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ORIGINAL PAPER

Effectiveness of some Bio-control Agents and Chemical Resistance Inducers Against Brown Stem Rot in Soybean (*Glycine max* (L.) Merrill)

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ABSTRACT

Brown stem rot (BSR) [*Phialophora gregata* (Allington & Chamberlain) Gams.] is a widespread and significant disease in all over world's soybean-growing regions. In isolation trials, eight isolates associated to soybean stem rot symptoms were collected from various governorates in Egypt. All the tested isolates could attack soybean plants of the cv. Giza 22 variety and cause brown stem rot. The results of pathogenicity tests showed that isolate of Mallawi from Minia governorate was the most virulent isolate. The isolates were identified as *Phialophora gregata*. Efficacies of some bio-control agents *viz*. *Trichoderma harzianum* and *Bacillus subtilis* in addition to chemical resistance inducers *viz*. salicylic acid (SA), humic acid (HA) and hydroquinone (HQ) compared with Topsin M-70 % tested individually or in combinations as seed treatments showed antifungal activities against *P. gregata in vitro* and *in vivo*. Linear growth *P. gregata in vitro* was severely reduced by chemical inducers and antagonistic bacteria to varying degrees as compared to the control. The combination of *B. subtilis* + SA was most efficient in inhibiting mycelial growth of *P. gregata*. *In vivo*, the application of all treatments used as seed soaking showed significantly reduced the percentage of internal stem discoloration (PISD). Employing chemical resistance inducers and bio-control agents together was more efficient than using each of them alone. Additionally, in two growing seasons after one another, biocontrol agents, chemical inducers and their mixtures significantly raised soybean growth and yield parameters. The best control of brown stem rot disease and the highest growth and yield parameters values was observed under combination of *B. subtilis* + SA. In soil analysis and biochemical changes in soybean plants, all treatments showed significantly increased in dehydrogenase (DHA), photosynthetic pigment content, total protein, total carbohydrate, total oil contents and nitrogen (N), phosphorus (P), and potassium (K) contents in yielded seeds, meanwhile the total phenols significantly increased in all treatments compared with untreated control treatment. In general, utilizing biotic inducers appears to be one of the solutions to replace, or to reduce, fungicides usage in the management of plant diseases.

Keywords: Soybean, *Glycine max*, *Phialophora gregata*, Brown stem rot, Hydroquinone, Humic acid, Salicylic acid, *Trichoderma harzianum*, *Bacillus subtilis*.

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INTRODUCTION

Soybean (*Glycine max* L.) is the most significant annual legume crops worldwide according to Prévost *et al*. (2010), it is a source of oil and vegetable protein. By supplying plant-based protein, vegetable oil, and animal feed, it significantly contributes to world food security. Soybean seeds provide a relatively high quantity of protein and numerous important amino acids. Also, it has 30% carbohydrate content with 10% total soluble sugars. Additionally, it is an excellent nutritional source of phosphorus and calcium (Nassiuma and Wasike, 2002 and Herridge *et al*., 2008). As a result of providing nitrogen fixation to the soil soybeans can also be regarded as an environmentally friendly crop. Infections by soil-borne fungal pathogens lead to production decrease because of damage occurring of roots, stems, leaves, pods and seeds discoloration. These fungi belong to the genera *Fusarium*, *Phytophthora*, *Pythium*, and *Rhizoctonia* (Sullivan, 2004) and *P. gregata*. Moreover, brown stem rot (BSR) is caused by *P. gregata* f. sp. *sojae* fungal pathogen Allington and Chamberlain (1948) W. Gams (syn. *Cadophora gregata* Abo El-Dahab, (1968) and Harrington and McNew, (2003).

The first record of brown stem rot (*Phialophora gregata*) in Egypt was by Abo El-Dahab (1968) who isolated *P. gregata* from seeds of infected plants. Abou-Zeid *et al*. (1987), Abd El-Al (1999) and Bahaa El-Din (2013) isolated *P. gregata* from six governorates in Egypt. They reported that *P. gregata* was the most

predominant fungus and high frequency was obtained from all inspected localities in all surveyed governorates.

Phialophora gregata infected susceptible soybean plants at the root level, colonized the vascular system before spreading to leaves (Allington and Chamberlain, 1948). The disease has already been documented worldwide. The fungus which is soil-borne and affects the vascular tissue of soybean plants, discolors the stem's vascular tissue and produces intervene necrosis. Under favor conditions the disease may cause 30% yield loss (Chen *et al*., 2000). Chemical pesticides are frequently utilized in modern crop protection (Cook *et al*., 1996). The need for increased sustainability in agriculture has arisen as a result of growing rendering the potential health and environmental risks linked by usage of these agrochemicals. Increased levels of antagonistic microorganisms in the soil are frequently associated with disease suppression in these fields. There has been substantial research into the processes by which these rhizobacteria mediate disease suppression (Haas and Keel, 2003).

Using biotic or abiotic inducers to induce acquired systemic resistance that enhances resistance of plants to upcoming pathogen infection appears to be the solutions to replace or reduce fungicides usage in plant disease control. As many resistance elicitors only give between 20 and 85% disease control, they rarely provide total control of infection. The resistance produced by these compounds has a broad spectrum against many pathogens and is long-lasting (Walters *et al*., 2013). As a complementary role in an integrated pest management strategy (IPM), the helpful rhizobacteria can be developed as biological pesticides to reduce the usage of chemical pesticides in agriculture a system using both contemporary, environmentally sound chemical and biological techniques. Since *B. subtilis* has a fungicidal impact on 26 additional fungi associated with soybean seeds as well as *Penicillium* sp., the use of biological control utilizing antagonistic bacteria has proven to be an effective method for eradicating a variety of plant infections Hashem *et al*. (2019). Additionally, soybean root rot-wilt disease complex syndrome infection was inhibited by *B. subtilis* (Yassin *et al*., 2019). According to Zian *et al*. (2019), root rot and wilt diseases, as well as growth promotion of lupine plants, were investigated for control of *in vitro* and *in vivo*, as well as *T. harzianum* and *B. subtilis* as biocontrol agents and chemical inducers hydroquinone (HQ) and salicylic acid (SA) as plant systemic inducers resistance. A number of chemicals could induce systemic resistance such as salicylic acid (SA), hydroquinone (HQ) and humic acid (HA) (Amin *et al*., 2007; Abd El-Hai *et al*., 2016 and Sarhan *et al*., 2018).

The present study aimed to controlling soybean brown stem rot disease during the use of bioagents (*B. subtilis* and *T. harzianum*) and chemical inducers (salicylic acid, hydroquinone and humic acid) either individually or in combination under greenhouse and field conditions compared to the fungicide treatment, and their impact on growth and yield parameters of soybean plants were studied.

MATERIALS AND METHODS

Soybean seeds source and tested materials:

The Department of Legume Crop Res., Field Crop Research Inst., Agric. Res. Centre, Giza, Egypt provided the soybean seeds of variety Giza 22 that are sensitive to brown stem rot infection Bahaa El - Din (2013).

Trichoderma harzianum and *B. subtilis* kindly provided by the Soil, Water and Environment Res. Inst. Agric. Res. Centre in Giza, Egypt Microbial Activity Unit.

Salicylic acid (SA) from Sigma Aldrich, USA, humic acid (HA), potassium humate soluble granule 85% WSG; Humus from Broadbeach Chemical International Co. Ltd., Inner Mongolia, China, and hydroquinone (HQ) from El-Nasr Co. for Intermediate Chemicals Egypt were employed as a chemical inducer at 10 mM.

TopsinM-70 %WP:

Common name: Thiophanate-methyl., Chemical name: dimethyl [1,2-phenylenebis (iminocarbonothioyl)] bis [carbamate]., Dose per kg seed: 3g/Kg Seed

Isolation and identification of the causal pathogen.

Soybean plants samples symptomatic with the brown stem rot disease were taken from three distinct governorates *viz*. Beheira, Beni-Suef and Minia governorate grown under natural infection. The infected plant samples with discolored stem and root tissues from each plant about five cm above and below the soil line were properly rinsed under water running, dried, and then cut to 0.5 cm pieces. The pieces surfaces were cleaned by submerging them for two minutes in a 2% sodium hypochlorite solution, then washing them repeatedly in sterilized distilled water and drying them between sterilized filter papers. Plant fragments were put on plates with PDA medium and cultured for seven days at 20 ± 2ºC. Basis on

the cultural, morphological, taxonomic, and microscopic characteristics, using the hyphal tip technique fungi were purified on PDA plates, and their identities were determined in accordance with Mengistu *et al*. (1986).

Pathogenicity test:

Eight isolates were examined for pathogenicity on the soybean Giza 22 cultivar. Under greenhouse circumstances, a described homogenized culture procedure was used to inject the causative pathogen into pots of disinfected soil (Tabor *et al*., 2006). Sterilized seeds of Giza 22 soybean cultivar were sown at the rate of five seeds per pot and four replicates were used for each isolate. Stems of 2-week-old plants were inoculated by injecting 20µl of inoculum suspension 2.7×10^7 spores/ml into a paste stem 2 cm above the soil line a syringe and 18- gauge needle. The percentage of internal stem discoloration (PISD) was used to measure the pathogenicity of the examined fungus. The most aggressive isolate was chosen for additional research.

Laboratory experiments:

1- Biocontrol agents biochemical activities:

Trichoderma harzianum and *B. subtillis* were examined for their biochemical activities in previous studies such as *T. harzianum* (gliotoxin activity), *B. subtilis* β- 1,3 glucanase (laminarinase and cellulase) activity; chitinase activity as reported by Zian *et al*. (2019).

