Journal of Phytopathology and Pest Management

eISSN: 2356-6507

Volume (1), Issue (2)

2014

Influence of certain carbon and nitrogen sources on antagonistic potentiality of *Trichoderma harzianum* and *Bacillus subtilis* against *Botrytis allii* the incitant of onion neck rot

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Abstract

It is widely known that certain sources of carbon and nitrogen may have an influence on the biological control efficacy against some pathogens. Gliotoxin fermentation agar (GFA) medium and nutrient glucose agar (NGA) medium amended with different carbon and nitrogen sources were used to study their antagonistic efficiency on *Trichoderma harzianum* and *Bacillus subtilis* against *Botrytis allii*. Results indicated that *T. harzianum* gave the highest inhibition % in growth of *Botrytis allii* when sucrose was used as a carbon source (61.04) while the lowest values of inhibition % were appeared by the application of mannitol as a carbon source (47.71). *T. harzianum* gave the highest inhibition % in growth of *B. allii* when uses potassium nitrate as a nitrogen source (53.75) while the lowest values of an inhibition% in growth of *B. allii* were obtained by application of beef extract as a nitrogen source (43.75). Results also showed that *B. subtilis* gave the highest inhibition% in growth of *B. allii* when mannitol was used as a carbon source (31.85) while the lowest values of an inhibition % in growth of *B. allii* were appeared by application of sucrose as a carbon source (28.14). *B. subtilis* gave the highest inhibition% in growth of *B. allii* when use glutamic as a nitrogen source (46.66) while the lowest values of an inhibition % in growth of *B. allii* were appeared by application of tryptophan as a nitrogen source (22.22). It was found that using sucrose and potassium nitrate enhanced efficacy of *T. harzianum* as a biological agent, also using mannitol and glutamic acid enhanced efficacy of *B. subtilis* as a biological agent.

Key words: : *Botrytis allii*, *Trichoderma harzianum*, *Bacillus subtilis*, carbon, nitrogen, neck rot

Introduction

Neck rot disease of onion caused by *Botrytis allii Munn* is a major disease of onion in Egypt (Kamel, 1952; El-Helaly et al., 1966; Abd-Elrazik et al., 1977; and Hussein et al., 1977). It causes tremendous losses for onion bulb and seed production. Onion (*allium cepa* L) is grown as a commercial crop in Egypt with approximately 54621.849 ha. planted annually and estimated yield of 129.083 tons for local consumption and export (FAOSTAT 2012). Traditional methods to control neck rot of onion (as fungicides) are harmful for human and environment. Biological control offers an environment-friendly alternative to the use of chemicals for suppressing the disease. *T. harzianum* and *B. subtilis* have an important role in biological control of soil-born fungi affecting onion in Egyptian soil (Abd El-Moity et al., 1978 &1981; Mousa et al., 1987; Sallam Nashwa, 2004).

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Various types of carbon sources are present in organic matter and organic amendments or in the exogenous substances. The carbon sources which are utilized by *T. harzianum*, plays an important role in the growth and bioactivity of the fungus. Danielson and Davey, (1973) found that *T. harzianum* better favors disaccharides. Germination of *Trichoderma* conidia must be dependent upon more than just the presence of an exogenous carbon source since better germination was obtained with complex materials, such as malt extract, yeast extract and peptone than with dextrose. The type of carbon source plays an important role on *T. harzianum* while its absorption and utilization varies from the carbon source to anther carbon sources in the organic matter in soil. The antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources (El-Banna, 2006). The present investigation was planned to study the effect of certain carbon and nitrogen sources *in vitro* on the antagonistic potentiality of *T. harzianum* and *B subtilis* against *B. allii*, the incitant of onion neck rot.

Materials and methods

Isolation and Identification of onion neck rot pathogen: Fourteen infected onion cultivar Giza 6 showing typical neck rot symptoms were collected from onion fields; two samples from Dronka, Assiut governorate, eight samples from Shandaweel and four from Thata, Sohag governorate.

