ROLE OF COMPOST MICROORGANISMS IN CONTROLLING POTATO BROWN ROT DISEASE

BY

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B.Sc. Agricultural Sciences (International Agriculture),
Fac. Agric. Cairo Univ. (2003)

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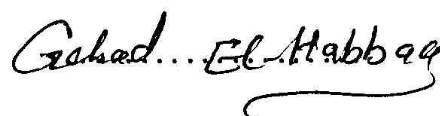

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ABSTRACT

Antagonistic bacteria of potatoes were isolated from different compost types. Based on *in vitro* screening, three bacterial isolates which effectively inhibited *Ralstonia solanacearum* (a bacterial wilt pathogen of potatoes) were characterized and identified. All the isolates were identified as *Bacillus spp.* In greenhouse experiments, the plants treated with *Bacillus* isolates (1, 2, and 3) reduced wilt incidence by more than 65%. All the selected isolates improved the growth of the plant. All the selected antagonists produced antibiotics which inhibited *R. solanacearum in vitro* and might be responsible for reducing wilt incidence *in vivo*. Also, production of siderophores in the culture medium by the antagonists was recorded, which could be involved in biocontrol and growth promotion in crop plants. From this study we concluded that *Bacillus* is a major antagonistic endophytic bacteria which have the potentiality to be used as a biocontrol agent as well as a plant growth-promoting rhizobacteria.

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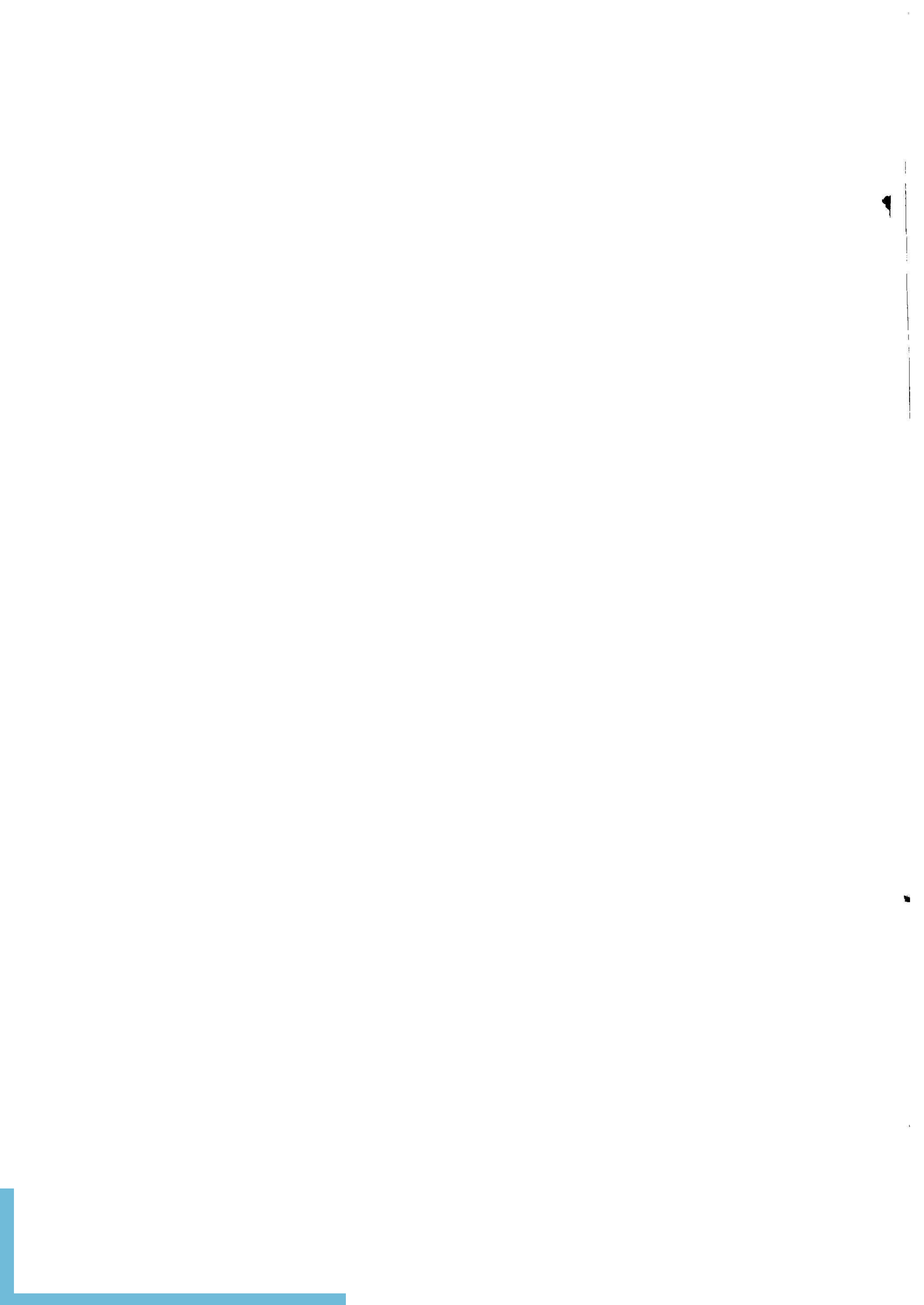
INTRODUCTION