2- Antifungal effects biotic and a biotic inducer on *P. gregata* under laboratory conditions

A. Biocontrol agents antagonistic effects:

T. harzianum and *B. subtilis* were tested, using PDA medium for their antagonistic to *P. gregata.* Agar disks (5mm in diameter) of antagonistic *T. harzianum* and pathogenic fungi were cut from periphery of 7 days old cultures or streak for bacteria were placed in Petri dishes (90mm diameter) containing PDA medium. Both of antagonism disc and pathogen disc was put in opposition to one another at 1mm from the plate edge and incubated at 22 ± 2°C for 24 h. Plates only inoculated with pathogenic fungi were represented as control, four plates were used for each treatment. When the tested fungus completely covered the medium surface in the control, the pathogen linear growth was assessed (Yeh and Sinclair, 1980). In each treatment four plates were used as replicate. Using Mohana and Raveesha (2007) formula, the inhibition percentage of *P. gregata* mycelial growth that was calculated as following:

$$\mathbf{I\ \% = \frac{C - T}{C} \times 100}$$

Where:

I = Inhibition percentage.

C = Average mycelial growth in control plate.

T = Average mycelial growth in treatment plate.

a- Chemical inducers antifungal effect:

On PDA medium, the impact of chemical inducers on the growth of the most severe virulent isolate was assessed. Ten ml of PDA medium that has been supplemented with,10 mM HQ, 10 mM HA and10 mM SA each alone were poured in the plate and a 5mm disc of the tested fungus was placed in the center before being incubated at 22±2°C. When the indicated pathogenic fungus full growth was seen in the check treatment, colony diameter was measured, and the growth inhibition percentage was calculated by using formula mentioned.

b- Antifungal effects of combining chemical inducers and biological controls:

Before this experiment, bioagents and chemical resistance inducers were tested in the laboratory to illustrate chemical inducers effects on growth of biocontrol agents to make sure that there is no inhibitory effects of chemical inducers on these microorganisms. *T. harizianum* and *B. subtilis* were cultured on potato dextrose broth (PDB) and nutrient broth in Erlenmeyer flask, respectively. At 25°C and 150 rpm for 48 hours on the rotary shaker, the flasks were incubated. The culture was centrifuged and sterilized using 0.22 Mm pore size membrane filters after being filtrated through filter paper. The filtrate results were stored in sterilized dark vials at 5°C until use.

Biotic filtrates were added to flasks (500 ml) containing 200 ml PDA medium to obtain 10% then, 10mM HQ, 10mM HA and 10mM SA according by (Abdel-Monaim, 2013; Abd EL-Hai *et al*., 2016 and Zain *et al*., 2019) were added separately and shacked gently to mix well, 10 ml of each treatment were poured in each Petri dish. As previously mentioned, the pathogenic isolate was inoculated into the plates. The pathogenic isolate alone was used as the control treatment and was plated on PDA medium. The plates with the inoculum were incubated at 22 ± 2°C until the control colony spread the plate. Colony diameter was now assessed, and a comparison between the pathogen and the control resulted in a calculation of the pathogen % growth inhibition.

Greenhouse experiment:

Bioagent treatments:

Once *T. harzianum* and *B. subtilis* had grown in the liquid culture media, these cultures were centrifuged under cooling (4°C) at 10000 rpm for

10 min. After that, the disinfected soybean seeds were steeped in the supernatant for 15 min. In a 20 cm Petri plate, *T. harzianum* and *B. subtilis* cells were individually collected were taken from each Erlenmeyer flask and mixed with 1-1.5 ml of 1% methyl cellulose (Sigma-Aldrich, Milwaukee, WI, USA) in sterile distilled water to create a bacterial slurry. Healthy soybean seeds were treated with slurry of bacteria after being pre-soaked in supernatant, then, to absorb the extra liquid, spread it out on a screen cloth with paper towels underneath, then before sowing, the treated seeds were allowed to air dry for 15 minutes. By using the plate dilution method and counting the number of colony-forming units (cfu/seed) on nutrient yeast dextrose agar (NYDA) medium, bacteria coated on seeds were counted (Atwa, 2016).

Chemical resistance inducer treatments:

Just before sowing, healthy soybean seeds were immersed in the inducer solutions for 15 minutes, hydroquinone (HQ), humic acid (HA) and salicylic acid (SA) at concentrations 10mM.

Combination among biocontrol agents and chemical inducers treatments:

The effects of chemical inducers (HQ, HA, and SA), biocontrol agents (*T. harzianum* and *B. subtilis*), individually and or their combinations against *P. gregata* were prepared by dissolving the chemical inducers in suspension of the biocontrol agents as seed soaking. In Plant Pathol., Res., greenhouse Inst., Agric., Res. Center, Giza, Egypt, experiments were conducted. Plastic pots (25 cm in diameter) were sterilized by soaking them in a 5% formalin solution for 15 minutes, then leaving them there for a week to allow the formalin to completely evaporate. Steam-disinfested sandy clay soil 1:2 (V.W) was used to fill pots. Each pot included five pretreated soybean seeds that were planted and immediately watered. For each specific treatment, there were five replicates used. A 0.1% 15:15:15 (NPK) fertilizer solution was added, and all pots were maintained in a greenhouse with natural lighting, irrigation as necessary, and weekly watering to nearly field capacity. The treatments were as follows:

1- *T. harzianum*; 2- *B. subtilis*
3- Hydroquinone (HQ); 4- Humic acid (HA)
5- Salicylic acid (SA); 6- *T. harzianum* + (HQ)
7- *B. subtilis* + (HQ); 8- *T. harzianum* + (HA)
9- *B. subtilis* + (HA); 10- *T. harzianum*+ (SA)
11- *B. subtilis* + (SA); 12- TopsinM-70 WP
13- Infested control

Inoculation Protocol:

The most aggressive *P. gregata* isolate was chosen based on a pathogenicity test. Spores of *P. gregata* were harvested from the green bean agar plates by washing the surface with sterile distilled water and gently scraping the cultures with a spatula. The fungus culture was grown on green bean extract medium containing 35 gm of green pods ground frozen *Phaseolus vulgaris* L., 20 gm agar and 50 mg of ampicillin/ liter. Fungus culture is incubated for 6 days at room temperature and ambient light. Spores concentration was determined using a spencer haemacytometer slide. Spores were suspended in a 1.2% water agar paste to obtain a final spore concentration of 2.7×10^7 spores/ml. Brown stem rot (BSR) disease severity was established on each plant using the severity scale recommended by Perez *et al*. (2010). Vigor of each individual plant was assessed using the scale from 1 to 7 described in Table (1) In this scale, a score of

1 - Corresponds to a dead plant
2- The plant with a green stem and no leaves
3- Chlorotic and necrotic leaves are prominent
4- Some stunting, mosaic, chlorosis and necrosis on leaves are observed
5- Leaf area is normal except for some yellowing
6 - Leaf area is normal, and plants are small but healthy
7- Plants are completely healthy.

Plant height from the soil to the top of the main stem was recorded in cm. Approximately 20µl of the suspension were injected into the soybean stems 2 cm above the soil line with an 18-gauge needle. Soil in containers was kept moist by watering the pots daily until saturation. The stem severity, plant height from the soil to the top of the main stem was measured and recorded in cm. Then plant stems were cut lengthwise from top to bottom. The amount of tissue discoloration and damage inside the vascular tissue was measured in cm upward from the inoculation point as indicated by (Tabor *et al*., 2003 and 2006). A plant was considered discolored if there was any visible dark brown discoloration on the vascular tissue or the pith. Stem severity was calculated as a ratio of the length of discoloration to total plant height expressed as a percentage. The percentage of internal stem discoloration (PISD) was used to assess soybean brown stem rot severity according to the following formula Sills *et al*. (1991).

$$\textbf{PISD (\%)} = \frac{\textbf{Height internal stem discoloration (cm)}}{\textbf{Plant height (cm)}} \times 100$$

Table (1): Brown stem rot (BSR) severity visual assessment scale in soybean plants

D*	Plant description
1	Dead plant
2	Green stem and no leaves
3	Chlorotic and necrotic leaves are prominent
4	Some stunting, mosaic chlorosis and necrosis on leaves
5	Leaf area is normal, with some yellowing
6	Leaf area is normal, plants are small but healthy
7	Healthy plants

*Degree is a measure of overall plant health.

Field experiments:

The effectiveness of biotic and abiotic inducers for controlling brown stem rot two field experiments were conducted under natural infection during the subsequent summer growing in two seasons of 2020 and 2021 at Etai El-Baroud, Agric., Res., Station, Behira governorate, Egypt, the history of the disease is known in this the field. The health of soybean seeds cv. Giza 22 were handled in the same way as in greenhouse conditions. Seeds were soaked in distilled water as the control, while Topsin-M70 % fungicide was utilized as seed dressing at 3g/Kg seeds. Seeds were planted in the field on May 27, of two seasons. The complete randomized block was designed in these trials (28 plots) with four replicates. Each plot had a surface area of 9 m^2 and five rows that were 3.5 m long and 0.6 m wide. The treatments were planted one seed per hill in 20 cm-apart hills on both sides of the one row ridge. All farming measures advised by the Egyptian Ministry of Agric. and Land Reclamation were applied. The percentage of internal stem discoloration (PISD) was used to assess soybean brown stem rot was determined as mentioned before after 60 days from sowing.

