0 corresponded to $1 \times 10^9$ CFU ml$^{-1}$ (Dilfuza and Gisela, 2004). Sterilized seeds of wheat cultivar (Sakha 95) were imbibed in bacterial suspended for 2 h at room temperature (Bashan and Levanony, 1985). Seeds imbibed in sterile 0.2% saline solution without bacterial cells under the same conditions were used as control.

The experiment was performed in plastic pots, each containing ~120 gm of dry wood reich that was watered and *Fusarium* inoculated. In each pot, 5 seedlings were sown at a depth of 1.5 cm, each seedling were further inoculated by adding 200µl of the bacterial suspension that were soaked in according to treatment to ensure that the seedling is surrounded with considerable number of living bacteria, control seeds received 200µl of sterile saline solution.

Data analysis

Means and standard errors of 3 replicates for each experiment of antagonistic action and enzyme activity were undertaken using the SPSS for Windows (Release 10.0.1) computer package. By analysis of variance and the significance of treatments was determined using LSD ($P < 0.05$).

Results

Two chitinolytic bacterial isolates that were isolated as endophytes from roots of wheat seedlings were selected based on their chitinolytic activity in agar plate when grown on DN amended with colloidal chitin
and the produced chitinase was shown as clear zone around the colony (Fig. 1C). *Azospirillum* isolates varied in some of their physiological characteristics as shown in Table 1 and 3.

### Table 1

**Tested Physiological Characteristics of *Azospirillum* Isolates**

<table>
<thead>
<tr>
<th>Biological parameters</th>
<th>E3</th>
<th>E12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase activity</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Pectinase activity</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Indole production</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Chitinase activity</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Nitrate reduction to nitrite</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+ve</td>
<td>+ve</td>
</tr>
</tbody>
</table>

### Table 2

**Antibiotics used to test the sensitivity of *Azospirillum* isolates**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock solutions concentration</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin(^a)</td>
<td>50mg/ml in water</td>
<td>100µg/ml (1/500)</td>
</tr>
<tr>
<td>Kanamycin(^a)</td>
<td>10mg/ml in water</td>
<td>50µg/ml (1/200)</td>
</tr>
<tr>
<td>Streptomycin(^a)</td>
<td>10mg/ml in water</td>
<td>50µg/ml (1/200)</td>
</tr>
<tr>
<td>Tetracycline HCl(^b)</td>
<td>5mg/ml in ethanol</td>
<td>50µg/ml (1/100)</td>
</tr>
</tbody>
</table>

The antibiotics were dissolved in: dH\(2\)O\(^a\), ethanol\(^b\), filter sterilized (0.2 µm pore size) and stored at −20 °C.

### Table 3

**Antibiotic sensitivity and nitrogenase activity of *Azospirillum* isolates**

<table>
<thead>
<tr>
<th>Index no.</th>
<th>T</th>
<th>K</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>S</th>
<th>Nitrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Re</td>
<td>+ve/+ve</td>
<td>+ve/+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/+ve</td>
<td>18.02</td>
<td></td>
</tr>
<tr>
<td>12Re</td>
<td>-ve</td>
<td>+ve/+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve/+ve</td>
<td>9.30</td>
<td></td>
</tr>
</tbody>
</table>

Nitrogenase activity was nmole C\(2\)H\(4\) h\(^{-1}\) per ml of bacterial suspension. T, tetracycline; K, kanamycin; A, ampicillin; B, benicillin; C, chloramphenicol and S, streptomycin.
Detection of antagonistic activity:

Cultures of *Azospirillum* 3Re or 12Re grown on PDA produced a metabolite(s) that was released into the agar and inhibited the hyphal growth of *Fusarium* spp. As shown in Fig. 2A, zones of inhibition were formed between the fungal colony and the antagonist. Width of the inhibition zone between fungus and the antagonistic isolates was used as indicator for the antifungal activities. Moreover, in Fig. 2B the culture filtrate of *Azospirillum* spp. had an inhibitory effect on growth of *Fusarium* sp. compared to control.