INTRODUCTION

Potato *(Solanum tuberosum L.)* is considered one of the four major and important food crops after wheat, maize and rice around the world (**Hawkes, 1992**). After the recent decline of cotton crop vegetable crops especially potatoes became a motivating crop for Egyptian producers and exporters where the cultivated areas of potatoes have reached approximately 350 thousand fed/year which yielded 3.5 million tons/year and out of this quantity, about 400-500 thousand tons were exported to many countries especially the EU countries and Russia. **(FAO Stat 2011)**

Potato plants are attacked with wide range of fungal, bacterial and viral diseases, where they cause serious losses in crop production and / or tuber quality. One of the most important bacterial diseases of potatoes is brown rot which caused by *Ralstonia solanacearum* where it predominates in tropical, subtropical and temperate regions and this disease is one of the major constraints to production of solanaceous crops in these regions (**Fahy and Persley 1983, Hayward 1991**). The brown rot disease was recorded for the first time in Egypt at El Gemmiza farm by **Briton Jones (1925)**, although the first introduction of the disease is not well documented, it has been assumed to coincide with the mass importation of potatoes at the time of Mohamed Ali Pasha (1805 ac), the Ottoman Viceroy, who imported cotton as well. The French invasion of Egypt by Napoleon Bonaparte (1798ac) might be another possible mean of introduction from Europe **(Barakat, 1963; Farag, 1976, Abd El-Ghafar *et al.* 1995, Gabr and Saleh 1997, Farag, 2000 and Balabel, 2006)**.

The causal agent of bacterial wilt was identified and described as *Bacillus solanacearum* by Erwin F. Smith **(Kelman, 1953).** In 1914, the name was changed to *Pseudomonas solanacearum* and for almost 80 years the pathogen was grouped within the genus Pseudomonas. In 1993, the new genus *Burkholderia* was validated and *P. solanacearum* become known as *Burkholderia solanacearum.* In 1996, *B. solanacearum* changed to *Ralstonia solanacearum* **(Hayward, 2000).**

R. solanacearum (**Yabuüchi *et al.* 1995**) is the causative agent of potato bacterial wilt. This pathogen is a heterogeneous and soil-borne plant pathogen, five races have been described according to the hosts affected and five biovars according to their ability to utilize and /or oxidize several hexose alcohols and disaccharides (**Hayward, 1991**). In Egypt, the dominant race of *R. solanacearum* is race 3, biovar 2 which is widely spreading in Europe, suggesting a possible origin of introducing to Egypt with seed tubers (**Farag, 2000**).