Analysis of rhizospheric soil dehydrogenase activity (DHA):

The soil rhizosphere around soybean roots assessed the activity of dehydrogenase (EC1.1) described by Glathe and Thalmann (1970) method. A 2 mL aliquot of 0.5% 2, 3, 5 triphenyl tetrazolium chloride (T.T.C) solution diluted in Tris buffer (pH 7.8) was added and mixed thoroughly with the rhizosphere soil sample (2 g). The T.T.C solution saturated and partially submerged the soil samples. The tubes were then sealed with rubber silicon stoppers and incubated in the dark at 30°C for 24 h. After that, 10 mL of pure acetone was added to each tube, and the tubes were shaken continuously for two hours in the dark to extract the resultant pink triphenyl formazan (T.P.F). After that, the suspension was passed through Whatman No. 1 filter paper. Using a UV spectrophotometer, the intensity of the filtrate's pink colour was assessed at a wavelength of 485 nm (Spekol UV-Vis Light, Westburg). Formazan concentrations were determined using a standard curve and expressed in µg TPF/g dry soil/day. Without a soil sample, a blank treatment containing all additives was considered and subtracted.

Determination of total phenol contents:

Five soybean plants were randomly selected from each treatment for biochemical evaluation of phenol contents at reproductive stages two and four (R2 and R4). Total phenol levels were estimated plant leaves using a method described by Zieslin and Ben-Zaken (1993). One g fresh plant sample was homogenized in ten millilitres of the 80% methanol were used, which was stirred for 15 minutes at 70°C. 250 µl of folin-Ciocalteau reagent (1 N), five ml of distilled water and one mL of methanol extract were added. One ml of saturated Na_2CO_3 solution, one ml of distilled water was added, and the mixture was then incubated at 25°C for one h. The mixture was then maintained at 25 °C, after 3 min. The measurement of the produced blue colour absorbance using a wavelength of 725 nm, was established. According to the standard curve created by a Folin-reaction with phenol solution, the amount of total phenols was determined and the result was the phenol equivalent in µg g^{-1} fresh tissue.

Evaluating photosynthetic pigments:

Using the method reported by Arnon (1949) to determine amounts of the total chlorophyll a, chlorophyll b, and carotenoid pigments were measured in soybean leaves. The method reported by Arnon (1949) to determined amounts of the total chlorophyll a, chlorophyll b, and carotenoids pigments were measured in soyabean leaves. For the purpose of extracting pigments, the pistil at 60 days after sowing homogenised a 1 mg sample of the first fully developed leaf apical with 10 ml of acetone 80% (V/V). To avoid the chlorophyll being degraded by light or enzymatic action, the procedure was carried out in low light and temperature. To determine of chlorophyll a, b, and carotenoids pigments, the reading was taken using a spectrophotometer (SPECTRONIC 20 D). The calculations were based on formulae of Hendry and Grime (1993) and the pigment content was given in mg FW^{-1}.

Chlorophyll a (mg g^{-1}) =
$[(12.7 \times A_{663} - 2.69 \times A_{645}) / 1000\ FW`] \times V$
Chlorophyll b (mg g^{-1}) =
$[(22.9 \times A_{645} - 4.68 \times A_{663}) / 1000\ FW] \times V$

Total chlorophyll (mg g^{-1}) =

$$[(8.02 \times A_{663} - 20.2\ A_{64}) / 1000\ FW] \times V$$

The carotenoids concentrations were determined by the equations of Hendry and Grime, (1993) for qualification of:

Carotenoids =

$$\frac{(A_{480} + 0.114\ A_{663}) - (0.638\ A_{645}) \times V}{112.5 \times FW}$$

Where:

V = volume of sample (mL)
A = absorbance
FW = fresh weight of leaf.

Growth and Yield parameters:

Ten soybean plants were collected by random samples from the inner rows at harvest stage of each plot to determine growth characters *viz*., plant height, number of branches) and yield parameters *viz*., number of pods / plant, seeds of weight / plant, weight of one hundred seeds and yield seed (Kg/feddan).

Seed quality:

Determination of total protein, total carbohydrate and total oil contents:

The dry seed samples were examined for total protein content according to the methods described in AOAC, (2000) total carbohydrate contents were determined by the phosphomolybdic acid method according to AOAC International (1990) and using petroleum ether in a Soxhlet instrument after extraction described method by 920.85 AOAC (1990) the total oil content was calculated gravimetrically and presented as dry matter (%).

Determination of chemical content:

The produced soybean seeds determined the amounts of nitrogen (N), phosphorus (P), and potassium (K) were measured according to Chapman and Pratt (1961) seed samples were wet digested in a solution of sulphuric and perchloric acids to assess the nutrients in aliquots. The Kjeldahl method was used to calculate nitrogen (Jackson, 1973). The stannous chloride decreased molybdo-phosphoric blue colour method was used to measure phosphorus (Jackson, 1973). Flame photometry was used to calculate potassium (Jackson, 1973).

Statistical analysis:

Using statistical analysis of completely randomised design (CRD) and randomised block design (RBD), in both the greenhouse conditions and the field conditions respectively. The data obtained was placed through computer statistical software (ASSISTAT) developed by (Silva and de Azevedo, 2009). ANOVA was used to analyse the data, and the least significant difference (LSD) test was used to compare mean values at the level of $P \leq 0.05$.

RESULTS

Isolation and identification of *P. gregata*: -

It has been found that eight fungal isolates of *Phialophora gregata* (Allington & Chamberlain) Gams. were isolated from rotted soybean roots and stems showing typically symptoms of brown stem rot disease from eight localities in different governorates, Egypt. *P. gregata* isolate representing each location to be using it for further studies.

Pathogenicity test:

Data in Fig. (1) show the values of pathogenicity test of eight *P. gregata* isolates on soybean plant cv. Giza 22, under greenhouse conditions by using the injection method which was recorded as percentage of internal stem discoloration (PISD). The results show that the isolates of *P. gregata* from Minia governorate were the highly pathogenic isolates, which were isolated from in four counties (Mallawi, Abo-Korkas, Maghagha and Samalout). The highest significant increase was recorded due to using the isolate from Mallawi, Minia governorate However, the lowest values were recorded by using the isolates of Beheira governorate especially in (Etai El-Baroud and Nubaria), while the isolates of Beni-Suef governorate, showed moderate activity in two regions (Sides and Biba). Depending on all the previous results of pathogenicity test the Mallawi isolate was selected for further studies because it was the most aggressive isolate among the isolated eight *P. gregata.*

Laboratory results (*in vitro* assay: Effect of antagonistic biotic and a biotic inducer on mycelia growth of *P. gregata* (direct confrontation):

Effect of the antagonistic activity of biotic, abiotic inducers individually and or their combinations on the mycelia radial growth of *P. gregata* compared with Topsin M-70% WP was studied *in vitro* as shown in Fig. (2).

Generally, all treatments showed some sort of antagonistic activity against the tested pathogenic fungus. Topsin M-70% WP prevented the growth of the pathogen. With regard to biocontrol agents, *B. subtilis* showed the best effect as compared with *T. harzianum*. In regard to the results of combining biocontrol agents and chemical inducers, the combination between *B. subtilis* and SA showed a highest effective on inhibition the pathogen mycelia growth more than other treatments, being 82.2 % reduction. Moreover,

the *B. subtilis* interacted with humic acid came in second order, being given 78.8% reduction. Also, using *T. harzianum* with any chemical inducers increased the efficiency of the bioagent in inhibiting the pathogen linear growth. In this respect, HA was the most effective followed by SA while HQ came at the end. On the other side, among the three-chemical inducers, SA was the most effective against *P. gregata* linear growth inhibition 62.2%, followed by HA 56.6%.

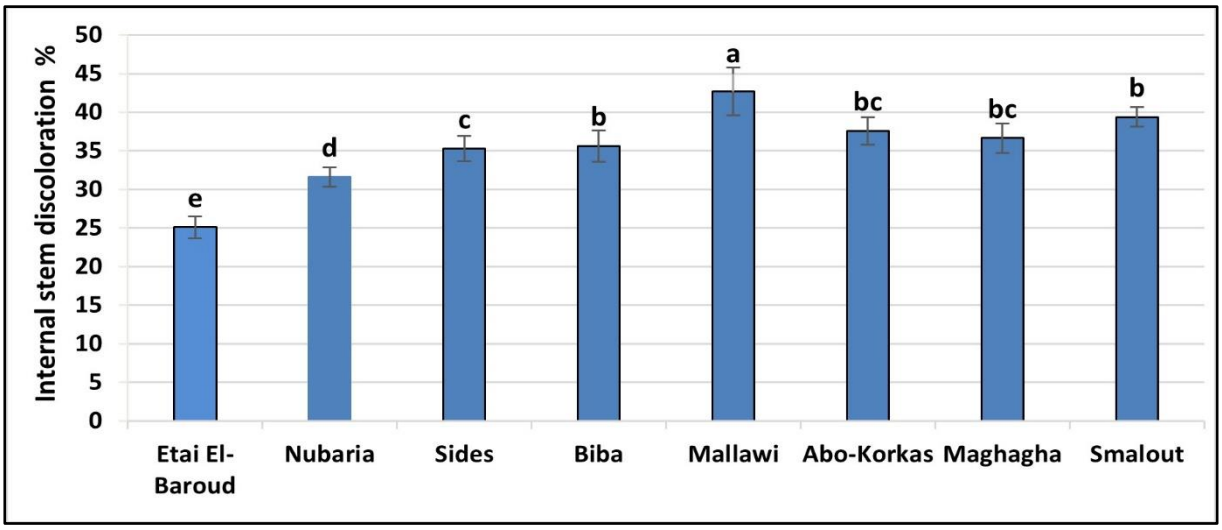

Figure (1): Pathogenicity test of eight isolates on soybean plant cv. Giza 22, percentage of internal stem discoloration (PISD). Significant treatment differences are indicated by different letters $p \leq 0.05$.