Production of diffusible and volatile

Both isolates of *A. brassicacearum* were found to produce diffusible as well as volatile antifungal compounds against two isolates of *Fusarium* and showed significant difference with control (P < 0.05) in respect of mycelial growth inhibition (Table 4). Mycelial growth of *Fusarium* was inhibited by diffusible antifungal compounds produced by both Azospirilla after 5 days of incubation and reached to 100% by *Azospirillum* 3Re against *F. oxysporum* (Table 4). *Azospirillum* 12Re showed the highest inhibitory effect against *Fusarium oxysporum* volatile by producing volatile compounds which was 29% at 5 days after incubation with respect to the control.

Table 4

Effect of volatile and diffusable nonvolatile compounds secreted by *Azospirillum* isolates on radial growth of *Fusarium* spp. after 5 days

<table>
<thead>
<tr>
<th>Pathogen</th>
<th><em>F. oxysporum</em></th>
<th><em>Fusarium</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% inhibition by volatile</td>
<td>% inhibition by non-volatile</td>
</tr>
<tr>
<td>Azospirillum 3Re</td>
<td>21a</td>
<td>100a</td>
</tr>
<tr>
<td>Azospirillum 12Re</td>
<td>29b</td>
<td>95a</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Means in a column with the same letter(s) don't differ significantly at 0.05 levels according to LSD (P < 0.05)

Enzyme production

The production of extracellular chitinase and β-1,3-glucanase by *Azospirillum*

The production of extracellular chitinolytic and β-1,3-glucanolytic enzymes by *Azospirillum* spp. in liquid culture medium amended with fungal mycelium as sole carbon source were determined at different
incubation periods (Figs. 3 and 4). The level of chitinase was sharply increased with time and started to decline after the first 24 hours (Fig. 3). The strain 3Re produced relatively higher level of chitinase (13.9 pkat ml$^{-1}$) at day 1 of the incubation period compared to 12Re (12.1 pkat ml$^{-1}$). Meanwhile, the highest levels of β-1,3-glucanase was found at the first 12 hours of the incubation period and subsequently decreased and reached the minimum at 24 hours (Fig. 4). Interestingly, 12Re was higher two folds in β-1,3-glucanase production (1.2 nkat ml$^{-1}$) than 3Re (0.62 nkat ml$^{-1}$) after 12 hours under the same conditions. Total protein was significantly higher in cultures inoculated with Azospirillum comparing with control containing fresh mycelium of Fusarium only (Fig. 5).

Extracellular cell wall degrading enzymes cause lysis and an abnormal change in the morphology of fungal hyphae

The finding of direct parasitism of Fusarium oxysporum mycelia by Azospirillum isolates were presented in figure (6), whereas, Azospirillum inhibited the growth of the phytopathogenic fungus Fusarium prompted the investigation of the effects caused by its metabolites on the growth and hyphal structure (as marked arrows in Fig. 6). Light microscope investigation revealed that extracellular metabolites of Azospirillum's isolate 12Re produced in culture medium against fresh mycelium of Fusarium oxysporum caused changes in hyphal morphology including hyphal swelling, distortion, cytoplasm aggregation, hyphal lysis with septa release, hypha ruptured near its tip. Moreover, an abnormal hyphal structure such as thickness and bulbous roundedness of the inhibited fungal hyphae was shown. Vacuolation, protein coagulation, cells empty of contents and finally massive lysis of hyphae after 28 hours were recorded (Fig. 6, A28). Azospirillum isolate 3Re exhibited the same pattern of lytic effect on F. oxysporum mycelium under the same culturing and incubation conditions. Control fungal mycelium after 28 h was shown with no morphologically or any cellular changes and normal fungal growth was observed (Fig. 6, C28).