Infected plants were cut into small pieces, thoroughly washed with tap water, surface sterilized by immersing for 2 minutes in 2 % sodium hypochlorite solution , rinsed several times in sterilized distilled water and dried with sterilized filter papers. The surface sterilized plant pieces were placed on potato dextrose agar (PDA) in Petri plates and incubated at 20ºC. After 4-5 days of incubation, the developed fungal colonies were purified by hyphal tip technique and single hyphal tips were transferred to PDA slant. The PDA slants were then incubated at 20ºC for 10 days. The pure fungal isolates were kept in 4ºC refrigerator for further studies. All Isolates were identified as *B. alli* by Assiut University Mycological Center, Egypt (AUMC).

Pathogenicity tests: To evaluate the virulency of fourteen isolates of *B. allii*, Pathogenicity tests using bulbs of onion cultivar Giza 6 were carried out under greenhouse conditions at the Shandaweel Agriculture Research Station, Sohag during the 2009- 2010 season. Healthy bulbs were surface sterilized by dipping in 0.1 % sodium hypochlorite solution for 3 minutes, rinsed several time sterile with distilled water and then left for drying at room temperature (about 30ºC). Pots (40cm in diameter) were sterilized by immersing in 5% formalin solution for 15 minutes then left to dry. Soil was sterilized by autoclaving at 121ºC for 3 hrs. The inocula of *B. allii* were prepared by washing the fungal propagules from 10-21 days old cultures. The spore suspension was adjusted to 10^3 spores /ml by using hemocytometer. The neck of the onion bulbs were sprayed with fungal suspension by using a handatomizer according to Kaufman and Lorbeer (1967). Control plants were sprayed with sterilized water.

The percentage of infection was recorded after 4 months from planting date according to the next formula:
Infection % = Number of infected plants / Total number of plants X 100

Isolation and identification of antagonistic fungi from onion plant rhizosphere: Several fungi were isolated from the rhizosphere of healthy and diseased onion plants obtained from different locations to test their antagonistic effects against *B. allii* according to Dhingra and Sinclair (1995). Plant roots were carefully dug out and the excess soil was gently shaken off and only the soil which was closely adhered to the root system was used. One gram soil was placed in a 250 ml flask containing 100 ml sterile water. The flasks were gently stirred until most of the adhered closely adhering soil released. Serial soil dilutions were prepared (1:100, 1:1000, and 1:10000). One ml of each dilution was spread onto the surface of (PDA) plates supplemented with 0.4 mg/ml streptomycin. The inoculated PDA plates were incubated at 20°C for 4 days. The isolated fungi were purified and identified according to Domsch et al., (1980).

Isolation and identification of antagonistic bacteria: One gram of soil samples was suspended in 99 ml of sterile distilled water and shaken vigorously for 2 min. then the soil suspensions were serially diluted in sterile distilled water, and one ml of different soil dilution (10^{-1} to 10^{-6}) was plated onto the surface of nutrient agar (NA) plate. The plates were incubated at 28˚C for 24-48 h. The isolated bacteria were identified according to Sneath et al., (1984).

Effect of carbon and nitrogen sources on antagonistic potentiality of *T. harzianum* and *B. subtilis* against *B. allii*

Carbon source: Six carbon sources namely glucose, sucrose, mannitol, fructose, lactose and starch were used to determine their effects on the antagonistic abilities of *T. harzianum* and *B. subtilis* on the highly pathogenic isolate of *B. allii* according the method described by El Banna (2006) .

Gliotoxin fermentation agar (GFA) medium was used as a basal medium. Different carbon sources were added to the replacement of the original carbon source (glucose) and the quantity of different carbon sources were calculated on the basis of their molecular weight. Different media were sterilized and poured in 9 cm Petri plates. One 0.5 cm in diameter disk of *B. allii* was placed at one side of the Petri dish near the periphery and on the other side of the same plate; 0.5 cm in diameter disk of *T. harzianum* was also placed at the same distance from the periphery of the plate.

Nitrogen source: Seven different nitrogen sources , *i.e*., potassium nitrate, ammonium tartrate, ammonium nitrate, glutamic acid, peptone, beef extract, and tryptophan were added to the basal medium (GFA) instead of ammonium tartrate that is the original nitrogen source according the method described by Hasanzadeh et.al., (2012). The amount of nitrogen source was calculated according to the molecular weight. Plates containing different nitrogen sources with antagonistic *T. harzianum* and the *B. allii* was done as previously described.

