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Effective Formulations to Improve the Efficiency of Bacterial Inoculum as Biological Control Agents against Some Root Diseases

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ABSTRACT

Fungi that cause root diseases are the most common pathogenic fungal groups, which affect a wide range of plant species and suits different environmental conditions. The most important of these pathogenic fungi are *Rhizoctonia solani* Kühn; *Fusarium semitectum*, and *Macrophomina phaseolina* which isolated from eggplant (*Solanum melongena*). This research was concerned with studying the effectiveness of some endophytic bacterial isolates, isolated from *Medicago sativa*; *Rosmarinus officinalis* and *Vicia faba,* as biological control agents. Bacterial isolates were identified as *Pseudomonas aeruginosa, Pseudomonas alcaligenes,* and *Pseudomonas mendocina* based on 16S rRNA and evaluated for their ability to inhibit these fungi individually or in mixtures under greenhouse and laboratory conditions. Eighteen different formulations of biological control agents were evaluated based on their base and additive combinations. The different bases used were Talk, Diatoms, and Clay, while the additives were Alginate, Arabic gum, and beef extract. The results showed that using of bacterial mixtures against pathogenic fungi was more effective than using each bacterial isolate individually. The most effective bacterial formulations were; 1- Diatoms with Arabic gum and Beef; 2- Clay with Alginate and Beef; 3- Clay with Arabic gum and Beef; 4- Clay with Arabic gum; 5-Talc with Arabic gum and Beef; 6-Talc with Arabic gum. All selected bacterial formulations significantly reduced root rot on Eggplant (*Solanum melongena*) under greenhouse conditions and positively affected the level of total phenolic compounds and total antioxidant activity in tested host plant. The study recommended carrying the tested biological control agents on ecofriendly materials to maintain the effectiveness of bacteria and reduce the incidence and severity of root rot diseases.

Keywords: *Medicago sativa*, *Rhizoctonia solani*, *Fusarium semitectum*, *Macrophomina phaseolina*, biocontrol..

1. Introduction

The fungi that cause root rot are the most widespread plant pathogens around the world, as it suits diversity in environmental conditions, as well as a wide host range of various plant species (Bodah, 2017). Fungi that cause root diseases cause great losses in yield in terms of quantity and quality (Gonzalez, *et al.,* 2010). Root rot represents a great danger to the plants, as its symptoms start from the bottom of the soil and increase in severity depending on the inappropriate environmental conditions. Some root diseases are caused by a single fungus, and sometimes by a group of fungi, resulting in a complex disease. Control by traditional methods for these types of pathogens is sometimes of weak feasibility in reducing infection and losses. As these fungi have a wide host range, some of them can survive in the soil for long periods. There are different fungal root rot pathogens in wide range of plant species, *Rhizoctonia solani* Kühn*; Fusarium semitectum*, and *Macrophomina phaseolina* are the most common root rot causal agents, these pathogens are found in different climate conditions (Lim *et al.,* 2002; Ganesan *et al.,* 2004; Koumoutsi *et al.,* 2004). These pathogens also are frequent diseases of many crops. Fusarium root rot is a common disease in several crops. Symptoms include brown lesions

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that progress to dark black on ground roots and stems, stunting and death. Fusarium species also responsible for a significant reduction in crop production, predominantly on various crops, including wheat, maize, barley, peas and beans and others (Eljounaidi *et al*., 2016). The intensive use of chemical pesticides to plant pathogens control has resulted in negative effects such as the release of toxic material in soil, reduced soil fertility and human health problems. Therefore, environmentally-friendly and sustainable replacement of chemical fertilizers or pesticides is highly challenging. Researchers have recently been interested in searching for sources of biological control of root rot diseases as safe, ecofriendly and cost-effective applications. As the use of biocontrol agents leads to the inhibition of pathogens through different mechanisms, including antagonism, hypo-virulence and induction of host resistance (Junaid *et al.,* 2013; Lugtenberg *et al*., 2015). Researchers have focused their efforts disease management using alternative tools to synthetic pesticides for controlling plant fungal diseases (Muthukumar and Venkatesh 2014). In plant diseases, biological control one of the most effective approaches to the use of microbial antagonists to suppress diseases by different groups of microorganisms. One of these groups is the endophytic bacteria which isolated from healthy plant tissues, and these bacteria are called endophytic bacteria and have no ability to cause any diseases and prove their effectiveness as biological control agents when they are able to inhibit other pathogens (McEvoy *et al.,* 1999; Junaid *et al.,* 2013; Das *et al.,* 2019; Strobel, and Daisy 2003). Several studies have proven that many plant species contain safe endophytic bacteria have the ability to inhibit pathogenic fungi and can be used in biological control. Many endophytic fungi and bacteria can be isolated from different plant species such as alfalfa (*Medicago sativa* L.) Stajković *et al.,* (2009); from Faba bean (Zaghloul *et al*., 2016); from a medical plant *Rosmarinus officinalis* (Abdulhadi *et al.,* 2020). Endophytic bacteria are present in all plant parts, such as leaves (Strobel and Daisy, 2003; Tan *et al.,* 2015), stems (Chung *et al*., 2015; Mao *et al*., 2019), root nodules (Martínez Hidalgo *et al*., 2015), roots (Potshangbam, *et al.,* 2017; Chen *et al*., 2019), rhizomes (Jasim *et al*., 2014) and seeds (Gond *et al*., 2015; Gupta *et al*., 2019). Biocontrol research consisted screening of antagonistic bacteria for their biological effects (Ryu 2013). Some endophytic bacteria also have the ability to induce resistance in host plant (Saijo 2018; Stringlis *et al.,* 2018). One of the potential objects of biological products for plant protection are endophytic strains of bacteria of the genus Pseudomonas spp. Previous studies were discussed the issues of the direct effect of bacterial metabolites on fungal pathogens and host plants through the activation of the phytoimmune system inhibiting the growth and development of pathogens. The genus Pseudomonas could be more effective in controlling root rot diseases than some chemical pesticides. The endophytic bacterial strains, especially Pseudomonas species, could turn on different signaling pathways against pathogens. Endophytic Pseudomonas species are superior bioactive agents against pathogens, induce systemic resistance, and controls the plant disease with resistance induction. Pseudomonas species are ubiquitously found in plants and members of this species have broad metabolites, which allows them to adapt different habitats and including plant endosphere (Ramírez-Bahena *et al*., 2015). Use of some ecofriendly materials as carriers of bacterial isolates lead to maximizing the potential for successfully developing and deploying a biocontrol product with developing mass production that optimize product quantity and quality (Saravanan *et al*., 2004; Jonathan *et al.,* 2004). A successful formulation of Pseudomonas spp. strain for use against plant disease is potentially more readily achieved than the bacterial spore (Senthil *et al.,* 2011). The present investigation was carried out to evaluate the efficacy of different formulations of Pseudomonas spp. for the management of some root diseases caused by *Rhizoctonia solani*; *Fusarium semitectum*, and *Macrophomina phaseolina*, on eggplant (*Solanum melongena*).