**Pot experiment**

The activity of Azospirillum on disease incidence and their biocontrol activities were investigated in a pot experiment (Fig. 7). Negative seedling control (infected pot without treatment) showed clear disease incidences, however, treated seedling with Azospirillum (3Re or 12Re), suppressed the Fusarium virulence on seedling of wheat plant. Seedlings of Azospirillum treated pots also recorded greater plant height as well as more number survivals compared with the other infested only with pathogen (Fig. 7). In genearlly, Azospirillum overcame pathogenicity of Fusarium on wheat seedling growth and showed 100% of seedlings growth compared with 30% growth of control infested only seedlings.

**Discussion**

Chitin is a structural component of cell walls of most fungi, insects, various crustaceans and nematode eggs. Chitin as an unbranched polysaccharide composed primarily of, B-1, 4 linked N-acetylglucosamine (NAG) residues. Chitinases are digestive enzymes that depolymerize the chitin polymer into N-
acetylglucosamine and chitobiose (Jolles and Muzzarelli, 1999). Chitin like cellulose has been thought of as abundant but difficult to digest (Akaki and Duke, 2005). In the soil, chitinases are produced by some actinomycetes (Mitchell and Alexander, 1962), fungi (Mian et al., 1982) and bacteria (Inbar and Chet, 1991), but chitinases are also released by many plants as part of their defense mechanism against various pathogens and plant-parasitic nematodes (Punja and Zhang, 1993).

Control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemicals. It is generally recognized that biological control agents are safer and more environmentally sound than is reliance on the use of high volumes of pesticides. Chitinases have been shown to be potential agents for biological control of the plant diseases caused by various phytopathogenic fungi and insect pests, because fungal cell walls and insect exoskeletons contain chitin as a major structural component.

Janisiewicz and Roitman, (1988) reported the failure of spores of *F. oxysporum* and *Colletotrichum gloeosporioides* to germinate in 2 days exposure to antagonistic bacteria indicated that the antimicrobial substances produced by these bacteria are fungicidal to the spore of the tested fungus. Antifungal activity of microorganisms is mostly due to the effect of lytic enzymes. These enzymes were found degrading the fungal cell wall (Sindhu and Dadarwal, 2001).

Our results indicated that both *Azospirillum* isolates had fungal cell wall degrading enzymes (chitinase and β-1, 3-glucanase) as well as other volatile and non volatile antifungal compounds and were able to hydrolyze the fungal chitin in 28 hours which magnifies its role as a soil bacterium and as an endophyte in the biological control of insects and pathogenic fungi. In our pot experiment, although soil was replaced with sterile wood reich which magnifies the virulence of *Fusarium* through the triggering of cellulase production by the fungus (since cellulose is the major component in wood reich), *Azospirillum* could combat the fungal infection to the wheat seedlings. This result could have a prime importance in organic farms where soils are amended with organic plant and animal residues which enhance the cellulose content in soil consequently, making microbial plant pathogen more aggressive. So, our *Azospirillum* isolates had proven successfully in combating *Fusarium* infection under the highest underground cellulose content, hence it is recommended as a successful biological fungicide especially in organic farms where the possibility of increase in fungal infection is magnified.

Previous report suggested that inoculation with *Azospirillum* can reduce bacterial leaf blight of rice with subsequent improvements in various yield components (Islam and Bora, 1998). Methods by which chitinolytic bacteria could be applied agronomically for crop protection could be through the direct application of the living immobilized bacteria to soil or the immobilized chitinase, also the chitin amended soils that could nonspecifically increase the population of chitinolytic microorganisms in soil, hence, it may be possible to use organic amendments to manipulate the soil micro-flora and induce desired changes in the endophytic microflora.