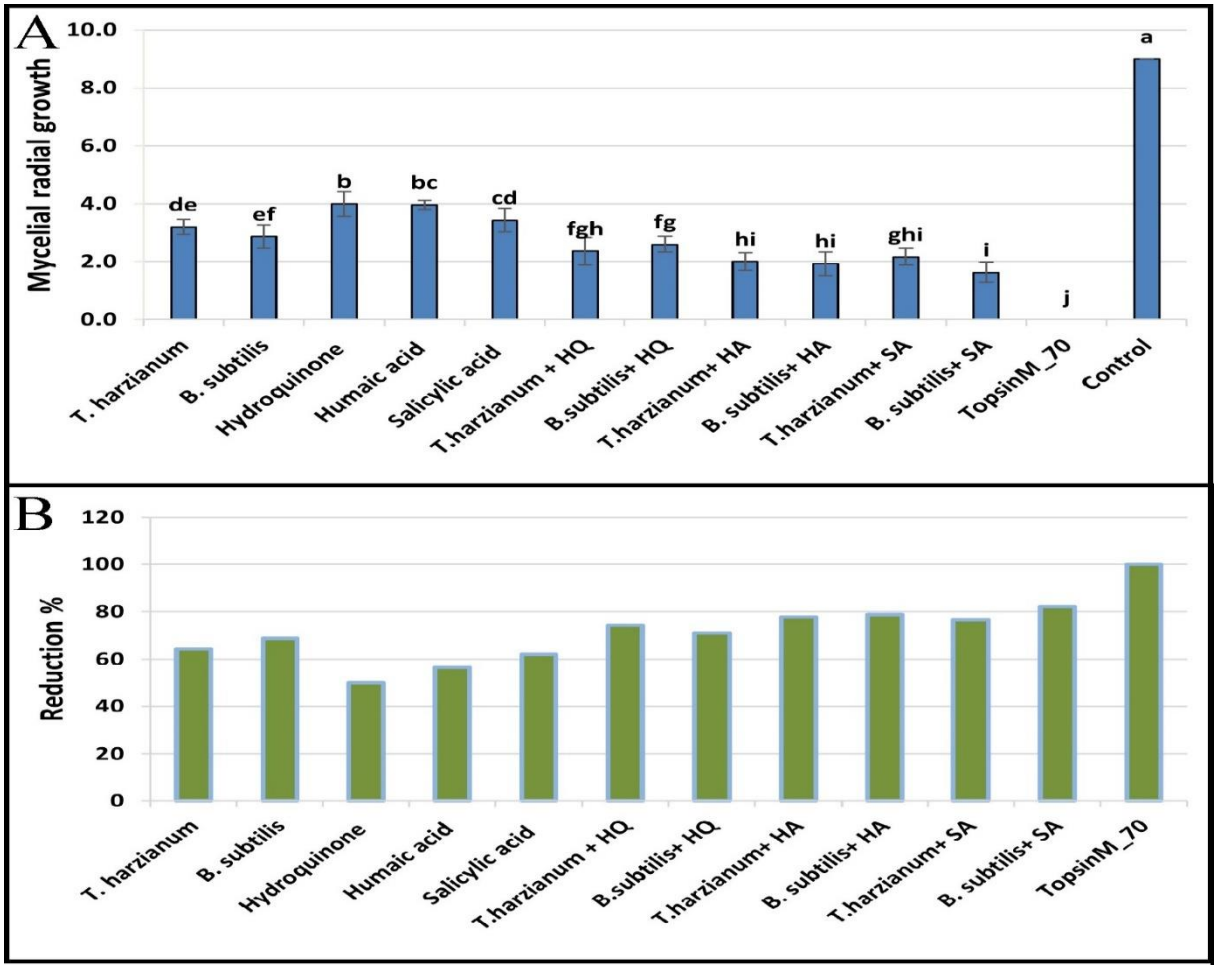

Figure (2): Effect of biotic and a biotic inducer of resistance on mycelial radial growth of *P. gregata* on PDA medium. Whereas A, mycelia growth and B, reduction of mycelium growth. Significant treatment differences are indicated by different letters at $p \leq 0.05$.

Greenhouse experiments:

Effect of biotic and a biotic inducer on percentage of internal stem discoloration (PISD) of soybean:

The impact of biotic and a biotic inducer and their combinations as seed soaking on disease incidence of brown stem rot in soybean plant was evaluated in pot experiment using the spores suspension of the most aggressive isolate of *P. gregata* (Mallawi isolate) based on pathogenicity test and injected into the soybean stems under greenhouse conditions. The cultures were grown, and spores were harvested and suspended in 1.2% water then the soybean stems were injected with the suspension. Data present Fig. (3) shows the measurements of discoloration inside the vascular tissue system and the damage to the amount of tissue in soybean plants. Determine the severity of the stems as a percentage using the ratio of length discoloration to total plant height.

The results in Fig. (3) show that all tested bioagents, inducers, individually and/or their combinations and Topsin M-70% WP treatment significantly reduced the percentage of internal stem discoloration (PISD) as compared with check treatment (infested control). The highest percentage of brown stem rot infection being 48.63%, was show in soyabean plants injected with the spore suspension (infested control). While the lowest disease incidence 6.37% was observed by using *B. subtilis* interacted with SA followed by the dual combination of *T. harzianum* + SA then Topsin M-70 % WP 8.17%. Taking into consideration that, the individual treatment of *T. harzianum* fungus was better than the bacterial strain *B. subtilis* in decreasing brown stem rot disease incidence were recorded 12.61% compared to 16.36%. Regarding the effect of chemical inducer, SA had the highest significant decrease of PISD giving 21.56% followed by HQ with recorded 25.33% then HA 27.30%. Generally, adding any chemical inducers to bioagent treatment led to an increase in the effectiveness against pathogen infection. SA was the more effective in this respect followed by HA. While HQ came late.

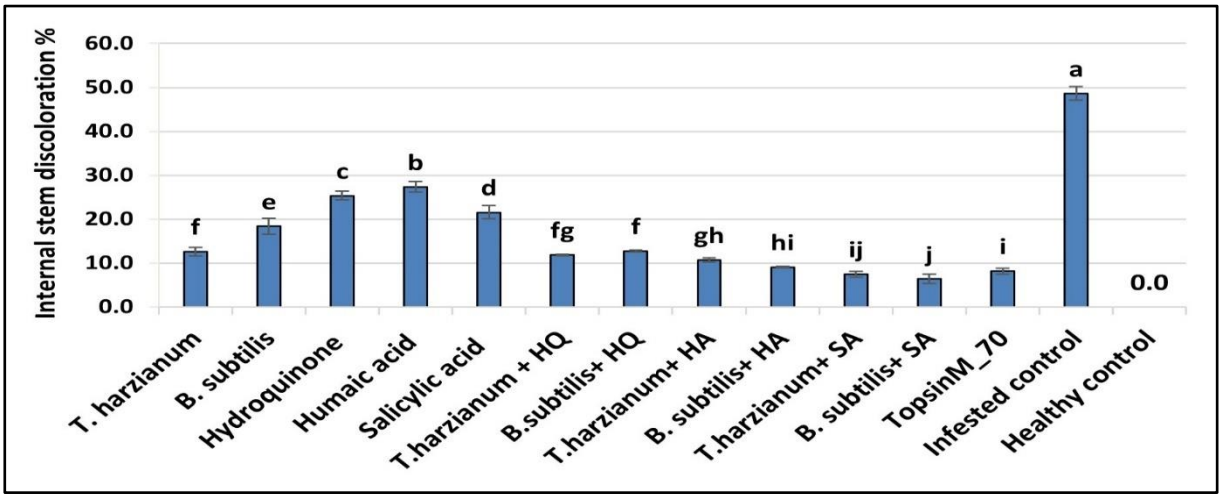

Figure (3): Effect of biotic and a biotic inducer on percentage of internal stem discoloration (PISD) of soybean under greenhouse conditions. Significant treatment differences are indicated by different letters $p \leq 0.05$.

Field trials:

Brown stem rot disease incidence:

Data of brown stem rot disease incidence of soyabean plants as affected by bio agents, inducers and their combinations under natural infection during summer growing seasons 2020 and 2021 in open field at Etai El-Baroud are illustrated in Fig. (4).

Soaking of soybean seeds with all tested bioagents, chemical inducers and their combinations significantly decreased the percentage of internal stem discoloration in both seasons compared with non- treated control. Generally, application of combination treatments gave the best protection against brown stem rot compared with individual treatments. The highest reduction in this parameter occurred due to combination of *B. subtilis* with SA followed by the combination *T. harzianum* + SA which recorded 2.64% and 2.93 % in the first season and 2.43% in the second one, respectively. It is worth mentioning that the fungicide came in the fourth order in the first season giving 3.42% and in the third order in the second season recording 3.44%. *Bacillus subtilis* was the least effective than the fungus *T. harzianum* under field conditions.

With respect to the chemical inducers effect SA was more effective in both seasons giving 4.54% and 5.50%, respectively. Humic acid came in the second order in the first season while HQ was better than him in the second season.

