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Interaction effects between *Trichoderma lixii* and *Azospirillum lipoferum* in vitro

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Abstract: Plant-beneficial microbes are used to promote crop growth, yield and are alternatives to chemical fertilizers. *Trichoderma* and *Azospirillum* are of the most important fungi and bacteria, respectively, which act as growth-promoters in plants. Twenty bacterial and two fungal isolates were isolated from samples collected from Biala, Kafrelsheikh Governate. One isolate from each bacteria and fungi was chosen on the basis of their response to a number of morphological and physiological tests. The bacterial isolate (S_2) was identified as *A. lipoferum* and the fungal isolate (T_4) was identified as *T. lixii*. The selected isolates showed a significant role in the extracellular chitinase and protease production, and showed a negative result in the in vitro dual culture interaction between them.

keywords: Trichoderma, Azospirillum, Interaction. In vitro

1.Introduction

Increasing the need for food for ever-increasing inhabitants has driven the scientists and society of agriculture to search for increased production of crops within the resources available [1] As well as, minimizing environmental damage [2]. One of the eco-friendly approaches is the use of plant growth-promoting micro-organisms (PGPM), which can play a significant role in plants' growth [3]. PGPM can be classified into two classes: plant growth-promoting rhizobacteria (PGPR), and plant growth-promoting fungi (PGPF).

Genus *Azospirillum* are bacteria which have been long recognized as (PGPR) [4, 5]. They have been isolated from the rhizosphere of several kinds of grass and cereals worldwide, in both tropical and temperate climates [6, 7]. *Azospirilla* are bacteria of the Gram-negative rhizospheric bacteria, which can live freely and fix nitrogen. They show an adaptable metabolism of C-and N that makes them best suited for establishing themselves in the rhizosphere's environment where microorganisms are competing with each other. Ammonium, nitrate, nitrite, amino acids and molecular nitrogen can act as nitrogen sources [8].

Trichoderma spp., are usually facultative aerobe present in huge communities in cultivated soils and media, as decaying wood. [9, 10]. They belong to the subdivision Deuteromycetes. The function of *Trichoderma* spp. isn't act only as a biological control agent; it can also stimulate rhizosphere's colonization, plant and root growth and increase plant defense responses [11, 12]. *Trichoderma* is an opportunistic, avirulent plant symbiotic fungus which really serves as a parasitic and antagonistic fungus against a lot of plant fungal pathogens, and offers defense against diseases of plants. *Trichoderma* spp. has been shown in lots of researches to be active biological control agents for diseases of plants' management, biological pesticides or products that can increase plant growth [11, 12, 13, 14].

1. Collection of samples

Wheat and grass samples and soil samples were collected from fields in Biala, Kafrel-sheikh. Samples were taken at depth of 6 feet away from the soil surface, preserved in plastic bags in a refrigerator at 4º C until used.

1.1. Preparation of samples

Five grams of each soil samples were put in 45ml sterilized saline solution and shaken for 2h. Roots were placed under a gentle stream of tap water for removing soils that attached to roots. When roots are free of adhering soils, it washed several times with sterilized distilled water. Then roots were soaked in 70% alcohol for two minutes, and washed with sterile distilled water.

2. Isolation,purificationandidentification of inocula

2.1. *Azospirillum*

Soil solution and root pieces were added in test tubes with 10ml of Nitrogen free bromothymol semi-solid malate (NFb) enrichment medium [6], then test tubes were incubated at 37°C for 72 h, then it serially diluted to 10-4 to 10-5 in sterile tap water. Loopfuls of the dilutions were streaked on plates of RC medium [15], which were incubated at 37 º C, for 72h. The small scarlet colonies were streaked on plates of RC medium to check the purity of isolates. Colonies have been transferred to solid malate medium slants containing 0.1 percent ammonium chloride. Cultures in the slants were streaked on the plates of malate agar medium containing 0.1% NH_4Cl to get pure colony. The pure colonies were transferred to the slants of the same medium and preserved.

2.2. *Trichoderma*

Quintuple serial dilutions of every soil sample were made in distilled sterilized water and samples were diluted with 0.5 ml, then poured on the *Trichoderma* specific medium surface (TSM) [16]. The plates were incubated at 28 ± 2°C for 96 h. Colonies appear different in shape on the plates. Then purified in the Potato Dextrose Agar (PDA) [17]. The purified isolates were kept at 4°C and applied in the study.

2.3. Biochemical characterization of the *Azospirillum* isolates:

2.3.1. Production of IAA

Production of IAA was determined by Salkowski's colorimetric method [18].