In case of the antagonistic *B. subtilis*, the different carbon and nitrogen sources were added to NGA media. One disk of *B. allii* was placed on one side of the Petri plates and

a loopful of 48h. Old culture of *B. subtilis* was streaked on the other side of the Petri plate.

The inoculated plates as well as the control plates that were inoculated with *B. allii* were incubated at 20˚C. Five replicates were used for each treatment. Data were recorded when the growth of *B. allii* completely covered the plate surface in control treatments.

The percentage of reduction in mycelial growth of *B. allii* was calculated using the following formula:

$$X = G_1\text{-}G_2/G_1 x100$$

Where:

X: % of reduction in growth.

G_1: growth of pathogenic fungus in control plates.

G_2: growth of pathogenic fungus in treated plates.

Statistical Analysis: Data were subjected to analysis of variance (ANOVA). Mean differences were separated according to Duncan's multiple range test ($p < 0.05$) (Duncan, 1955).

Results

Pathogenicity tests: Table (1) indicate that all the tested *B. allii* isolates were able to infect onion plants with different levels of virulence. The most virulent isolates were BA-8, BA- 9, BA-12, and BA-13 causing the highest percentage of infections (100). Isolates BA-10, BA-11 and BA- 14 showed moderate virulence (90, 83, and 83). The lowest virulent isolate was BA-7 (46.66).

Effect of certain carbon sources on antagonistic potentiality of *T. harzianum* and *B. subtilis* against *B. allii*: Table (2) indicate that sucrose as a sole carbon source showed the highest percentage of inhibition of growth of *B. allii* (61.94) followed by starch and lactose (59.79 &51.04). The lowest percentage of inhibition in the growth of *B. allii* was shown when mannitol was used as a carbon source (47.71). There was no significant difference between the percentage of mycelial inhibition in case of glucose and fructose.

Table 1: Pathogenicity tests of *B. allii* isolates on onion cultivar Giza 6 under greenhouse conditions*.

Isolate No.	Infection (%)
BA-1	63.30^{f}
BA-2	53.33^{h}
BA-3	56.66^{g}
BA-4	73.33^{d}
BA-5	66.66^{e}
BA-6	63.33^{f}
BA-7	46.66^{i}
BA-8	100^{a}
BA-9	100^{a}
BA-10	90^{b}
BA-11	83^{c}
BA-12	100^{a}
BA-13	100^{a}
BA-14	83^{c}
Control	0^{j}

* Means followed by the same letter are not significantly different according to Duncan's multiple range test at 0.5%

The effect of different carbon sources on the antagonistic capability of *B. subtilis* indicated that the highest growth inhibition of *B. allii* was shown when mannitol was used as a sole carbon source (31.85) (Table 3). There are no significant differences in growth inhibition of *B. allii* under other tested carbon sources (Table 3).

Table 2: Antagonistic capability of *T. harzianum* against *B. allii* using certain carbon sources*.

Carbon source	Inhibition growth%
Glucose	51.04[c]
Fructose	50.41[c]
Mannitol	47.71[d]
Sucrose	61.04[a]
Lactose	58.96[b]
Starch	59.79[ab]

* Means followed by the same letter are not significantly different according to Duncan's multiple range test at 0.5%

Table 3: Antagonistic capability of *B. subtilis* against growth of *B. allii* using certain carbon sources*.

Carbon source	Inhibition growth%
Glucose	29.63[b]
Sucrose	28.14[b]
Fructose	28.51[b]
Lactose	28.88[b]
Starch	28.51[b]
Mannitol	31.85[a]

* Means followed by the same letter are not significantly different according to Duncan's multiple range test at 0.5%.

Antagonistic capabilities of *T. harzianum* and *B. subtilis* against growth of *B. allii* using certain nitrogen sources: Table (4) indicate that the highest inhibition in the growth of *B. allii* was detected when potassium nitrate was used as a nitrogen source (53.75) followed by tryptophan (50.83), ammonium tartrate (50.42) and ammonium nitrate (49.37). The lowest mycelia growth inhibition of *B. allii* was appeared under the application of beef extract as a nitrogen source (43.75).

Table 4: Antagonistic capability of *T. harzianum* against growth of *B. allii* using certain nitrogen sources*.