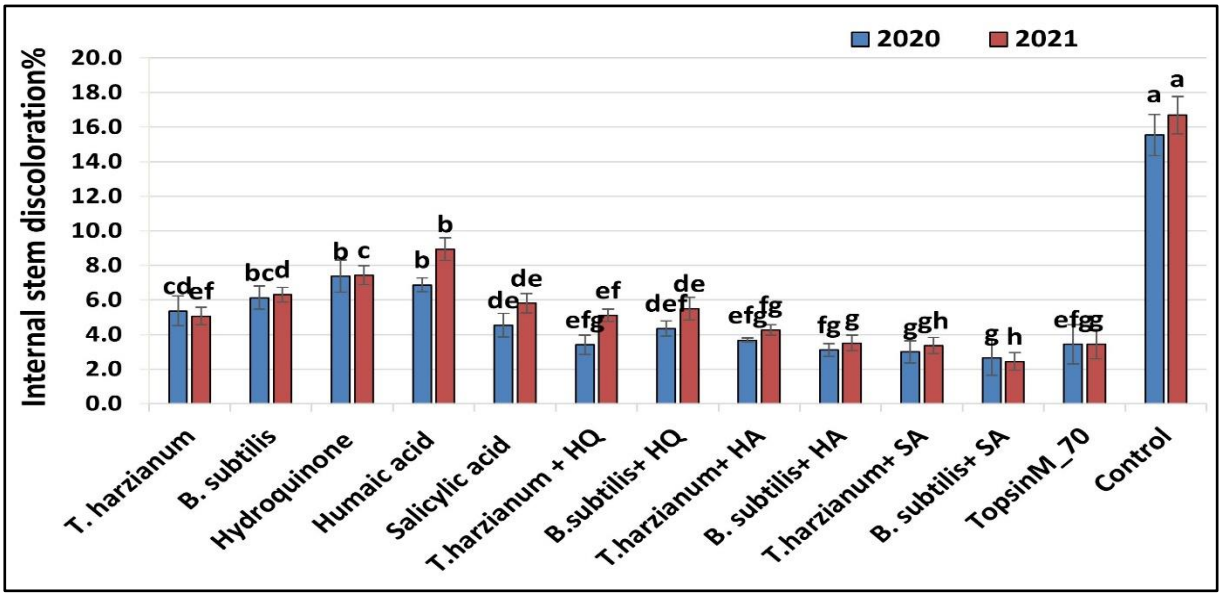

Figure (4): Effect of biotic and a biotic inducer on percentage of internal stem discoloration (PISD) of soybean plants under field experiment at Etai EL-Baroud during summer growing seasons 2020 and 2021. Significant treatment differences are indicated by different letters $p \leq 0.05$.

Analysis of rhizospheric soil dehydrogenase activity (DHA):

As shown in Fig. (5) the impact of individual biocontrol agents and chemical inducers, as well as combinations of these agents. Enzyme activity of dehydrogenase (DHA) in the rhizosphere soil of soybean plants in Etai El-Baroud during the summer growing season of 2021. The enzyme activities in the rhizospheric soil of the uninoculated soybean plants were significantly suppressed by the control treatment, as demonstrated by the results. Surprisingly, the DHA activity in the soil of *B. subtilis* + HA treated plants increased to record the highest activity among the other treatments (137 g TPF/g soil/day), while the activity of the same enzyme was only marginally increased in *T. harzianum* + SA inoculated plants (122 gTPF/g soil/day) compared to their un-inoculated counterparts (61 g TPF/g soil/day). While inoculation with *T. harzianum* + HA and *B. subtilis* + SA promoted the DHA activity by 82%, for each treatment compared with the un-inoculated treatment.

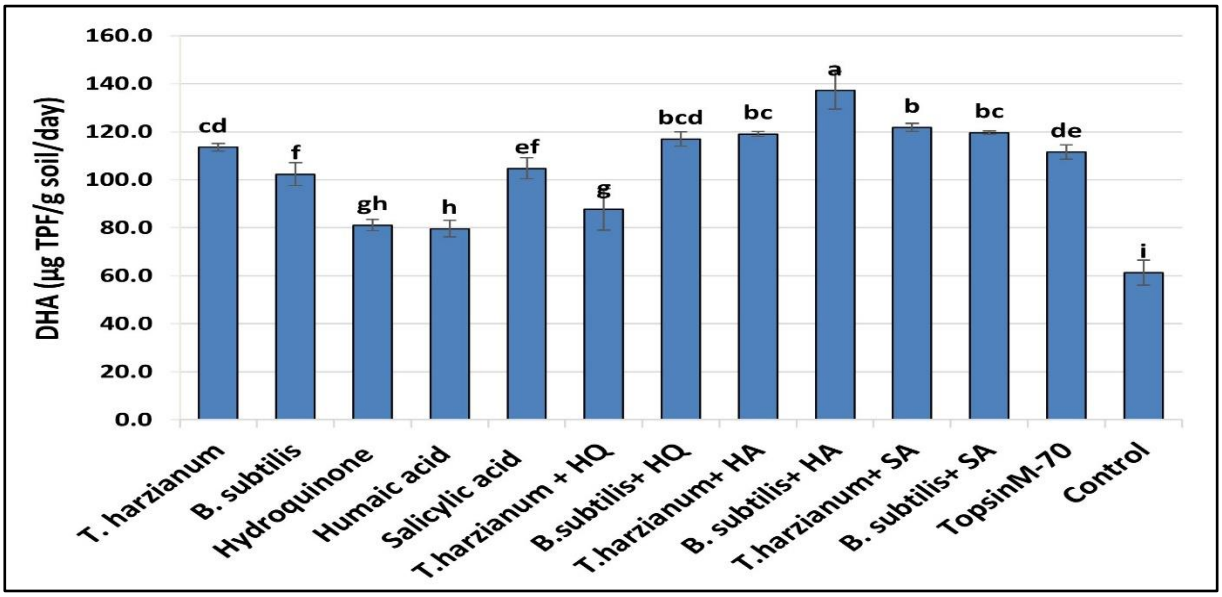

Figure (5): Effect of biotic and a biotic inducer on the activities of soil dehydrogenase (µg TPF/g soil/day) in the rhizosphere of soybean plants under field conditions at Etai El-Baroud during summer growing season 2021. Significant treatment differences are indicated by different letters $p \leq 0.05$.

Determination of total phenol and photosynthetic pigments contents:

The contents of total phenol and photosynthetic pigments in soybean leaves of the un-inoculated and inoculated plants grown under field experiment at Etai El-Baroud during summer growing season 2021 are presented in Fig. (6) and Table (2). As shown from the results illustrated in Fig. 6, in all treated soybean plants the total phenol contents significantly increased compared to the untreated control treatment 0.75 mg/g FW.

Salicylic acid and *T. harzianum* + HQ followed by Topsin M-70, *T. harzianum* + HA, *T. harzianum* + SA, *B. subtilis* + HQ, *B. subtilis* + HA, *B. subtilis* + SA, *T. harzianum*, being 1.74, 1.42, 1.35, 1.32, 1.28, 1.19, 1.11, 1.04 and 1.00 (mg/g FW), respectively, whereas the lowest value of phenol content was recorded in, Humic acid, Hydroquinone and *B. subtilis* being 0.99, 0.88 and 0.79 mg/g FW, respectively compared to the untreated control treatment 0.75 mg/g FW**.**

Photosynthetic pigments as response of seed inoculation compared with un-inoculated 60-days-old soybean plants are presented in Table (2). As shown from the results, the contents of chlorophyll a, chlorophyll b and carotenoids were significantly reduced in response to disease in control treatment. Where, the leaves of the un-inoculated plants had the least amounts of chlorophyll a, chlorophyll b and carotenoids, which were 0.50, 0.44 and 0.14 mg/g FW, respectively. The inoculation with *T. harzianum* + HA, *T. harzianum* + HQ significantly reversed the negative effects of disease; where the recorded increase in of chlorophyll a were 2.7-fold and 2-fold, respectively, in chlorophyll b it was about 1.6-fold and 1.4-fold, in carotenoids it was 3-fold and 2.6-fold, respectively, compared with the un-inoculated plants. Meanwhile, the *B. subtilis* + SA significantly affected carotenoids, chlorophyll a, and chlorophyll b, where it increased them by 1.7-fold, 1.9-fold, and 4-fold, respectively compared with un-inoculated ones. However, the different biocontrol agents and chemical inducers in all inoculation treatments differed significantly from one another.

Table (2): Effect of biotic and abiotic inducer on pigment content (mg/g FW) of leaves of soybean grown under field experiment at Etai El-Baroud during summer growing season 2021.

Treatments	*Ch. a	*Ch. b	** Ca
T. harzianum	0.81cde	0.98 a	0.64 a
B. subtilis	0.63 fg	0.48 f	0.15 g
Hydroquinone (HQ)	0.94 bc	0.52 ef	0.33 de
Humic acid (HA)	0.84 cde	0.61 de	0.31 ef
Salicylic acid (SA)	0.72 ef	0.60 de	0.26 f
T. harzianum + HQ	1.01 b	0.68 cd	0.36 de
B. subtilis + HQ	0.90 bcd	0.60 de	0.38 d
T. harzianum + HA	1.35 a	0.69 cd	0.47 c
B. subtilis+ HA	0.80 cde	0.71 c	0.60 ab
T. harzianum + SA	0.76 def	0.77 bc	0.51 c
B. subtilis + SA	0.87 bcde	0.83 b	0.60 ab
Topsin M-70	0.80 cdef	0.80 b	0.57 b
Control	0.50 g	0.44 f	0.14 g

* Chlorophyll, ** Carotenoids

Significant treatment differences are indicated by different letters at $p \leq 0.05$.