2.3.2. Determinationofphosphatesolubilization ability

Phosphate solubilization ability was determined by Pikovskaya's agar method [19].

2.3.3. Efficiency of N_2 fixation (total nitrogen)

The efficiency of N_2 fixation was determined by micro-kjeldahl analysis method.

3. Enzyme production:

3.1. Extracellular chitinase production:

3.1.1. *Trichoderma* :

Made by using a solid agar medium [20]; plates that inoculated were incubated at 28°C in a dark place and checked daily for 7 days to see the clear area.

3.1.2. *Azospirillum*:

Inoculated plates with solid agar medium [21] were incubated at 30°C in a dark place and checked daily for 7 days.

Using iodine is a Proof of extracellular chitinase activity, clearing of the opaque agar medium is the Evidence.

3.2. Extracellularprotease production:

Made by using skim milk agar medium [22], inoculated plates were incubated at 30°C in dark place and checked daily for 7 days to see the clear area.

4. <u>In vitro</u> dual culture bioassay of *Azospirillum* and *Trichoderma* isolates interactions:

A single streak was made bisecting a Petri dish with a diameter of 90 mm with potato dextrose agar (PDA). A 4x4 mm diameter agar disk colonized with *Trichoderma* was placed at the plate's border on either side of the bacterial streak. The dual culture bioassay was replicated three times and incubated at 28°C for 7 days.

Results and Discussion

1. Determination of qualitative characters of *Azospirillum* isolates:

In the present study, isolation of twenty bacterial strains was made from roots and soils of the rhizosphere of grass and wheat. All bacterial isolates were growing perfectly on the NFb medium that is the specific medium for *Azospirillum* [23], and in RC medium [1].There were 17 from 20 isolates

showed the ability of phosphate solubilization, and there were 3 isolates from 20 isolates had high ability in the production of ammonia as shown in **Table (1)**. There are two phosphate solubilization mechanisms; produce organic acids or the production of enzymes. *Azospirillum* usually uses organic acid, doesn't produce it [8]. But *Azospirillum* does produce gluconic acid in the presence of glucose as a carbon source. *Azospirillum brasilense* cannot grow in the glucose as a source of carbon. Consequently, gluconic acid is only formed in the media when the glucose is amended with fructose. Such gluconic acid solubilizes the soil phosphorous [24] and thus can help plants in improving phosphorus nutrition.

2. Determination of quantitative characters of *Azospirillum* isolates

Results in **Table (2)** showed that the IAA concentration ranged from (2.8 µg/ml - 31.8 µg/ml). Crozier *et al*. [25], which estimated production of IAA in the same species, assumed that values of production of IAA ranging from 7.99 to 140.97 µM for *A. brasilense* and 0 – 85.9 µM for *A. lipoferum*, and with Venieraki *et al*. [26] who assumed that production of IAA ranging from 29.8 and 194.8 mg L^{-1} by two *A. zeae* strains. Kuss *et al*. [27] assumed that total N values in *Azospirillum* ranged from 5.56 to 12.99 µg mL^{-1}.

Table 1: Ammonia production and phosphate solubilization as qualitative characters of *Azospirillum* isolates.

Isolates	Ammonia production	phosphate solubilization
S_1	++	+
S_2	++++	+
S_3	+++	-
S_5	+	+
S_6	+++	+
S_7	+	+
S_8	+++	+
S_9	++++	+
S_{10}	+++	+
S_{11}	++	+
S_{12}	+	-
S_{13}	+++	+
S_{14}	+++	+
S_{15}	+	+
S_{16}	+++	+
S_{17}	++++	+
S_{18}	+++	+
S_{19}	+	-
S_{20}	+++	+

Table 2: IAA and Total nitrogen as quantitative characters of *Azospirillum* isolates.

Isolates	IAA(µg/ml)	Total nitrogen (mg/ml)
S_1	24.3	0.005285
S_2	23.7	0.00945
S_3	10.2	0.00497
S_5	28.2	0.00595
S_6	23.1	0.0091
S_7	20.6	0.005355
S_8	12.2	0.00765
S_9	22.2	0.005005
S_{10}	8	0.00938
S_{11}	3.2	0.004795
S_{12}	30.9	0.005075
S_{13}	70.3	0.00784
S_{14}	7.1	0.004865
S_{15}	31.8	0.00476
S_{16}	7.2	0.00525
S_{17}	4.4	0.007105
S_{18}	2.8	0.004305
S_{19}	14.8	0.00525
S_{20}	24.3	0.00441