Ralstonia solanacearum is very difficult pathogen to control. The main methods recommended includes cultural technique, crop rotation, resistant varieties and biological control which are not sufficient to reduce the losses due to the location of the pathogen in the xylem where it is protected against conventional control measures, also the ability of the pathogen to survive in the soil and colonize high number of host species. Therefore, it is necessary to study alternative methods of control such as use of compost and compost microorganism to suppress potato brown rot **(Hoitink and Fahy, 1986; De Ceuster and Hoitink, 1999; Hoitink, *et al.* 2001; Abd El-Ghafar, *et al.* 2004; Mikhail, *et al.* 2005 and Yousef, 2007).**

The present work aimed to: 1) isolation and identification of the causal organism of potato brown rot disease using traditional and advanced methods for detection and identification like, PCR and fatty acids profiling. 2) Isolation of compost microorganisms and studying the efficiency of some of these microorganisms against brown rot causal agent. 3) Identification of these microorganisms which having the abilities to inhibit the growth of *R. solanacearum.* 4) Studying the ability of these isolates to produce siderophores and antibiotics against *R solanacearum.* 5) Studying the effect of these isolates on disease severity under quarantine greenhouse conditions.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

1- Symptoms and isolation of potato brown rot pathogen:

King *et al.* (1954) found that isolation of *R. solanacearum* from infected plant materials such as potato tubers on ordinary bacteriological media such as King's B medium is being quite successful.

On tubers, external symptoms may or may not be visible according to the state of disease development, which depends highly on age of the host plants and environmental factors. In the later stage of disease development, bacteria pass through the vascular tissues and will emerge from the eyes as sticky, dirty-white, often bubbly masses to which the soil readily adheres. Cutting the diseased tubers revealed a browning and necrosis of the vascular ring and immediately surrounding tissues up to 0.5 cm in each side of the ring. A creamy fluidal exudates usually appears on the vascular ring of the cut surface **(Harrison, 1961; Fahy and Persley, 1983 and Shekhawat *et al.*, 1992b)**

Fahy and Persley (1983) reported that the first visible symptoms are wilting of the leaves at the ends of the branches during the warm weather of the day with recovery at night, eventually, infected plants fail to recover and die. A streaky brown discoloration of the stem may be observed on diseased stems up to 2.5 cm or more above the soil surface and the leaves have a bronze tint, as the disease develops. They reported also that isolation and identification of a phytopathogen are among steps of Koch's postulates of diagnosis of a plant disease.

Hayward (1991) stated that, tuber symptom was described as brown rot, cut tubers showed brownish discoloration of the vascular ring, and slight squeezing forces opus-like slime out of the ring, or it may exude naturally.

Shekhawat *et al.* (1992a) reported that the brown rot pathogen affects both above and below ground plant parts and damage can occur in two ways; premature wilting of foliage top growth namely bacterial wilt of potato plants, and rotting the tubers in the soil or during storage namely brown rot of potato tubers.

Ghimire (1993) reported that the disease caused by *Pseudomonas solanacearum* is the second most important constraint to potato production (after *Phytophthora infestans*) in Nepal. Field observation, tuber inspection, biovar typing and serological methods were used in detecting the disease and the pathogen.

Engelbrecht (1994) improved a semi selective medium (SMSA) in South Africa then modified by **Elphinstone *et al.* (1996, 1998)** to detect the pathogen from soil, water and potato tuber tissues. The limit of detection of the modified SMSA medium was 10^2 CFU per ml infected tuber homogenate **(Elphinstone *et al.*, 1996).** The described colony appearance on SMSA was found similar to that on triphenyltetrazolium chloride (TTC) medium **(Kelman, 1954)**.

Elphinstone *et al.* (2000) stated that symptomless (latent) infections are common, particularly at low temperatures. In this phase, the pathogen can survive in storage potato tubers for long distance transport and cause the disease in new environments whenever conditions favor its multiplication

Shambhu *et al.* (2001) performed the characterization of *Ralstonia solanacearum* strains, the causal agent of potato bacterial wilt disease from Nepal and Thailand based on pathogenicity, biochemical, physiological and serological tests. Fifteen *R. solanacearum* strains isolated from wilt infected potato plants and tubers grown in Nepal were characterized as race 3, biovar 2 based on the pathogenicity on different host plants.

Adipala *et al.* (2002) reported that *Ralstonia solanacearum* caused losses on a number of crops, especially solanaceaous crops in many countries in Sub-Saharan Africa. The disease affects potato, tomato, tobacco, eggplant, groundnuts and capsicums.

Balabel (2006) suggested that plating bacterial suspensions of *R. solanacearum* from different sources revealed virulent and avirulent forms. The first is described as milky, white, flat, irregular and fluidal with red coloration in the center. Avirulent form developed less fluidal or a fluidal colony which is completely pink to red.