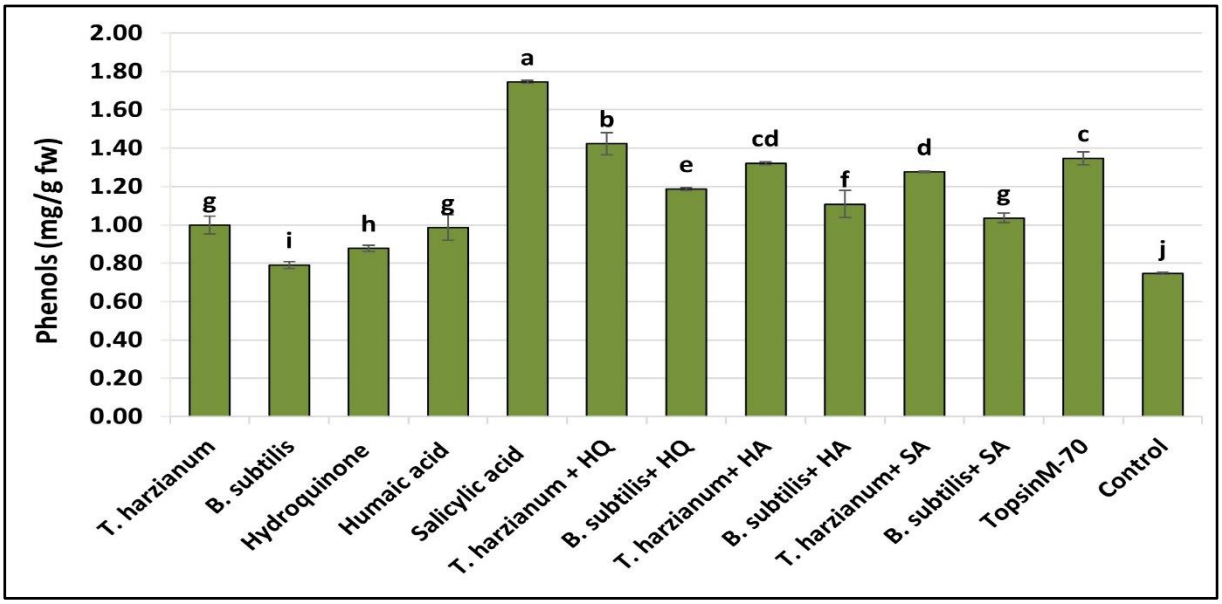

Figure (6): Effect of biotic and a biotic inducer on total phenol content (mg/g FW) of leaves of soybean grown under field experiment at Etai El-Baroud during summer growing season 2021. Significant treatment differences are indicated by different letters $p \leq 0.05$.

Soybean growth, productivity and yield:

The results show that Table (3) demonstrates that there were significant differences between the tested biotic and a biotic inducer and their combination regarding soybean growth parameters and yield components. It was observed that the reduction in disease assessment reflected in plant growth, productivity and yield. All individual and combined application of treatment as well as fungicide Topsin M-70 % WP increased significantly soybean growth parameters *viz.* plant height and branches number constituently increasing the productivity and seed yield. In this respect, the combination between *B. subtilis* and SA gave the highest values of growth parameters compared with other treatments. The application of *T. harzianum* and SA came in the second order followed by *B. subtilis* + HA in increasing of plant height, while *T. harzianum* + HA came next in branches number. Concerning the effects of chemical inducers, HA followed by HQ was better than SA on plant height. Among bioagents, *T. harzianum* was more effective than *B. subtilis* in increasing growth parameters in both seasons.

Data concerning yield and its components, it can be easily noticed that the highest values of pods number/ plant occurred under *B. subtilis* interacted with HQ followed by the individual treatment of HQ the dual combination of *T. harzianum* +HQ. Among bioagents, *B. subtilis* was more than *T. harzianum.* While, of chemical inducers, HQ was better than SA, but HA came late. Application of *T. harzianum* + HQ recorded the highest plant seed number in the first season followed by *B. subtilis* + HQ then SA alone. Moreover, in the second season SA came first followed by *B. subtilis* + HQ then *B. subtilis* +HA. In both seasons the bacterial addition was more effective than the fungal addition in increasing pods and seeds number. Generally, all applications of bioagents, chemical inducers and their combinations increased significantly yield parameters *viz*. 100- seed weight, plant seed wight and seed yield /plant in both seasons compared with control. The combination treatment was more effective than individual treatments.

Seed quality:

Determination of total protein, carbohydrate and oil contents:

The effect of biocontrol agents and chemical inducers on protein content, total soluble carbohydrate and total oil of soybean plants grown under field conditions at Etai El-Baroud during summer growing season of 2021 are illustrated in Fig. 7 (A-B-C). The amounts of protein in the yielded seeds of the un-inoculated and inoculated soybean grown under field conditions as response of bio control agents and chemical inducers are illustrated in Fig. (7A) there was often a considerable increase in seed oil content and a decrease in seed protein content compared to the untreated control. Protein and oil had a negatively correlation with one another. The highest increase of the total protein was recorded in the *T. harzianum* + HA inoculated seeds recorded 20% relative to the un-inoculated ones. Likewise, the inoculated treatments with *T. harzianum* + HQ, *B. subtilis* + HQ and *B. subtilis* + HA had higher protein content while the protein contents were less affected among other inoculated treatments, compared with un-inoculated counterparts.

The biocontrol agents and chemical inducers inoculation notably increased the accumulation of carbohydrates in the seeds of soybean without significant difference among the treatments. *B. subtilis* + HQ, *B. subtilis* + HA and *T. harzianum* + HQ were recorded as the highest of oil content compared the control.

Similarly, the biocontrol agents and chemical inducers inoculation notably increased the accumulation of carbohydrates in the seeds of soybean without significant difference among the treatments. Whereas the inoculated and treated *T. harzianum* seeds had lower accumulation of the total carbohydrate content which was 24.7%. However, seed inoculation with biocontrol agents and chemical inducers slightly raised the amount of total carbohydrates in seed yield compared with the un-inoculated counterparts. The least amount of total carbohydrates was recorded in the control, which was 22.7%.

Determination of chemical contents:

The effects of seed inoculation with biotic and a biotic inducer on nitrogen (N), phosphorus (P), as well as potassium (K) contents in yielded seeds of soybean grown under natural infection in field conditions at Etai El-Baroud during summer growing season 2021 are illustrated in Fig. (8). In the case of nitrogen, the lowest values occurred under control treatment 2.06%. Meanwhile, relative to the un-inoculated samples, biocontrol agents and chemical inducers inoculation resulted in an increase in the nitrogen content, where the inoculation with *B. subtilis* + HA had the highest increase of nitrogen by 73%. The phosphorus contents in yielded seeds were 0.32% in control, however, the inoculation with *B. subtilis* + SA and *B. subtilis* + HA resulted in a significant increase in the phosphorus contents by 53% in respect to the un-inoculated counterparts.

Potassium contents of yielded seeds had a decrease in control samples, which was 0.12%, while biocontrol agents and chemical inducers inoculation had an increase in the potassium content of seeds compared with the un-inoculated ones. The highest increase in K content was recorded in HA-inoculated seeds by 50%, compared with un-inoculated counterpart. Moreover, biocontrol agents and chemical inducers inoculation as well as control had no significant difference in potassium contents in yielded seeds among each other.

Table (3): Effect of biotic and abiotic inducer on growth parameters of soybean plants under field conditions at Etai El-Baroud during summer growing seasons 2020 and2021.