1. Enzyme production

Results in **Figures (1,2,3)** illustrate that *Azospirillum* and *Trichoderma* isolates showed enzymatic activity. *Azospirillum* showed clear zone around the colonies with a diameter of 3 cm in chitinase enzyme and 3.5 cm in protease enzyme. On the other hand, *Trichoderma* showed a clear zone around the colonies with a diameter of 3.2 cm in chitinase enzyme and 1 cm in protease enzyme.Microbes' production of extracellular hydrolytic enzymes can do a function in the inhibition of harmful fungi of plants. Chitin and b-1,3-glucans are the main components of numerous cell walls of fungi [28]. Various extracellular enzymes such as protease, chitinase, cellulase and 1,3-β-glucanase have been involved in the biocontrol of plant pathogens [29, 30, 31, 32].

Figure 1: Extracellular chitinase activity by *Trichoderma lixii.* Clear zones around fungal

colonies are indicative of chitinase enzyme activity.

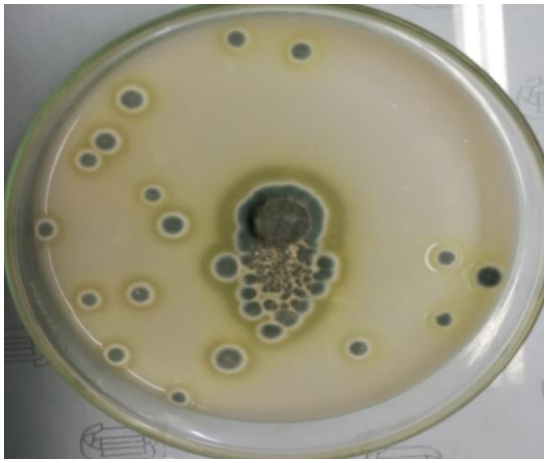

Figure 2: Extracellular protease activity by *Trichoderma*. Clear zones around fungal colonies are indicative of protease enzyme activity.

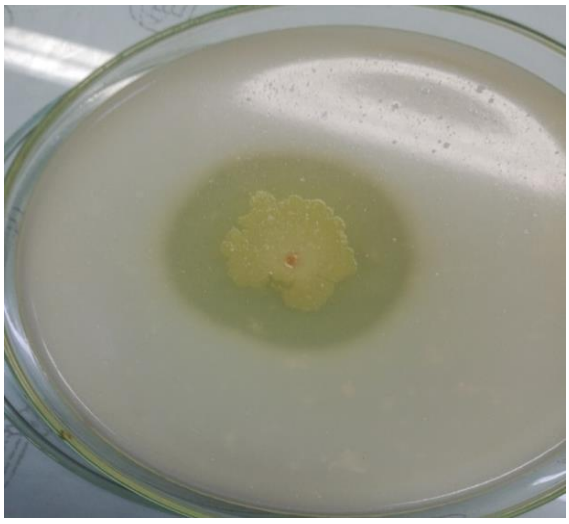

Figure 3: Extracellular protease activity by *Azospirillum lipoferum.* Clear zones around bacterial colonies are indicative of protease enzyme activity.

1. <u>In vitro</u> dual culture bioassay of *Azospirillum* and *Trichoderma* isolates interactions:

Figure 4 showed the positive result of the interaction between *Trichoderma lixii* and *Azospirillum lipoferum*. Using two groups of micro-organisms together has been recommended as one approach for improving plant growth and enhancing biological control [30, 33, 34, 35]. The benefits of using these combinations for the promotion of plant growth and biological control include enhancing crop yields and mineral uptake [33, 36].

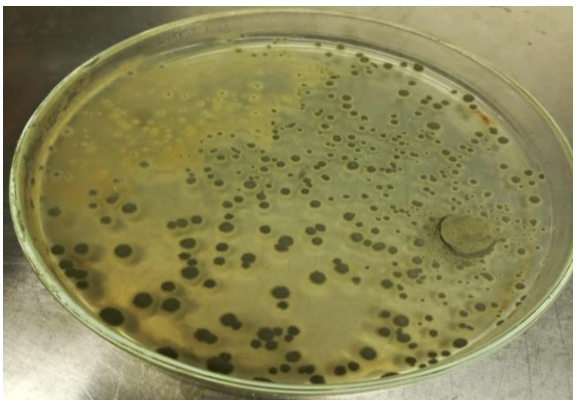

Figure 4: Dual culture bioassay of *Trichoderma lixii* (T) and *Azospirillum lipoferum* (A) showing the over growth of *Trichoderma* on *Azospirillum*

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