Dean *et al.* (2006) suggested that early symptoms were wilting of the lower leaves with rolling of the leaf margins, subsequently leaves showed sectorial chlorosis and eventually papery brown necrosis. Sometimes only one part of the stem showed wilting symptoms.

Aissata (2007) stated that potato was one of the most important cash crops in Mali. Bacterial wilt caused by *Ralstonia solanacearum* has become a major problem causing important damage in production. The race and biovar of the bacterium present in Mali were identified as Race 1 biovar 3.

Abd El-Ghany (2010) surveyed the causal brown rot disease in many localities of potato cultivations in 4 Egyptian governorates where intensive potato cultivation was chiefly prevalent during season 2003-2004. The survey was carried out on potato tuber, water, soil and on some volunteer plants grown in potato fields and on borders of irrigation canals. The occurrence of potato brown rot disease was confirmed in all surveyed governorates with variable. In general, the highest percentage of positive samples was in Menoufia followed by Gharbia and Kalubia while the lowest one was in Ismailia governorate.

Siri *et al.* (2011) evaluated 28 strains of *R. solanacearum* isolated from major potato-growing areas in Uruguay, including 26 strains isolated from potato tubers and 2 from soil samples. All strains belonged to phylotype IIB, sequevar 1 (race 3, biovar 2).

Bader (2012) stated that infected potato plants with brown rot disease (caused by *Ralstonia solanacearum*) under greenhouse and field conditions exhibited yellow leaves or sudden wilting of leaves then dead plants, whitish exudates seen on the cut surface on tubers, a wet breakdown inhibited at the point of attachment of the stolon and the eyes of tubers. A light-brown breakdown of water-conducting tissues could be seen in tuber crosses. Milky fluid light is squeezed from this discolored area in infected potato tubers.

2- Identification of isolated bacteria:

Elphinstone *et al.* (1998) compared several methods for the detection of *R. solanacearum* in potato tubers, including, immunofluorescent antibody staining (IFAS), indirect ELISA, polymerase chain reaction (PCR), semi-selective culture and tomato bioassay methods.

2.1 Cultural and morphological characteristics:

Hayward (1991) reported that *Ralstonia solanacearum* is a strictly aerobic, gram-negative, short rod, non-spore forming and non capsulating bacterium. The bacterium's colony has an irregularly round form with fluidal, white and red center slime on SMSA and TTC media **(Kelman, 1954 and Elphinstone *et al.,* 1996)**.The bacterium highly motile, bear 1-4 polar flagella **(Kelman and Hruschka, 1973)**.

Balabel *et al.* (2005) suggested that plating bacterial suspensions of *Ralstonia solanacearum* from different sources on 2,3,5 triphenyltetrazolium chloride revealed virulent and avirulent colony forms. The first is described as milky, white, flat, irregular and fluidal with red coloration in the center. A virulent form developed less fluidal colony which is completely pink to red.

Atta (2008) stated that detection methods by plating on the SMSA medium showed that colonies with irregularly round shape and slimy white color with pink centers was considered the typical morphology of bacterial colony.

Bader (2012) isolated ten isolates of *R. solanacearum* (*Rs*) from the diseased potato tubers and soil collected from the tested fields in Qalubiya (Beltan and El-Hadaden) and Beheira (El-Tawfikia and Hosh-Eysa) governorates during growth seasons 2009-2011. All testes trails for identification of bacteria based on morphological, physiological and biochemical characteristics confirmed that all ten isolates were *R. solanacearum*.

2.2 Physiological and biochemical tests:

Buddenhagen *et al.* (1962); Pegg and Moffett (1971) and He *et al.* (1983) reported that five races of *R. solanacearum* were characterized according to host range as follows: Race 1: Attacks solanaceous plants. Race 2: Attacks musaceous plants. Race 3: Attacks potatoes only. Race 4: Attacks ginger. Race 5: Attacks mulberry.