2. Materials and Methods

2.1. Isolation, Identification and frequency of the causal pathogens

Samples of Eggplant showed root rot symptoms were collected from different fields. Ten plants with signs of infection are collected to laboratory for isolation and identification. Diseased part from plant roots were washed with tap water three times, then the affected parts cut into small pieces 2-5 mm, surface sterilized for 2 min in NaOCl (1%), then rinsed several two times with sterilized distilled water and dried between sterilized filter papers. The surface-sterilized samples were plated onto potato dextrose agar (PDA) medium and incubated at 28±2 °C for seven days after incubation; the developed fungal colonies were purified by single spore and/or hyphal tip isolation technique. The appeared fungi were purified and maintained on the same medium (PDA) and were used for morphological

identification by microscopy as described by Nelson *et al.,* (1983) at the Nano-Phytopathology Lab. (NPPL), Desert research Center, Cairo, Egypt. Subcultures of the obtained isolates were kept on PDA slants and stored at 5°C for further studies.

2.2. Frequency of isolated fungi

To calculate the percentage of fungi presence in each of isolation areas, 25 Petri dishes containing potato dextrose agar (PDA) 4 plant pieces in each plate, and the frequency for each fungi present calculated using the following formula:

$$\text{Frequency for isolated fungi} = \frac{\text{Times the fungus appears in isolation dishes}}{\text{total pieces in all dishes}} \times 100$$

2.3. Pathogenicity of obtained fungal pathogens

Fungal pathogenic isolates in this study from infected eggplant roots were found to be pathogenic and had the ability to infect eggplant seedlings 10 days after inoculation.

Inoculation were performed at the four-true-leaf stage of eggplant seedlings, the root system was cut to 5 mm below before being dipped in fungal pathogen suspensions to be tested. Inoculated plants were replanted to pots after 10 minutes. The test was performed a rate of 10 replicates for each isolate, and ten plants were inoculated in distilled water as control, Percentage of infection was recorded after every seven days where inoculated seedlings were uprooted and the lower stem and tap root were examined of internal tissues. After 21 days, the pathogenic isolates with their aggressiveness were determined. Symptom evaluation was made based on scale of symptoms proposed as:(0 = No symptoms; + = Light yellowing of leaves, moderate root rot and/or crown rot; ++ = severe yellowing of leaves and severe rot root, +++=severe rot root and crown rot ++++ = Dead seedlings). The tested isolates caused 30 and 90% root rot compared to the control treatments. These results are in greenhouse.

2.4. Isolation of endophytic bacteria as biocontrol agents

Different plants with economic importance (*Medicago sativa*; *Rosmarinus officinalis* and *Vicia faba.*) were randomly collected from different geographical locations in Egypt, put into plastic bags andkept on ice for the isolation of potential endophytic bacteria. Different Plant parts were surface sterilized using methodology described by Petrini *et al.,* (1992) where samples were immersed two times in 70% ethanol for three minutes and immersed twice in 2-4% aqueous solution of sodium hypochlorite for five minutes and again immersed for one minute in70% ethanol, finally, washed two times in sterile distilled water for five minutes for removing surface sterilization agents with further drying in sterilized paper in a laminar flow hood. Subsequently, sterilized plant samples were crushed under sterile conditions and the resulting juices were plated on King's agar medium (1 ml of the macerated tissue was serially diluted up to 10^{-3} using sterile10 mM potassium phosphate buffer, pH 7). About 1 ml from each dilution of intercellular fluid of its tissue was plated on King plates in triplicate and kept in an incubator at 28°C for 48 hours. After incubation, the grown bacterial colonies were selected.

2.5. Survey of endophytic biocontrol agents against pathogenic fungi

Selection of bacteria dependent on their potential as biocontrol agent inhibiting the growth of the three pathogenic fungi *Rhizoctonia solani* Kühn; *Fusarium semitectum*, and *Macrophomina phaseolina* were investigated *in vitro* using dual culture technique (Alabouvette *et al*., 1993, Coskuntuna and Özer, 2008). For survey of the most effective isolates of bacteria, one disc (0.5 cm in diameter) of 7 days-old culture of each pathogenic fungus was separately placed in center of sterile Petri dish of potato dextrose agar (PDA), each of bacterial isolate was streaked 1 cm from the edge of each side of the same Petri dish. Petri dishes of PDA medium inoculated with fungal cultures and free of bacteria were used separately as control. All plates were incubated at 28±2°C until the growth of each pathogenic fungus in the control treatment reached to the edge of Petri dish. The reduction of pathogenic fungi mycelia growth rate were calculated according to the sizes of the inhibition zones (distance between fungal mycelium and bacterial colony). Therefore, the most effective bacterial strains were selected for further investigation.

2.6. Enzymatic activities of the bioagents

For each bacterial isolate, three enzymes (Chitinase, cellulase and protease) were assayed as follow:

Total Chitinase Activity; Chitinase activity was assayed by measuring the release of reducing saccharides from colloidal chitin according to Wang and Chang (1997) with minor modification. Colloidal chitin was prepared from the commercial chitin by the method of Mathivanan (1995).The selected isolates were inoculated into the colloidal chitin broth medium, composed of (g/l) K_2HPO_4, 0.7; KH_2PO_4, 0.3; $MgSO_4.5H_2O$, 0.5; $(NH_4)_2SO_4$; colloidal chitin 20; and pH 7.0 and were incubated for 5 days at 30 °C. One milliliter of cultures supernatant was mixed by 0.5 ml of 1% solution of dinitro salicylic acid and heated at 100 °C for 5 min. Absorbance of the reaction mixture was measured at 582 nm after cooling to room temperature. One unit of the chitinase activity was defined as the amount of enzyme, which yields 1 µmol of reducing sugar as N-cetyl-d-glucosamine equivalent per minute.

Protease Assay; Protease activity of the three bacterial strains supernatants was measured according to the techniques described by Pokhrel *et al.,* (2014), with slight modifications, 1 ml of each supernatant was incubated with 0.1 M phosphate buffer (pH 7.0) and 2 ml 1% casein solution at 50 °C for 2 h. The reaction was stopped by the addition of 3 ml trichloro acetic acid and subsequent centrifugation, 1 ml of this solution was mixed with 2.5 ml of 0.5 M sodium carbonate and incubated at room temperature for 30 min. One milliliter of Folin phenol reagent was added and incubated again for 15 min at room temperature before being measured at 660 nm. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 µmol of tyrosine per min.

Cellulase Assay; Quantitative assay of cellulase activity was carried out using the 3.5-dinitrosalicylic acid (DNS) method (Miller,1959), 1 ml of each bacteria culture was taken and inoculated into100 ml of 1% CMC liquid media, and incubated for 24-30 hours at room temperature above a shaking incubator. Three ml of culture was centrifuged for 10 minutes at 6000 rpm. The centrifuged supernatant was separated from the pellet and the supernatant was used as crude extract enzyme to measure its activity using DNS reagent. The substrate used in the measurement of cellulase activity was 1% CMC dissolved in 0.1 M phosphate buffer with pH 6.0 and the incubation time was 30 minutes. The standard sugar used as control was glucose. The reducing sugar produced from the reaction was measured by a spectrophotometer with a wavelength (λ) of 540 nm. One unit of the enzyme activity cellulosic substrate defined as 1 µmol of reducing sugar released per min.