Application of chitinolytic *Azospirilla* to soil not only has a benefit in fighting the fungal and insectal pests but also as a nitrogen fixer and a plant growth promoter, it can cause the following aboveground
plant responses to *Azospirillum* inoculation in cereals and non cereal species were often reported increases in; total plant dry weight, in the amount of N in shoots and grains, and in the total number of tillers, fertile tillers, and ears; earlier heading and flowering time; increased number of spikes and grains per spike; increased grain weight; plant height and leaf size; and higher germination rates (Bashan, 1986; Mertens and Hess, 1984; El-Katatny and Idres, 2012). A positive inoculation effects on various root parameters, including increase in root length, particularly of the root elongation zone (Kapulnik et al., 1985), increase in number and length of lateral roots, which increases the root volume, increases in root dry weight, increase in the number, density, and early appearance (Morgenstern and Okon, 1987).

The biological control of plant diseases is one of the viable alternatives to chemical control in sustainable agriculture (Killani et al. 2011). The various reasons for the inhibitory activity of *Azospirillum* may have been toxic metabolites or the active enzymes that can damage the fungal cellular walls. Bacterial exo-chitinases and glucanases may have an important antagonist role against fungi (Machado et al. 2010).

Since *Azospirillum*, strains had no preference for crop plants or weeds, or for annual or perennial plants and can be successfully applied to plant that have no previous history of *Azospirillum* in their roots, it appears that *Azospirillum* is a general root colonizer and is not a plant specific bacterium. *Azospirillum* is a universal bacterium found almost everywhere (Okon et al., 1985) hence it could have a wide spectrum of application to many plant kinds as a plant growth promoter and/or fungal and insect pest control.

**Declaration**

Competing interests: The authors declare no competing interests.

**References**


Figures
Figure 1

A: Azospirillum growth in semi solid DN medium; B: The characteristic colony type of Azospirillum on RC medium; C, Azospirillum 3Re and 12Re grown on DN amended with colloidal chitin, chitinase degraded chitin appears as a clear zone around the colony.
Figure 2

Antagonistic activities of an *Azospirillum* isolates against *F. oxysporum*, (A) *F. oxysporum* inhibited by cells of *Azospirillum* 3Re and 12Re (after 5 days); (B) *Fusarium* sp. inhibited by culture filtrate of 3Re and 12Re (after 4 days).

![Graph showing chitinase activity](image)

**Figure 3**

Time-course experiments related to chitinase activity produced by *Azospirillum* 3Re and 12Re in presence of *F. oxysporum* fresh mycelium as sole carbon source. Values given in the figure represent the mean value, and the error bars indicate the standard deviation.
Figure 4

Time-course experiments related to β-1,3-glucanase activity produced by *Azospirillum* 3Re and 12Re in presence of *F. oxysporum* fresh mycelium as sole carbon source. Values given in the figure represent the mean value, and the error bars indicate the standard deviation.
Figure 5

Time-course experiments related to total protein produced by *Azospirillum* 3Re and 12Re in presence of *F. oxysporum* fresh mycelium as sole carbon source comparing with control. Values given in the figure represent the mean value, and the error bars indicate the standard deviation.
Figure 6

Direct exploitation of *F. oxysporum* mycelium by *Azospirillum* 12Re as a carbon and nitrogen source after 28 h incubation (A4 = 4h, A10 = 10h, A20 = 20h, A28 = 28h). C0 is control of healthy hyphal growth at zero time, whereas, C28 is control after 28 h. Morphological effects of the bacterium on the lysis of phytopathogen hyphae (arrows), where; A4, early stages of hyphal distortion and lysis in occurrence of bubbles and swelling in hyphae; A10, vacuolation and protein coagulation; A20, hyphal lysis, septa release and empty cells of contents; A28, massive lysis of hyphae after 28 hours, hypha ruptured near its tip and hypha with coagulated cytoplasm. (320 x).
Figure 7

Inhibition of *Fusarium* in pot experiment. A, Control seedling (dead and alive); B and C *Azospirillum* treated seedlings (3Re and 12Re, respectively).