Nitrogen source	Inhibition growth%
Ammonium tartrate	50.42[b]
Glutamic acid	47.70[c]
Potassium nitrate	53.75[a]
Ammonium nitrate	49.37[b]
Beef extract	43.75[d]
Peptone	46.25[c]
Tryptophan	50.83[b]

* Means followed by the same letter are not significantly different according to Duncan's multiple range test at 0.5%.

Table (5) indicate that highest percentage of inhibition in the growth of *B. allii* was occurred with glutamic acid (46.66) followed by potassium nitrate (30.22), ammonium nitrate (29.25) and ammonium tartrate (28.14). The lowest values of inhibition in the growth of *B. allii* were appeared by the application of tryptophan as a nitrogen source (22.22).

Table 5: Antagonistic capability of *B. subtilis* against growth of *B. allii* using certain nitrogen sources*.

Nitrogen source	Inhibition growth%
Beef extract	29.07[b]
Peptone	24.81[c]
Ammonium nitrate	29.25[b]
Potassium nitrate	30.22[b]
Ammonium tartrate	28.14[b]
Tryptophan	22.22[d]
Glutamic acid	46.66[a]

Means followed by the same letter are not significantly different according to Duncan's multiple range test at 0.5%

Discussion

Neck rot of onion caused by *B. allii* is one of the most serious disease that attack onion plants grown for seed or bulb production. During the onion storage, fungus can attack the bulbs causing severe losses. (Abd-El-Rahman, 1984; Bedlan, 1991; Hayden et al., 1997; Nielsen et al. 2002). The results reported herein indicated that the neck rot disease caused by *B. allii* is the main factor of decreasing onion production in Assiut and Sohag governorates, Egypt. Pathogenicity tests on onion cultivar Giza 6 showed that *B. allii* isolates had different levels of virulence. Such results are in agreement with similar works (Ahmed et al, 1992; El-zawahry et al.1997; Chilvers et al., 2004; Saleh, 2004; Chilvers & Dutoid, 2006; and Jorjandi et al., 2009) those found different levels of virulence between tested isolates of *B. allii* which may be due to the genetic structure of each isolate. Many studies have been conducted to examine the ability of different *Trichoderma* isolates to utilize a variety of different carbon and nitrogen compounds, Hasanzadeh et al. (2012) used different carbon sources, including mannitol, sorbitol, arabinose, and fructose, and found that the best growth of *Trichoderma* isolates was on media amended with sorbitol or arabinose as a sole carbon source. For the nitrogen compounds the maximum growth of *Trichoderma* isolates was on ammonium nitrate followed by sodium nitrate and potassium nitrate.

The antagonistic capabilities of *T. harzianum* isolate grown on different sources of carbon showed that the highest inhibition in the growth of *B. allii* was achieved when sucrose was the sole carbon source; the lowest value of growth inhibition of *B. allii* was occurred with application of mannitol. Such results are in agreement with those reported by Chattannavar et al., (2005). Sucrose consists of one molecule of glucose and another of fructose linked together by β- glucosidal bond, *T. harzianum c*an produce sucrase enzyme to utilize sucrose, also it is needed for maximum efficiency for biological control. The influence of using different nitrogen compounds on the antagonistic capability of *T. harzianum* against *B. allii* showed that the highest inhibition in the growth of onion pathogen was achieved when potassium nitrate was the main nitrogen constituent, followed by tryptophan. While the lowest values of inhibition in the mycelial growth of *B. allii* were produced under application of beef extract. The antagonistic capabilities on different sources of carbon of *B. subtilis* against *B. allii* showed that the highest growth inhibition was achieved by mannitol followed by glucose. The lowest values of growth inhibition of *B. allii* were produced by the application of sucrose as the sole carbon source. Using different nitrogen compounds, the antagonistic capability of *B. subtilis* against *B. allii* showed that highest growth inhibition of *B. allii* was obtained by the application of glutamic acid followed by potassium nitrate, this may be agree with Jitendra et al., (2012) who stated that glutamic acid as an organic nitrogen source of nitrogen supported enzyme activity of the microorganism as compared to the other tested sources.. The lowest values of growth inhibition of *B. allii* were produced when tryptophan was the sole nitrogen source. The current study allows us to choose the best carbon and nitrogen source for further studies as formulations.

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