2.7. Molecular identification of promising endophytic bacteria as biocontrol agents

Three selected bacterial isolates were identified to molecular level by direct extraction of genomic DNA from the colonies grown on NA medium and was used as template for PCR by using forward and reverse universal primers, F (5′ AGA GTT TGA TCC TGG CTC AG-3′) and R (5′-GGT TAC CTT GTT ACG ACTT-3′), according to the modified method of Ishikawa *et al.,* (2000). The selected isolates were sequenced, using 16S rRNA sequencing and assembled in the BioEdit software (Hall 1999). The molecular identification was carried out by Humanizing Genomics-Macrogen Comp. Geumcheon-gu, Seoul, South Korea (https://dna.macrogen.com). The phylogenetic tree was constructed, using the neighbor-joining NJ method (Saitou and Nei 1987).

2.8. Preparation of bacterial mixture for bioformulations

For each bacterial strain, the inoculums were produced by transferring one loopful from bacterial culture to 100 mL of Nutrient Broth (NB) medium in 250 mL Erlenmeyer flask and incubated at (28±2°C) on a rotary shaker at 150 rpm for 48 h. Then, mixture of equal volume of the three bacterial strains broth was developed, then, the mixture containing10^8 CFU mL^{-1} was used in preparation of formulations.

2.9. Formulation of Bacterial Bio-Agents

Different carrier materials for bio-agents formulation were used; each bacterial formula consists of one basic material (Carrier) and additives in the ratio of 3:1, respectively. The basic materials used in the preparation of formulations were talc, clay and diatom, while arabic gum and alginate were used as additive. Combination of the carriers and the additives were incorporated to develop form nine bacterial formulations. For each prepared formula, beef as enrichment material at the concentration of 1% was incorporated or not to the prepared formula, so, nine formulas with beef and 9 formula without

beef were developed. The inert carriers, enrichment and additive materials were mixed and sterilized by autoclaving. For each of the 18 prepared formula, mixture of the three bacterial bioagents broth were mixed with each formula separately at the ratio of 40% of net formula volume were mixed well under aseptic conditions, then the mixtures were air dried in a laminar-flow chamber for48 h. After drying, one gram sample was removed for initial population count. Then powder formulations were placed in petri plates, sealed with parafilm, stored at room temperature, and sampled for viability assessment (Omer, 2010).

2.10. Selection of the most efficient bacterial formulations against phytopathogenic fungi

The suppressive effect and antagonistic activity of each of the prepared formula against the three phytopathogenic fungi *Rhizoctonia solani* Kühn; *Fusarium semitectum*, and *Macrophomina phaseolina* was demonstrated using the same technique previously (inoculate 0.025 gm of formulation instead of disc) used for recording the antagonistic activities of bacterial isolates against fungi.

2.11. Observation of bacterial adhesion to formula surfaces

Adherent bacteria were fixed with glutaraldehyde (2.5%) on each of the selected formula surfaces for 4 h at 4 °C. The adherent bacteria were dehydrated in 70% ethanol concentration. Then, samples were sputter-coated with gold using a (S150A) sputter coater and then dried under vacuum and observed using high-resolution SEM (Quanta FEG250- FEI) at 12000X magnifications (Kammoun *et al*., 2020). For microbiological analysis, soil dehydrogenase activity (µg TPF/g dry soil/24 h) was analyzed by the reduction of triphenyl tetrazolium chloride (TTC) to triphenyl formazan (TPF) as described by (Friedel *et al.,* 1994).

2.12. Chemical analysis

Antioxidant of leaf extract: The plants were separated, washed, air dried for complete drying and powdered. The powder was extracted with methanol and filtered, the filtrate was concentrated in a rotary evaporator at 40°C then oven dried at 40°C till dryness, the dried plant extract was dissolved in methanol to a final concentration of 1 mg/ml as described by Essawi and Srour, (2000). Antioxidant activity of plant extracts were measured using free radical scavenging activity as described by Singleton *et al.,* (1999). To 500µL of the cell free extract, 3.0 mL of freshly prepared solution of 2,2-DiPhenyl-2-Picryl hydrazyl hydrate(DPPH) at a concentration of 5mg/100 mL(ethanol) was added. Control was prepared using 500µL of ethanol added to 3mL DPPH solution, mixed in dark and incubated for 30 min. Absorbance was recorded at 517nm after 30 min of incubation in the dark with methanol serving as blank. The readings were recorded in triplicates and the average absorbance value was calculated. Ascorbic acid (100 µg/mL) was used as natural standard. The percentage of radical scavenging activity was calculated according to the equation [Abs517 control – Abs517 sample / Abs517 control] x100

Total phenolcontents in whole plant were carried out using the Folin-Ciocalteu reagent using the method of Singleton *et al*., (1999). One ml of previously prepared plant extract was taken and added to 0.5 ml of distilled water and 0.125 ml of Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 minutes before addition of 1.25 ml of 7% Na2CO3. The solution was adjusted with distilled water to a final volume of3 ml and mixed thoroughly. After incubation in the dark for 30 min, the absorbance at 650 nm was read versus the prepared blank. A standard curve was plotted using different concentrations of Catechol (standard, from 0-1000µg/ml). Total phenol contents (TPC) were expressed as Catechol equivalent /mg of dry weight.

2.13. Propagation of inoculum of isolated fungi for greenhouse experiment

Inoculum of each isolated fungi was prepared on autoclaved barley medium (75 g washed dried barley grains, 100 washed dried coarse sand and 75 mL tap water) in 1 liter Erlenmeyer flask, ten flasks for each fungus. Each flask was inoculated by five discs (0.5 cm in diameter) of seven-day-old cultures of each pathogen. During incubation period, the grains were shaken daily to prevent them from sticking together due to hyphal growth, then the bottles were incubated at 28±1°C for 15 days (until the fungal growth covered the media).