Hayward (1964) and He *et al.* (1983) found that biovars classification of *R. solanacearum* is based on some biochemical properties *i.e.*, their ability to produce acids from hexose alcohol and sugars as follows:

Table (1): Biovars classification of *R. solanacearum* is based on some biochemical properties

Biovar	1	2	3	4	5
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-
Maltose	-	+	+	-	+
Lactose	-	+	+	-	+
Cellobiose	-	+	+	-	+

The dominant race in Egypt is race 3, biovar 2 **(Farag *et al.* 2004)**, being characterized by low virulence to tobacco and has a lower optimal temperature than other biovars **Buddenhagen and Kelman (1964); Hayward (1964); and Krieg & Holt (1984).**

2.3 Fatty acid profiling:

Bacteria contain lipids in concentrations of 0.2-50%, usually 5-10% of dry weight. For fatty acid analysis (FAA) the lipids containing esterified fatty acids are important. These are mainly: phospholipids,

(in the cell membrane), glycolipids, (in the cell membrane but less common than phospholipids), lipid A, (in the outer membrane of Gram-negative bacteria) and lipoteichoic acids, (cell wall components of Gram-positive bacteria). Bacterial fatty acids contain usually 9-20 carbons in a chain. They may be linear, branched, cyclic, saturated, unsaturated (in most cases mono-unsaturated) or containing 2- or 3-hydroxy-groups. Bacteria have the straight chain fatty acids in common with eukaryotes. The mono-unsaturated forms of these straight chains, however, usually have the double bond located at seven, instead of nine carbons from the terminal methyl group. Unique bacterial fatty acids are the hydroxy, cyclopropane and branched chain fatty acids. In Gram-negative bacteria the main saturated acids are 16:0, 18:0 and often also 14:0, most important unsaturated fatty acids are 16:1 cis or trans 9 and 18:1 cis or trans 11 and hydroxy acids (as part of the LPS) are common. Gram-positive bacteria usually contain important quantities of branched fatty acids **(O'Leary, 1967; Ratledge and Wilkinson, 1989)**.

The fatty acid content of the lipids and the occurrence of free fatty acids in the bacterial cell may differ qualitatively and/or quantitatively according to the genetic composition and physiological age of the bacterium, nutrients and oxygen available and temperature **(Casano *et al.,* 1988 and Rose, 1989)**. When grown and harvested under standardized conditions, bacteria show a total, whole cell fatty acid profile which differs from even closely related bacteria. Fatty acid patterns have shown to be

especially valuable tool for the classification and identification of bacteria.

Stead (1992) stated that classification of *P. solanacearum* and other pseudomonads by fatty acid profiling based on nucleic acid homology. Classification of Taxa in rRNA homology group 1, 2 and 3 was made on the basis of the types of hydroxy fatty acid found. Fatty acid group 1 members (the fluorescent pseudomonads) all had 10:0 30H while, group 2 had 12:0 30H. Whereas, group 3 members (now largely reclassified in *Comamonas* and *Acidovorax*) where all had 10:0 30H. *P. solanasearum* and its allies were further subdivided according to the additional presence of 16:0 30H, present in *P. cepaci* and its allies but absent in *P. solanacearum, P. syzygii* and the banana blood disease bacterium. Differentiation of races and biovars of *P. solanacearum* was not sufficiently accurate under the cultural condition selected. The use of slightly older cultures on other media may allow accurate race determination. However, fatty acid profiling showed that several strains of *P. solanacearum* tested had atypical profiles although they undoubtedly belong to this sub-group. Fatty acid profiling offers a rapid, accurate method for diagnosis of disease caused by *P. solanacearum.*

Stead *et al.* (1998) differentiate between the Gram-positive genera and Gram-negative ones based on fatty acids. The evidence that most fatty acids in the gram-negative bacteria have an even number of carbon atoms. While, branched acids with odd numbers of carbon atoms are common in the Gram-positive bacterium that is being contrary to the Gram-negative ones. Cyclopropane acids are almost

exclusively found in the Gram-negative bacteria. Unsaturated acids are found in most bacteria and most of these are mono-unsaturated, cis-forms although tri-saturated and trans-isomers are occasionally found.

Farag (2008) found that extracts of tubers and weed isolates are similar to large extent in FA content, with only slight variation. The extracts of the weed isolates, however, were poor in C15: ω6C, C15:0 and C17:0 compared to tuber ones. It may be noticed, however, that the extracts of both isolate sources did not show C13:0 and C17: 1ω8C compared to soil and water isolates. The absence of these fatty acids in the extracts may be indicative to the plant origin. While for soil and water isolates, the extract of the first was poor enough in the majority FA detected, compared to the second. The soil isolate extract of FA was lower in 18 FA out of 24 detected. It is important to note that extracts of water and soil isolates did not show C12:0 and C19:0 Iso, which may be indicative to their origins.