Treatment	Plant height (cm)	Branches number /Plant	No. of pods/ plant	100 seed weight (g)	seed weight /Plant (g)	Seed yield Kg /feddan
	Season 2020					
T. harzianum	65.2	3.5	80.8	17.83	40.7	1565.7
B. subtilis	66.8	3.3	84.2	18.13	43.5	1647.6
Hydroquinone (HQ)	69.8	3.6	114.4	19.80	45.0	1723.6
Humic acid (HA)	73.5	2.8	99.7	19.00	41.1	1565.2
Salicylic acid (SA)	65.1	2.8	110.1	18.75	42.5	1645.6
T. harzianum + HQ	70.6	3.2	117.0	20.03	52.6	1822.5
B. subtilis + HQ	68.4	3.8	119.5	21.52	55.3	1875.6
T. harzianum + HA	69.8	3.9	114.8	18.04	47.8	1680.5
B. subtilis + HA	75.7	3.1	116.7	19.76	55.2	1750.4
T. harzianum + SA	86.8	3.8	106.7	19.33	49.9	1690.6
B. subtilis + SA	89.1	4.0	113.4	20.29	52.5	1702.7
TopsinM-70	89.1	3.7	112.0	19.48	49.4	1680.75
Control	56.8	2.5	75.1	15.95	38.7	1430.23
	Season 2021					
T. harzianum	69.4	2.5	85.5	18.90	41.8	1670.4
B. subtilis	61.5	2.7	90.8	18.86	44.5	1770.0
Hydroquinone (HQ)	63.8	3.3	119.8	20.95	48.7	1830.6
Humic acid (HA)	67.8	2.7	97.7	20.01	45.90	1650.5
Salicylic acid (SA)	60.1	3.0	115.7	19.45	47.50	1695.6
T. harzianum + HQ	62.6	3.2	122.0	21.56	55.60	1920.0
B. subtilis + HQ	72.7	3.5	124.5	22.06	59.00	1975.5
T. harzianum + HA	72.9	3.1	113.7	19.50	48.90	1710.7
B. subtilis + HA	68.5	2.7	116.5	20.65	58.50	1898.5
T. harzianum + SA	81.5	3.9	110.1	19.80	54.30	1750.5
B. subtilis + SA	80.4	3.8	118.1	21.64	57.10	1800.5
TopsinM-70	84.9	2.7	115.3	20.85	55.10	1776.9
Control	61.8	2.1	75.9	16.61	39.40	1475.6
	Mean					
T. harzianum	67.3	3.0	83.2	18.36	41.25	1618.0
B. subtilis	64.2	3.0	87.5	18.49	44.01	1708.8
Hydroquinone (HQ)	66.8	3.5	117.1	20.37	46.85	1495.0
Humic acid (HA)	70.7	2.8	98.7	20.60	43.30	1607.8
Salicylic acid (SA)	62.6	2.9	112.9	20.34	45.60	1670.6
T. harzianum + HQ	66.6	3.2	119.5	20.79	54.08	1871.2
B. subtilis + HQ	70.6	3.7	122.0	21.29	57.16	1925.5
T. harzianum + HA	71.4	3.5	114.3	19.16	48.35	1695.6
B. subtilis + HA	72.1	2.9	116.6	20.20	57.16	1824.4
T. harzianum + SA	84.2	3.9	108.4	20.56	50.45	1720.5
B. subtilis + SA	84.8	3.9	115.8	21.46	54.80	1753.1
TopsinM-70	87.0	3.2	113.7	20.16	52.25	1698.8
Control	59.3	2.3	75.5	16.28	39.03	1453.0
L.S.D.5%						
Treatments (T)	1.54	0.09	0.51	0.49	0.70	41.17
Seasons (S)	6.38	0.56	1.00	1.26	1.79	70.34
T × S	5.67	0.32	1.84	1.78	2.53	148.45

Significant treatment differences are indicated by different letters $p \leq 0.05$.

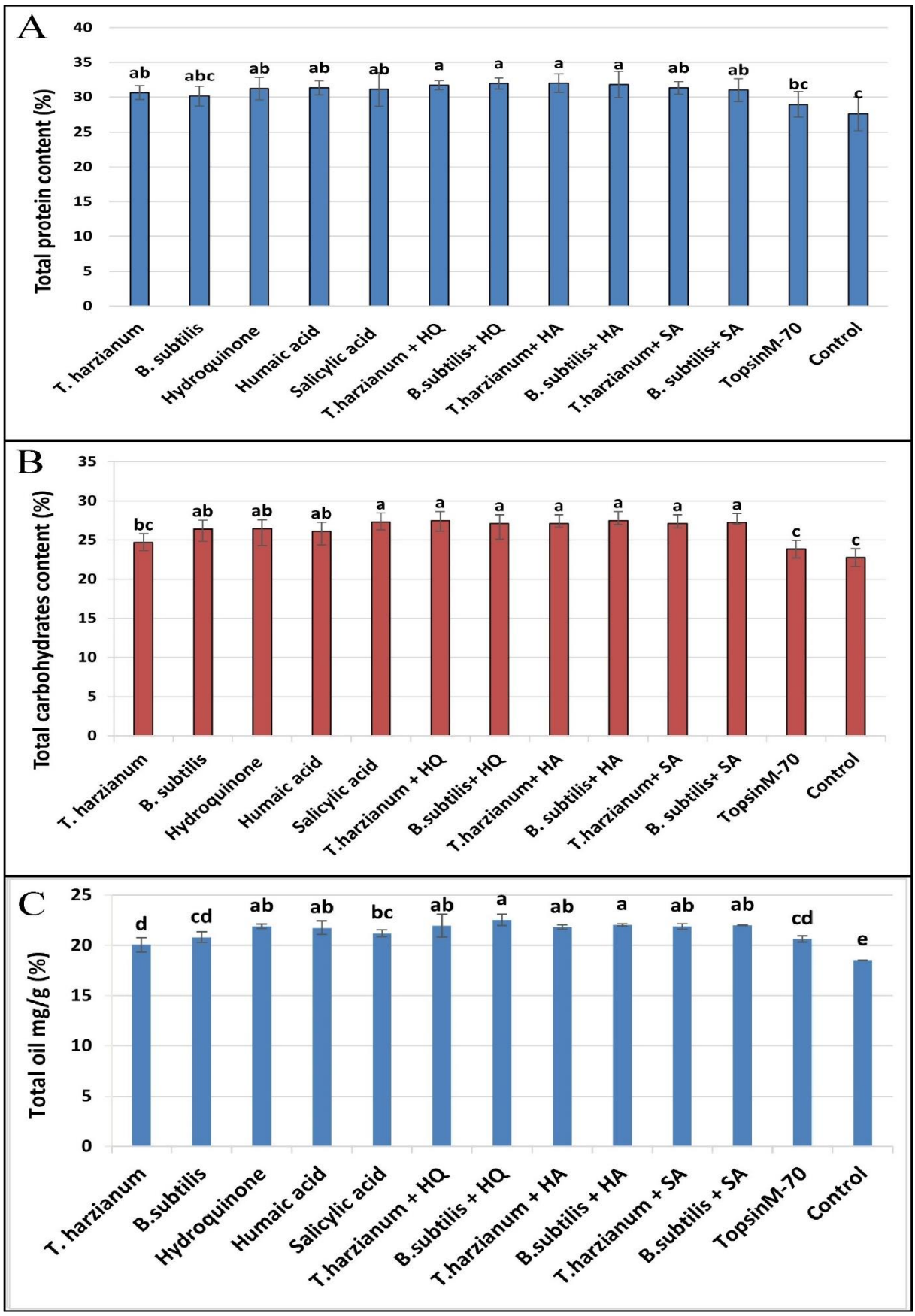

Figure (7): Effect of biotic and a biotic inducer inducer on total protein, carbohydrate contents and total oil (%) of yielded seeds of soybean grown under field conditions at Etai El-Baroud during summer growing season 2021. Whereas A is total protein, B, total carbohydrate and C, total oil. Significant treatment differences are indicated by different letters $p \leq 0.05$.

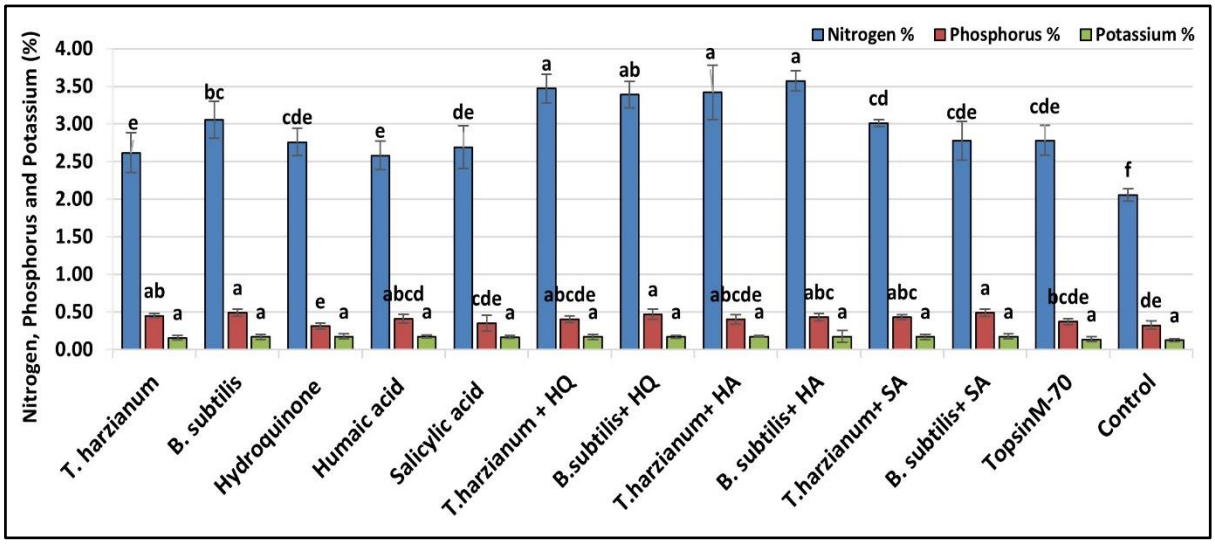

Figure (8): Effect of biotic and a biotic inducer on chemical contents (N, P and K %) of yielded seeds of soybean grown under field conditions at Etai El-Baroud during summer growing season 2021. Significant treatment differences are indicated by different letters $p \leq 0.05$.

DISCUSSION

Phialophora gregata, the causal pathogen of brown stem rot disease was obtained from roots, stem base, stem and soybean straw and seeds of infected plants Abo-El-Dahab (1968), Lai (1968), Sayed-Ahmed (1988) and Eathington *et al*. (1993). Moreover, *P. gregata* is serious pathogen that attack another hosts (adzuki bean and mung bean) Kobayashi *et al*. (1991), Gray and Pataky (1994) and Bahaa El-Din (2013) found that isolation from soybean stems collected from the surveyed governorates indicated that *P. gregata* was the most frequent fungi.