2.14. Assessments of root rot incidence and severity under greenhouse condition

A pot experiment was designed using plastic pots (16cm diameter) containing sterilized soil (sandy loam). Fungal pathogens grown on barley grains were mixed with the soil in plastic pots at the

rate of 3% of soil weight 4 days before planting, 6 treatments × 3 replicates for each treatment + 3 replicate infested control in completely randomized design. Control pots inoculated with non-infested barley medium. As well as a mixture of fungi used in a ratio of 1:1:1 in another pots. The experimental soil characterized as sandy soil in texture with alkalinity, low salinity and low contents of available nutrients as shown in Table (1). The pots were irrigated periodically. Each pot was planted with three seedlings of eggplant. Seedlings root rot was determined. The experiment included 35 treatments namely non-infested soil (control), soil treated with phytopathogens; *Rhizoctonia solani*, *Fusarium semitectum*, and *Macrophomina phaseolina* individually and soil treated with mixture of fungal pathogens, as well as soil treated with promising formula which included mixture of *Pseudomonas* spp. Also soil treated with both fungal pathogens and bioagents. Pots were kept under greenhouse conditions till the end of the experiment (6 weeks of sowing). The severity and incidence of eggplant root rot were determined at the end of the experiment.
Disease incidence and severity was calculated according to the following formula:

$$\text{Diseaseincidence (D.I)} = \frac{\text{Number of infected plants}}{\text{Total of plants}} \times 100$$

$$\text{Diseaseseverity (D.S) \%} = \frac{\text{Total number of plants of class } 0\times 0+\text{Total number of plants of class } 4\times 4\text{nts}}{\text{Total number of plants examined} \times \text{highest degree}} \times 100$$

Table 1: Chemical and physical analysis of the experimental soil

pH (1:1) suspension	EC dS m^{-1} (1:1) extract	Soluble cations (Meq L-1)				Soluble anions (Meq L-1)			
		Ca^{++}	Mg^{++}	Na^{+}	K^{+}	$CO3^{--}$	HCO_3^{-}	Cl^{-}	SO_4^{--}
8.8	0.65	0.86	0.62	4.36	0.21	-	2.13	3.10	0.85
Texture class	**CEC Meq/100 g soil**	**$CaCO_3$ %**	**OM %**	**Available nutrients (mg kg^{-1})**					
				N	**P**	**K**	**Fe**	**Mn**	**Zn**
Sandy	5.17	0.15	0.06	27.2	3.21	67.1	2.60	1.01	0.33

2.15. Statistical analysis

Analysis of variance (ANOVA) was used to compare microbial inhibition of fungal pathogen growth in relation to not inoculate with endophytes. Treatment means were compared using least significant difference test (LSD) at 0.05%. All experiments were repeated at least two times. Where repeated tests were resulted similar data, data from a single representative experiment are presented. Variations in absolute results among the greenhouse experiments were analyzed.

3. Results and Discussion

3.1. Isolation of fungal pathogens from infected plants and their frequency

During the seasons from 2019 to 2021, many fields in 3 new reclaimed regions (Baloza, North Sinai Governurate; Salheya, Sharkiya Governurate; New Benisuif, Benisuif Governurate) were visited, from nursery and open fields. From each site, 40 samples (plants and rhizosphere soil) were collected where plants showed symptoms of root rot. A total of 22*Rhizoctonia solani*; 15 *Fusarium semitectum* and 28 *Macrophomina phaseolina* isolates were isolated from 120collected samples.

3.2. Morphological Identification of fungal phytopathogens

Depending on the morphological characteristics of the fungal isolates using microscopic examination, the fungal isolates were defined as follows:

Rhizoctonia solani; The obtained isolates were multinucleate, containing 4 - 8 nuclei with an average of 4.97 nuclei per hyphal cell, and hyphae width (about 2.26 µm), and average of Cells Diameter 12.50 x 25.80 µm. after incubation of tested isolates in the dark at 24 ± 1˚C, Sclerotia were produced by all the isolates where the average of sclerotia diameter recorded (1.24 x 1.12 to 2.8 x 3 mm). The colony colors were started with white creamy mycelium to dark brown in isolates, Macroconidia are sickleshaped. Microconidia are abundant, oval to kidney-shaped, with long monophialides. According

to the microscopic examination, the information obtained from this study, 22 isolates were identified as *R. solani*. These results are consistent with what was mentioned by (Ke *et al*., 2016; Al-Fadhal *et al.,* 2019).

Fusarium semitectum, for identification, the isolates were cultured on PDA for observation of primary characteristics, such as shape of microconidia and macroconidia. All Fusarium isolates obtained from diseased eggplant fields in the four locations were identified as *F. solani*, *F. oxysporum* and *F. semitectum,* based on frequency and pathogenicity test, *Fusarium semitectum* isolates appeared to be more virulent compared to others, where resulted the same symptoms on treated plants in growth chamber. Morphological characters showed that, Hyphae are septate and hyaline, spindle-shaped macroconidia, conidiophores simple and branched monophialides and polyphialides. Formation of orange and light sporodochia was also observed. aerial mycelium are mostly straight, 3-5 septate, and measure 6.8-33 x 2.4-3.8 µm. possess a foot cell, are 3-6 septate. Chlamydoconidia are brown, and in short chains.15 isolates were identified as *F. semitectum*, these characteristics are in agreement with what is stated in The Fusarium Laboratory Manual (Leslie and Summerell, 2006).

Macrophomina phaseolina; the fungus was isolated from some eggplant plants and characterized by growth on the PDA, as fungal mycelium started transparently to white colony, hyaline hyphae with thin walls to dark brown hyphae with septa, spherical or oval microsclerotia, started with light brown to darker brown or black in old cultures, the fungus was identified according to the morphological and cultural characteristics observed and confirmed by the description of (Lakhran *et al*., 2018; Pandey *et al*., 2020).

3.4. Pathogenicity of obtained fungal isolates

The pathogenicity tests were conducted at greenhouse of Nano-Phytopathology Lab., Desert Research Center, Cairo, Egypt. Pathogenicity test of *Rhizoctonia solani, Fusarium semitectum,* and *Macrophomina phaseolina*, were carried out using isolated and identified fungi. The obtained results are showed in the Table (2) most of the fungal isolates used in the experiment proved to be pathogenic with various degrees. The inoculated seedlings were examined after every seven days. In case of soil inoculations with fungal pathogens individually were moderate effect, while the maximum effectiveness on eggplant resulted with mixture of the three pathogenic isolates and with pathogenicity by *F. semitectum* alone in compared with control. One of the most virulent isolates of each fungal genus was selected to further experiments for evaluation the biocontrol agents and their ability to suppress the pathogens.

Table 2: Effect of Pathogenicity test after inoculation of different fungi on eggplant

Pathogens	Effect of pathogenicity after inoculation/days			
	7th day	14th day	21th day	effectiveness
R. solani	++	++	+++	More effect
F. semitectum	+	++	++++	highly effect
M. phaseolina	-	+	+++	More effect
Mixture	++	+++	++++	highly effect
Control	-	-	-	

3.5. Isolation and identification of endophytic bacteria

In this study, endophytic bacteria were isolated from parts of different plants. Out of 35 isolated endophytic bacterial strains, three strains showing gram negative rode shape were selected for further characterization considering identified as *Pseudomonas* spp. Identified bacterial isolates are (*Pseudomonas aeruginosa*; isolated from root of *Medicago sativa plant. Pseudomonas alcaligenes*; isolated from stem of *Rosmarinus officinalis* plant*,* and *Pseudomonas mendocina;* isolated from leaves *of faba bean "Vicia faba",* were identified based on the 16S rRNA gene sequence with identical percent of 100%, 97.94% and 97.7% respectively (Table 3) and (Fig 1a-1c).

Table 3: Source and locations of identified endophytic bacteria as biocontrol agents

Code	Endophytic bacteria	Host plant	Part/plant	Site	Location
RMr	*Pseudomonas aeruginosa*	*Medicago sativa*	Root	Ras sudr, South Sinai Governorate	29°32'39.1"N 32°45'23.7"E
MRs	*Pseudomonas alcaligenes*	*Rosmarinus officinalis*	Stem	Moghra, Matrouh Governorate	30°29'05.5"N 29°14'11.8"E
KVl	*Pseudomonas mendocina*	*Vicia faba*	Leaf	Kharga, New Valley Governorate	25°23'37.1"N 30°32'36.6"E

3.6. Enzymatic activities of the three selected endophytic bacteria

Enzymatic activities of the most effective bacterial isolates from *Medicago sativa; Rosmarinus officinalis* and *Vicia faba* were assayed. The results showed that cellulase, chitinase and protease were detected in the three tested isolates, *P. alcaligenes* was the highest one in cellulase and protease activities (14.83 and 83.31 U) respectively, while high chitinase activity was observed in *P. mendocina* from stem of *Vicia faba*. Also the cellulase activity was mostly expressed in isolates from stem of *Rosmarinus officinalis* (Table 4). Similar studies on enzyme production by bacterial endophytes from leaves and stem of various species of plants such as *Rhizophora apiculata, Excoecaria agallocha*, and *Aegiceras corniculatum* showed presence of chitinases, proteases and cellulase which isolated from Mangrove plants (Gayathri *et al.,* 2010), endophytic bacteria isolated from leaves of the common bean *Phaseolus vuglaris* (Araújo, 2012), bacterial endophytes isolated from Mentha spicata *(*Adewale *et al*., 2015), from Curcuma longa (Kumar *et al.,* 2016); *Bacillus* and *Paenibacillus* strains from medicinal plant Lonicera japonica (Zhao *et al*., 2015), endophytic bacteria associated with leaves of switchgrass *Panicum virgatum* L. (Aliferis *et al.,* 2013). The presence of cellulases, chitinase and Protease activities were exhibited by isolates identified to be Pseudomonas spp. This is in line with previous studies that have shown the presence of cellulases in bacteria endophytes (Scherwinski *et al.,* 2008), *Pseudomonas plecoglossicida* (Nishimori *et al.,* 2000), *Pseudomonas putida* in carrot (Surette, 2003). Some of isolates were successfully identified with 16S rDNA sequences and continued to evaluate them as biocontrol agents against root rot as the most common diseases of eggplant and other host plants.

Table 4: Enzymatic activities of the three selected endophytic bacteria

Endophytic bacteria	Cellulase U 1 µmol of reducing sugar min^{-1}	Chitinase U 1 µmol of reducing sugar min^{-1}	Protease U (1 µmol of tyrosine min^{-1})
P. alcaligenes	14.83	0.95	83.31
P. aeruginosa	6.30	1.35	55.98
P. mendocina	0.73	1.92	83.20

3.7. Antagonistic activities of bacterial strains against disease causal agents

All the tested bioagents inhibited the radial growth of pathogenic fungi as antagonistic effects compared with control (Table 5). *Pseudomonas aeruginosa* exhibited the maximum antagonistic activity causing an inhibition of *R. solani* 82.3 %, followed by *F. semitectum* which inhibited by 74.5% and *M. phaseolina* by 70.2%, while the *Pseudomonas alcaligenes* causing inhibition percentage (53.6, 68.4 and 55.8) for *R. solani*, *F. semitectum* and *M. phaseolina* respectively. The less effect of inhibition resulted with *Pseudomonas mendocina* which inhibited the tested pathogens by 45.2, 52.7 and 46.2% for *R. solani*, *F. semitectum* and *M. phaseolina* respectively. The inhibitory effect of these endophytic bacteria against was probably due antibiosis effect. Pseudomonas exerts its biocontrol activity through direct antagonism of phytopathogens this antagonistic activity of endophytic bioagents resulted in the present study is similar to the finding of (Cartieaux *et al*., 2003; De La-Funte *et al*., 2006) who reported effective inhibition of different fungal pathogens in vitro.

Table 5: Effect of endophytic bacteria on Growth reduction of tested pathogenic fungi (%)

Antagonistic Microorganism	Growth reduction of tested soil-borne pathogenic fungi (%)		
	R. solani	*F. semitectum*	*M. phaseolina*
Pseudomonas aeruginosa	82.3	74.5	70.2
Pseudomonas alcaligenes	53.6	68.4	55.8
Pseudomonas mendocina	45.2	52.7	46.2
Control	100	100	100

Mean values within columns for each organism followed by the same letter are not significantly different ($P \leq 0.05$).

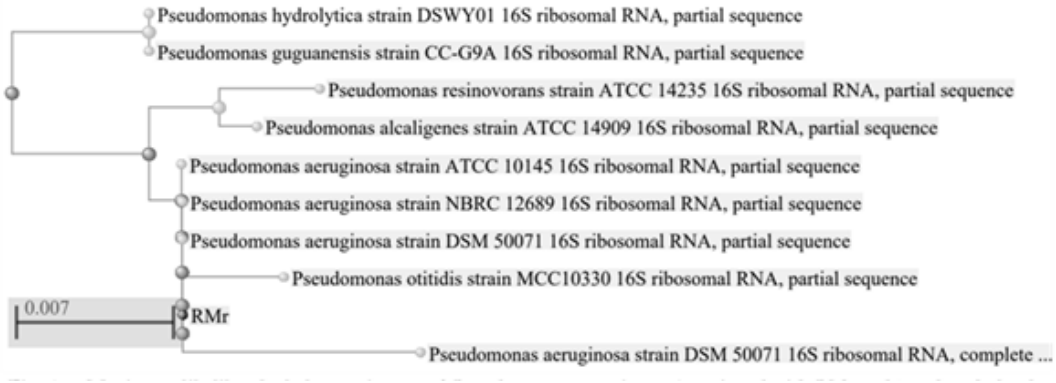

Fig. 1-a: Maximum-likelihood phylogenetic tree of *Pseudomonas aeruginosa* (mentioned with RMr code) and and closely related species of the genus Pseudomonas.

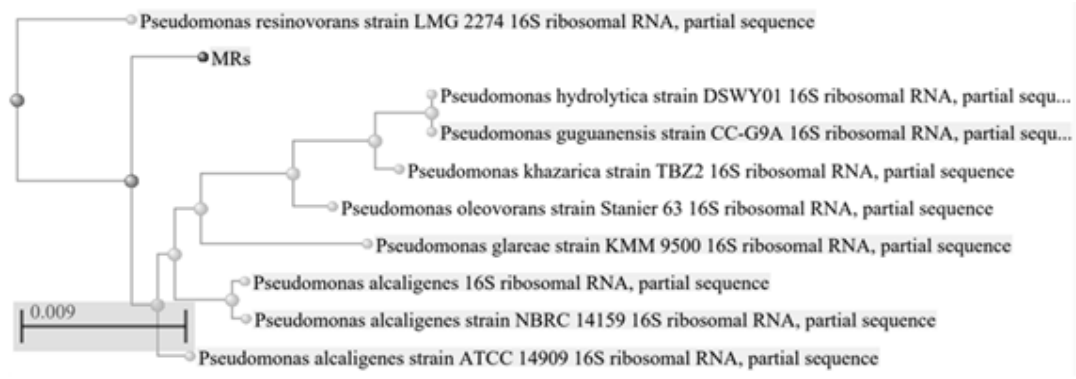

Fig. 1-b: Maximum-likelihood phylogenetic tree of strain BSTT44T and the type strains of *Pseudomonas alcaligenes* (mentioned with MRS code) and closely related species of the genus Pseudomonas.