In the present investigation, using identifying keys, *P. gregata* was purified and identified, and a tested isolate has the potential to attack roots of soyabean Giza 22. Pathogenicity tests revealed that every cause is discoloration of internal stem tissues and foliar symptoms of wilt, interveinal chlorosis (yellowing between leaf veins) and necrosis, a reduction of healthy plants. All characteristics of brown stem rot were revealed by Abo El-Dahab (1968), Abd El-Al (1999), Grau *et al*. (2004) and Malvick and Impullitti (2007).

This study shows the possibilities of reducing brown stem rot disease infection in soybean utilizing biotic (*T. harzianum* and *B. subtilis*) and abiotic inducers (SA, HA and HQ) individually and/or in combination, *in vitro* findings demonstrated that both biocontrol agents and chemical inducers, separately and/or in combination, reduced the mycelia growth of the tested pathogenic fungus by varying degrees. The mixture of biotic and a biotic inducer inhibited the mycelia growth than using each alone especially in case of *T. harzianum* + HA and *B. subtilis* + SA. These results are in agreement with those reported by Raaijmakers *et al*., (2002). They reported *Trichoderma* produces some toxic metabolites against phytopathogens. Including antibiotics, massoilactone, gliovirin, viridian, heptelidic acid, alamethicins, harzianic acid, peptaibols, glisoprenins, tricholin, and 6-penthyl-α-pyrone. Moreover, Meena, (2014) explained the bio-control mechanisms of *T. harzianum* and *B. subtilis* to inhibition the linear growth of pathogens is due to different mechanisms, such as hyphal interactions, mycoparsitism, enzyme secretion, antibiosis and competition. On the other side, according to Giridhar and Reddy (2001) found that the use antioxidants may reduce and subsequently alter the membrane permeability and oxygen tension in fungus-containing media, which causes mycotoxin production which increase in the medium. The oxidation of membrane lipids, which interferes with many enzyme functions, including protein, DNA and RNA synthesis, may also be a contributing factor to the antioxidant's antimicrobial effects (Nesci *et al*., 2003). Additionally, according to Abdel-Monaim (2013) and Sarhan *et al*. (2018) whom reported that *T. viride* and salicylic acid, either separately or together, prevented pathogenic fungi from growing and attacking the roots of faba bean or common bean and Zian *et al*. (2019) showed that the biocontrol efficiency of antagonists *T. harzianum* and *B. subtilis* may be stimulated by SA and HQ resulting in a significant increase in their population density and antagonistic effect against the tested pathogens.

The mechanism of bio-agent *viz.* pathogens during different stages of interactions, it can be summarized in the following stages:

First stage: An antagonistic fungus is drawn to a pathogenic fungus by its chemical stimulus, which causes the antagonist to respond chemotropically.

Second stage: Lectins help distinguish between the pathogen and bioagents.

Third stage: the process of mycoparasitism of the antagonist (*Trichoderma*) hyphae either such as chitinase, glucanase, and pectinase and grow alongside the host hyphae or coil around them and secrete various lytic enzymes. This is followed by the interactions between the pathogen and antagonist mycelium. Reithner *et al*. (2011). Moreover (Harman, 2006) reported that three methods of different mechanisms of *Trichoderma* spp. that inhibit fungal growth through first, competition for nutrients and space; second, parasitism (using the host's nutrients), and third, antibiosis, which involves the production of an antibiotic or inhibitory metabolite.

The activity of bacteria against fungal pathogens may be due to producing antibodies and siderophores which inhibit fungal growth Sarhan *et al*. (2018). In addition, it has presented several solutions. By stimulating the formation of increased phytoalexin amounts in the plant and raising the activity of increased lytic enzymes, *Bacillus subtilis* can help plants become resistant to disease. Sailaja and Podile (1998). Moreover, it was discovered that *B. subtilis*, *B. cereus*, *B. pumilus* and *B. amyloliquefaciens* were antagonistic to *F. solani in vivo* Ajilogba *et al*. (2013).

In addition, all treatments significantly reduced the infection of the brown stem rot disease *in viv*o or in the field tested compared to the control treatments. The percentage of internal stem discoloration (PISD) was reduced more effectively using combinations of biotic and a biotic inducer than when each was used alone. The best results in this study were obtained when HA and/or SA were combined with *T. harzianum* and *B. subtilis*. These findings are in accordance with those of Nafie and Mazen. (2008) and Bahaa El-Din (2013).

In field experiments, the application of biotic and a biotic inducer alone or in combination as seed soaking of soybean in two summer growing seasons of 2020 and 2021 at Etai El-Bourd Res. Stations Farms showed a significant increase in soybean growth parameters and component yield to produce a good seed quality. In comparison to applying each agent alone, the use of biocontrol agents and chemical combination a biotic inducer resulted in significant improvements in all growth characteristics, yield parameters, and seed quality. *T. harzianum* + HA and *B. subtilis*+ HA were the most efficient. The increment in soybean growth and productivity may be back to the role of these applications on controlling brown stem rot disease and enhancing total phenol and photosynthetic pigments. Photosynthetic pigments are the best indicator reflecting in plant healthy as well as carotenoids content had antioxidants content. Any factor that triggers a rise in photosynthetic pigments will also increase the amount of carbohydrates in plant organs, which include structural polysaccharides like pectin that serve as a barrier against pathogen invasion Hamideh *et al*. (2013). In their crucial role as antioxidants, carotenoids quench (deactivate) singlet oxygen, an oxidant created during photosynthesis Halliwell and Gutteridge (1999). Under some circumstances, carotenoids can also prevent the oxidation of fats (lipid peroxidation) Nafie and Mazen, (2008). Moreover, there is a correlation between photosynthesis and seed protein concentration because it causes sucrose to go from leaves to seeds, where it is converted to protein precursors Smith *et al*., (1989). Total phenol is considered to be an antifungal substance, and when it rapidly accumulates at the site of the infection, it stops or slows the pathogen growth Lamba *et al*. (2008). According to Farkas and Kiraaly (1962), phenols content is oxidized to produce quinones or semi-quinones, which are more toxic and have a significant impact on the fungal pathogens as antimicrobial agents. Furthermore to, *Trichoderma* produces an increase in the number and biomass of nodules, which in turn promotes an increase in growth and production and also causes an increase in root size and root depth Adams and Lynch (2007). According to Dwivedi *et al.* (2009), some bacterial strains produce antifungal substances such phenazine and di-acetyl-phloroglucinol (DAPG), which have a good impact on mycorrhizal colonisation and soybean growth characteristics.

Shahda (2000) explained the mode of action of antioxidants in one or more of the following mechanisms: A. Scavenger activity of free radicals, B. Quencher activity of singlet excited states, C. Chelation of transition metals and D. In activator/activator activity of enzymes. Moreover, the involvement of salicylic acid in reducing the adverse effects of the growth of pathogens as well as enhance the plant growth and productivity, Soybean seeds soaking in salicylic acid that increased the amounts of

pectin, cellulose, lignin, and phospholipids in either the shoots or the roots as well as the soluble sugars in the roots (Al-Hakimi, 2006) in turn improve overall plant heath and increasing the plant ability to resist the pathogens. El-Mougy (2002) added that SA and hydroquinone were highly toxic to fungal mycelial linear growth and has a direct antifungal activity and induced systemic resistance.

Many sectors of plant production, humic acid (HA) can be used successfully as a soil conditioner or plant growth stimulant to increase natural resistance to plant diseases. It is a suspension based on potassium humates (Scheuerell and Mahaffee, 2004). Results substantially support by Abd El-Kareem, (2007) assumptions that HA acts as a signal to cause systemic resistance, whether directly or indirectly. (Scheuerell and Mahaffee, 2004) found that HA is a potassium humate-based solution that can play a role successfully in many aspects of plant production as a plant growth stimulant or soil conditioner to improve natural resistance against plant pathology and incest and increase plant output.

Generally, these anti-oxidants might control flowering, plant growth, and the development of disease resistance (Dmitrier *et al*., 2003). Antioxidants enhance the phytohormones gibberellins and auxin, give the plant hormones like cytokinins and auxin, and inhibit the enzyme IAA-oxidase, which plays the breakdown of plant growth hormone (El-Bassiony *et al*., 2010).

Results obtained showed that the application of the treatments used an increase in dehydrogenase (DHA) enzyme activities in the rhizospheric soil of soybean plants under field conditions, which leads to an increase in nitrogen fixation by rhizobia bacteria and promoted plant growth and productivity. Rhizobial metabolites may indirectly reduce soil-borne fungi by fostering plant development. Rhizobia greatly improves plant development and yields by reducing soil-borne diseases, according to a number of earlier research Sheikh *et al*., (2006) and Mazen *et al*. (2008).

Therefore, it could be concluding that the combined application of *B. subtilis* and SA as seed treatment after sowing is recommended as safe alternative method for reducing brown stem rot disease as well as improving the productivity and seed quality of soybean plants.

CONCLUSION

From the outcome of our investigation, it is possible to conclude that treatments with biotic and abiotic in combination with seed soaking significantly have been very successful than using each individually effective in decreasing brown stem rot disease caused by *Phialophora gregata*, as well as, improved plant growth, seed yield, and seed quality as total (protein, carbohydrate, oil, nitrogen (N), phosphorus (P), and potassium (K)) contents were increased, meanwhile, the activity of dehydrogenase (DHA) enzymes increasing that useful in the fertilization of the soil. It can be recommended that these biotic and abiotic could be used as antimicrobial agents such as total phenols contents, stimulate photosynthetic pigments can be suggested as a part of usage in the management of brown stem rot disease of soybean at least to decrease the use of fungicides for the health of humans, animals, and the environment.

CONFLICTS OF INTEREST

The author(s) declare no conflict of interest.

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