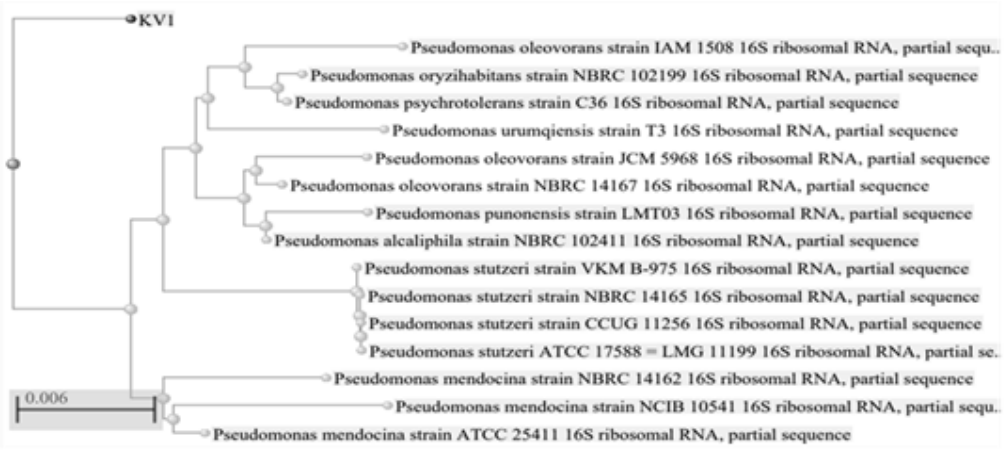

Fig. 1-c: Maximum-likelihood phylogenetic tree of *Pseudomonas mendocina* (mentioned with KVI code) and and closely related species of the genus Pseudomonas.

3.8. Evaluation of well-developed bio-formulations *in vitro*

The inhibitory effects of 18 different bioagent formulas (Table 6) against linear growth of pathogenic fungus *in vitro* were evaluated (Fig. 1). All bioformulations inhibited the linear growth rate of the tested fungi to varying degrees. The highest efficient formulations were ([1]Diatoms + Arabic gum + Beef; [2]Clay+ Alginate + Beef; [3]Clay + Arabic gum + Beef;[4]Clay + Arabic gum;[5]Talc+ Arabic gum+ Beef; and [6]Talc+ Arabic gum).The highest mean inhibition values were obtained against *F. semitectum* followed by *R. solani*, while *M. phasolina* showed more resistance to the tested formulas than other fungi.

Table 6: Evaluation of well-developed bio-formulations

Components of Formula			Inhibition zone (cm)		
			R. solani	***F. semitectum***	***M. phaseolina***
Diatoms	**Alginate**	Beef	-	-	0.6
		Without	-	0.4	0.5
	Arabic gum	Beef	1.4	1.3	0.9
		Without	1.1	1.2	0.9
	Alginate + Arabic gum	Beef	0.2	0.2	-
		Without	1.2	0.9	0.6
Clay	**Alginate**	Beef	1.5	0.9	0.9
		Without	-	1.6	0.6
	Arabic gum	Beef	1.5	1.5	0.8
		Without	1.5	1.1	0.6
	Alginate +Arabic gum	Beef	0.5	1.4	0.5
		Without	0.7	0.6	0.9
Talc	**Alginate**	Beef	0.4	1.1	0.9
		Without	0.7	1.9	0.8
	Arabic gum	Beef	1.5	1.3	0.7
		Without	1.4	1.3	0.9
	Alginate +Arabic gum	Beef	0.8	1.7	0.6
		Without	0.8	1.1	0.7

3.9. Observation of bacterial adhesion to formula surfaces

The results of the examination with a scanning electron microscope SEM confirmed the presence of cells bacterial density in the most effective six tested formulations where bacterial cells appeared

during the microscopic examination, which indicated their vitality and the ability of these carriers to keep bacteria alive and thus their effectiveness in biological control. Electron microscopy examination revealed that the most formulas in terms of containing bacterial cells were formula 3 (Clay + Arabic gum + Beef); then formula 2 (Clay+ Alginate + Beef), followed by formula 5 (Talc+ Arabic gum+ Beef) as presented in (Fig.2). Electron microscopy results agreed with the results of the microscopic examination to count bacterial cells and confirm their vitality in the tested formulas. The microscopic examination also indicated the effectiveness of all tested compositions in preserving bacterial cells and their vitality for ninety days. The results showed that the formula 3 (Clay + Arabic gum + Beef) showed a significant superiority over all other formulations in extending the shelf life of consortium with highest number of colonies, but at each stage of the examination the number of bacterial colonies was decreasing, while it was a noticeable superiority in maintaining the vitality of cells compared to other formulations. The results also indicated that the best tested formulations were those that contain beef compared to other formulations that do not contain it. This is in accordance with the findings of Amer and Utkhede (2000) were formulated *B. subtilis* and *Pseudomonas putida* using various carrier materials such as talc and kaolin. In all carrier formulations, the tested bacteria survived up to 45 days. Also the obtained indicators are in accordance with the findings of Ruthvan (1984); and Whereas Carrera *et al.,* (2007); Jayasudha *et al*., (2017) who indicated some materials as carriers are effective in trapping water inside the micropores and hence, the water needed for the germination of spores in these carrier–endospore formulation might had been physically separated from the spores, where water is absorbed in the large pores and open channels were in contact with enough water to be germinated and hence proliferated.

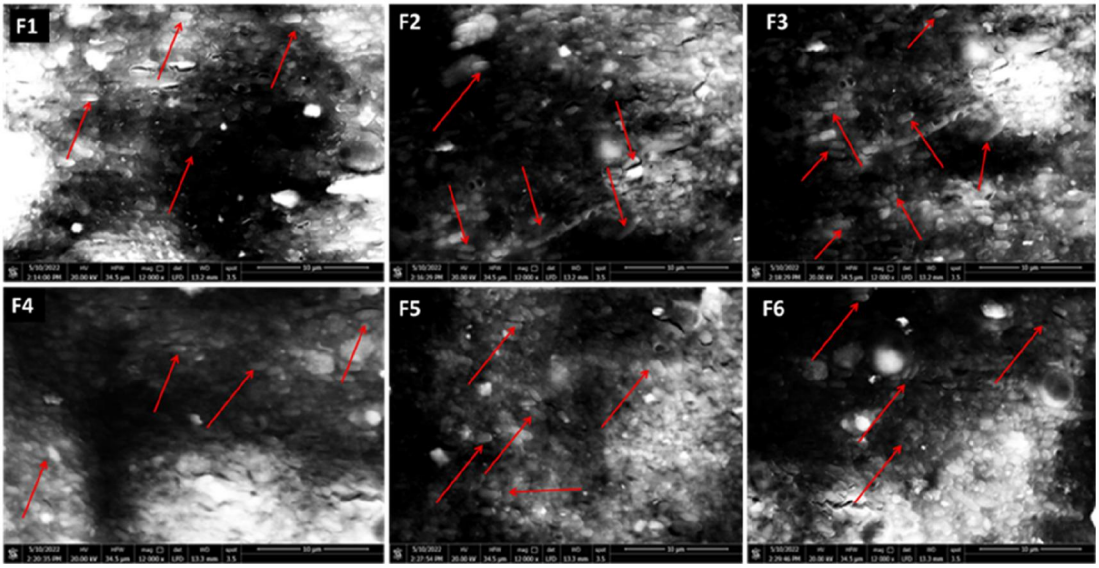

Fig. 2: SEM observations of bacterial adhesion on selected bioformulations; F1:(1) Diatoms + Arabic gum + Beef; (2) Clay+ Alginate + Beef; (3) Clay + Arabic gum + Beef; (4) Clay + Arabic gum; (5) Talc+ Arabic gum+ Beef; and (6) Talc+ Arabic gum.; - The arrows in the pictures indicate the bacterial cells as shown by the microscopic examination

3.10. Effect of bacterial formula on eggplant root rot disease in pots

In this experiment, the efficiency of six formulations and free suspension of pathogenic fungi in controlling root-rot diseases caused by *R. solani, F. semitectum* and *M. phasolina* and their mixture were evaluated. Data in Table (7) show that all bacterial formulations decreased damping root-rot incidence and disease severity compared with the controls. Data indicated that, soil infested with pathogenic fungi as control showed the highest rate of root rot infections compared with non-infested soil. Also the eggplant plants treated with mixture of fungal pathogens showed the highest diseases severity compared to fungal pathogen individually, generally, the formulations 2, 3 and 5 application significantly reduced the root-rot infection as disease incidence and severity caused by all tested fungi

(Mixture of pathogens). The maximum reduction in disease severity was recorded with formula 5 (18.2%) followed by formula 3 (20.6%) and formula 2 (23%) while the less of effectiveness was recorded with formula 6 (60.4%) compared to control (70.3%). *R. solani* was more sensitive to all tested formulas than other fungal pathogens. The obtained results are in the same direction with results in previous studies where bacterial formulations have significant potential as biocontrol agents against *Phytophthora* spp., *Pythium* spp., *Rhizoctoniasolani*, *Colletotrichum acutatum* and *Fusarium oxysporum* (Sangita and Shah, 2000).

Table 7: Effect of different bioagents formula on disease incidence and disease severity in eggplant under greenhouse condition at 60 days after transplanting under greenhouse conditions

Fungi	* Formulations	Disease Severity (%)	Disease Incidence (%)
Non-Pathogen	Control	3.8 q	5.3 p
	Formula1	1.5 qr	2.3 q
	Formula2	0 r	0 r
	Formula3	0 r	0 r
	Formula4	1.9 qr	2.2 q
	Formula5	0 r	0 r
	Formula6	2.7 qr	2.4 q
	Mean	1.41	1.74
R. solani	Control	45.2 d	38.4 e
	Formula1	15.5 klm	21.5 j
	Formula2	6.8 p	17.3 kl
	Formula3	8.2 op	9.2 o
	Formula4	13.5 mn	20.5 j
	Formula5	8.4 op	12.4 n
	Formula6	20.5 ij	23.6 i
	Mean	16.8	20.46
F. semitectum	Control	47.2 d	44.6 c
	Formula1	25.3 h	28.2 h
	Formula2	11.7 n	16.4 lm
	Formula3	11.2 no	16.2 lm
	Formula4	22.6 hi	30.5 g
	Formula5	14.2 lmn	15.1 m
	Formula6	30.2 g	32.8 f
	Mean	23.2	26.26
M. phaseolina	Control	53.8 c	40.4 d
	Formula1	35.5 f	29.5 gh
	Formula2	15.7 klm	17.8 kl
	Formula3	12.6 mn	16.8 klm
	Formula4	32.2 g	28.2 h
	Formula5	16.8 kl	18.5 k
	Formula6	30.8 g	27.7 h
	Mean	28.2	25.59
Mix of pathogens	Control	70.3 a	64.8 a
	Formula1	39.2 e	52.2 b
	Formula2	23 hi	23.4 i
	Formula3	20.6 ij	22.4 ij
	Formula4	45.4 d	37.5 e
	Formula5	18.2 jk	20.7 j
	Formula6	60.6 b	40.5 d
	Mean	34.5	32.8

***Formulations:** (1) Diatoms+Arabic gum+Beef; (2) Clay+Alginate+Beef; (3) Clay+Arabic gum+Beef; (4) Clay +Arabic gum; (5) Talc+Arabic gum+Beef; and (6) Talc+Arabic gum.

Bioformulations of *Pseudomonas aeruginosa, Pseudomonas alcaligenes, and Pseudomonas mendocina* proved to be more effective than its free spore suspension in soil treated with pathogens. Clay+Alginate+Beef; Clay+Arabic gum+Beef and Talc+Arabic gum+Beef based formulations recorded the highest reduction of disease severity and incidence for all tested fungi. No significant

variations between Diatoms+Arabic gum+Beef; Clay+Arabic gum; and Talc+Arabic gum based formulations were detected in reducing all the traits studied. The most effective formula was (Clay+Arabic gum+Beef) based formulation which reduced root rot incidence by 18.2 %. Root-rot severity reached the highest rate when mixture of fungi was applied to the soil comparing with control.

3.11. Plant-growth promoting activity of endophytic strains

In this study the role of bacterial isolates of *Pseudomonas aeruginosa, Pseudomonas alcaligenes*, and *Pseudomonas mendocina* were evaluated in terms of their effectiveness as a plant growth promoters and resistance inducer against root rot pathogens in eggplant, where all bacterial isolates proved to be effective as growth stimulating factors, all treatments with bacterial formulations recorded the best readings on the level of plant fresh weight, shoot and root length of plants. Formulations 3 and 5 showed the best effect on growth parameters of plants not treated with fungi or plants inoculated with pathogens, on plant fresh weight, F3 recorded (6.1g, 7.9g, 9.0g) with pathogenicity by *R. solani, F. semitectum* or *M. phaseolina* compared to control (3.6g, 2.4g, 2.7g), while pathogenicity by a mixture of fungi, F3 was recorded (4.5g) compared to the control (2.8%). In estimating some of the vital components in the plant that are related to resistance and growth improvement, such as the effect of bacteria on each of the total phenolic compounds (ppm) and antioxidant activity (scavenging effect %). Data showed in Table (8) revealed that, when plants were treated with bacterial isolates carried on different substances without infecting plants with any pathogenic fungi, the bacteria-treated plants recorded the best results, compared to the untreated plants, in total phenolic where formula 5 recorded (1248.5 ppm) compared to the untreated plants (1067.8 ppm), while antioxidant activity (scavenging effect %) was in the treatment with formula 3 (66.7 %), followed by formula 5 (64.7%) compared to untreated plants (57.7%). The results showed that plants infected with pathogenic fungi when treated with bacterial formulations had better results in total phenolic compounds and antioxidant activity (scavenging effect %) compared to the control, that is in the case of infection with fungi *R. solani, F. semitectum, M. phaseolina* individually or a mixture. The most effective formula was F3, which recorded in total phenolic compounds (848.9 ppm; 953.3 ppm; 1031.1 ppm) with pathogenicity by *R. solani, F. semitectum, M. phaseolina* compared to control (655.3 ppm; 612.6 ppm; 624.3 ppm). Also, when industrial infection with a mixture of fungi, F3 was recorded (763.4 ppm) compared to the control (470 ppm). With the antioxidant activity (scavenging effect %), the most effective formula was F3 also, which recorded in total (48.3 %; 53.5 %; 58.3 %) with pathogenicity by *R. solani, F. semitectum, M. phaseolina* compared to control (35.7 %; 30.7%; 29.4 %), with pathogenicity by a mixture of fungi, F3 was recorded (40.3%) compared to the control (23.2%). The results were in the same direction with all tested characteristics, where the best bacterial formulation was F3 followed by F5 compared to control.These obtained results were consistent with the results of several studies that were concerned with evaluating the efficacy of Pseudomonas spp. as biological control agents and plant inducers of diseases resistance such as Cartieaux *et al.,* 2003 who mentioned that pseudomonads have received particular attention as biocontrol agent of choice. Pseudomonas exerts its biocontrol activity through direct antagonism of phytopathogens and induction of disease resistance in the host plant. Weller 2007 mentioned that, there are several ways in which different plant growth-promoting Pseudomonas have been reported to directly facilitate the proliferation of their plant hosts.

Pseudomonas spp. produce Phytohormones which helps in stimulating plant growth and known to be involved in root initiation and cell enlargement. Some microorganisms increases root length and surface area which in turn enables plants to increase systemic resistance and increase in shoot and root length as well as other growth parameters and induce resistance (Salisbury 1994; Patten and Glick 2002; Asghar *et al.,* 2002; Jain *et al.,* 2011).

Table 8: Effect of bacterial application on total phenolic, antioxidant activity and dehydrogenase in soil as well as growth parameters of eggplant plants grown in soil infested pathogenic fungi

Fungi	Formulation	Total Phenolic Compounds ppm	AntiOxidant activity (Scavenging Effect %)	Dehydro-genase in Soil	Plant Fr. Wt. gm	Shoot length cm	Root length cm
	Control	1067.8	57.7	2.5	8.8	27.3	6.5
	Fo rmula1	1187.8	62.7	11.1	10.6	29.4	6.9
	Formula2	1192	62.7	11.8	10.9	31.7	7.4
Non-Pathogen	**Formula3**	1635.8	66.7	14.6	12.7	33.6	7.6
	Formula4	1118.3	60.7	10.5	10.1	28.4	6.9
	Formula5	1248.8	64.7	13.3	11.6	31.7	8.2
	Formula6	1083.5	59.7	10.4	9.9	28.2	7.4
	Mean	1219.1	62.1	10.6	10.7	30	7.3
	Control	655.3	35.7	3.6	3.6	18.2	3.3
	Formula1	780.7	43.3	4.8	5.5	22.6	4.8
	Formula2	827.9	45.3	4.9	5.7	23.4	4.9
R. solani	**Formula3**	848.9	48.3	5.3	6.1	26.3	5.4
	Formula4	779.5	43.3	4.7	5.3	21.2	4.9
	Formula5	835.3	46.3	5	5.9	24.9	5.5
	Formula6	766.8	42.3	4.6	4.9	20.3	4.8
	Mean	691.3	38.4	4.7	4.8	19.8	4.3
	Control	612.6	30.8	4	2.4	11.6	2.3
	Formula1	901.3	50.3	6.6	6.8	25.1	5.5
	Formula2	911.3	51.3	6.7	7.3	25.5	5.6
F. semitectum	**Formula3**	935.3	53.5	7.9	7.9	26.7	5.9
	Formula4	893.9	49.3	6.5	6.6	25.1	5.5
	Formula5	923.3	51.3	6.7	7.5	26	6.2
	Formula6	871.1	49.3	6.5	6.2	24.8	5.2
	Mean	864.1	48	6.4	6.4	23.5	5.2
	Control	624.3	29.4	3.8	2.7	12.6	2.8
	Formula1	965.7	54.3	5.7	7.1	26.9	6.1
	Formula2	967.6	55.3	5.8	8.2	27.2	6.3
M. phaseolina	**Formula3**	1031.1	58.3	5.8	9	28.9	6.9
	Formula4	957.4	54.3	5.7	6.2	25.9	5.9
	Formula5	1003.7	57.3	5.8	8.6	28	6.4
	Formula6	942	53.3	5.3	6.6	25.2	5.5
	Mean	927.4	51.8	5.4	6.9	24.9	5.7
	Control	470	23.2	4.2	2.8	13.5	3
	Fo rmula1	704.5	35.3	8.4	3.4	17.9	3.6
	Formula2	739.7	37.3	8.8	3.6	18.1	4.2
Mix of pathogens	**Formula3**	763.4	40.3	9.7	4.5	18.5	5.1
	Formula4	688.2	34.3	8.3	3.3	17.2	3.6
	Formula5	743.7	38.3	9.4	4.1	22.5	4.8
	Formula6	634.2	31.3	8.2	3.2	15.2	3.6
	Mean	610.5	31	8.1	3.2	15.6	3.6
	LSD 5%						
Pathogen		3.57	0.63	0.35	1.32	2.13	0.99
Formulas		4.22	0.75	0.42	1.56	2.52	1.17
Interaction		0.44	1.67	0.94	n.s	5.63	n.s

4. Conclusion

Endophytic bacteria; Pseudomonas *Pseudomonas aeruginosa, Pseudomonas alcaligenes*, and *Pseudomonas mendocina* isolated in this study shown their potentiality in reduction of the root rot severity and incidence of eggplant seedlings caused by *Rhizoctonia solani, Fusarium semitectum*, and *Macrophomina phaseolina*. In addition, the evaluation of different formula of endophytic bacteria showed the, the most effective formulas were Clay+Alginate+Beef; Clay + Arabic gum + Beef and Talc+ Arabic gum+ Beef based formulations, were reduced the root rot as severity and incidence. The endophytic bacteria have ability to induce the plant resistance and positively affect the enzymes. *In vitro*

and greenhouse experiments were carried out to determine the antagonistic activity of endophytes. Further studies may be required to confirm the effectiveness of endophytes under field conditions as well as the antagonistic effects on other diseases of economic importance. This study showed the possibility of isolating the endophytic *Pseudomonas* spp. from different plant species, and the ability of these endophytic bacteria to reduce the infection of root rot on eggplant in different formula, and have a role in promoting plant growth.

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