Khakvar *et al.* (2011) used FAME (Fatty acid methyl esters) analysis with *R. solanacearum* strains and showed that fatty acid composition were very variable. Nine types of fatty acids were identified and quantified among all 47 strains. Fatty acids 12:0 and 16:1 2OH were just found in the non-pathogenic strains in low quantities (always <2%). Concentrations of fatty acid (16:0) in the pathogenic isolates were always higher than non-pathogenic isolates. The dendrogram based on all fatty acids composition formed nine major clusters. All clusters were highly correlated with host and pathogenicity of bacterial strains but low correlation was found between biovar and FAME clusters. All 9 isolates that were clustered

in groups A, B and C were nonpathogenic with two main characteristics; two types of fatty acids (12:0 and 16:1 2OH) and lower concentration of one of main fatty acid (16:0). Also, there is a significant correlation between these FAME clusters and three non-pathogenic groups in PFGE (Pulsed Field Gel Electrophoresis) clustering. The rest (D to I) were included with all pathogenic strains. Correlation between FAME clusters and biovars in nonpathogenic groups was higher than pathogenic groups. Pearson's correlation tests showed that there is highly significant correlation between pathogenicity and concentration of one of individual fatty acid (14:0). Also, low but significant correlation was found between biovar type and 14:0 and 17:0 cyclo.

2.4 Immunofluorescence antibody stain (IFAS)

Janse (1988) used an IFAS and obtained high levels of sensitivity for detection of *R. solanacearum* in potato tissue. This method is usually used in conjunction with the tomato bioassay test. The detection level by the polyclonal antibodies in IFAS is 10^4 cells ml^{-1} and the false positive reaction due to cross reacting bacteria was limited to only 2.3% using two polyclonal antiserum against whole cells of *R. solanacearum.*

De Boer *et al.* (1996) stated that IFAS is considered as one of the most sensitive serological tests for detecting bacteria.

Matter (2008) stated that detection level of polyclonal antibody in IFAS is recorded of 10^4 cells ml^{-1} where the false reaction was limited to only 2-3%

Bader (2012) found that identification of tested bacterial isolates using immunofluorescent antibody staining technique (IFAS) gave

positive results which mean that these tested isolates are *R. solanacearum*. The morphology of bacterial cells appeared as short rod shape and green fluorescent with specific fluorescent-labeled antiserum.

2.5 Polymerase chain reaction (PCR) technique:

Tsai and Olson (1991) reported that the significant advantage of PCR is the potential for detection of a very small numbers of bacteria in the environment. However, detection of *R. solanacearum* from soil with PCR is quite difficult; one reason for this is due to the property of PCR that requires DNA free from soil materials particularly humic-materials that inhibit Taq polymerase in PCR. Another general problem with PCR technique is the inability to differentiate between dead cells and viable cells.

Picard *et al.* (1992) reported that the majority of laboratories became dependent mainly on PCR techniques in spite of the inhibition of the amplification reaction by compounds found in crude bacterial extracts which give false negative results or low detection sensitivity.

Schaad *et al.* (1995) developed the Bio-PCR technique to resolve the problems for detecting viable cells of *R. solanacearum* in soil. In this technique, an agar plating step prior to PCR analysis provides benefit of biological amplification of the PCR target. These combinations between a selective medium for amplifying viable cells of *R. solanacearum* and PCR for amplifying specific fragments of *R. solanacearum* DNA may improve the efficiency of the detection of viable cells of this pathogen in soil.

Weller *et al.* (2000) stated that a fluorogenic (Taq-Man) PCR assay was developed to detect *Ralstonia solanacearum* strains. Two fluorogenic probes were utilized in a multiplex reaction, one broad-range probe (RS) detecting all biovars of *R. solanacearum*, and a second more specific probe (B_2) detecting only biovar 2. Amplification of the target sequence was measured by the 5` nuclease activity of Taq DNA polymerase on each probe, resulting in emission of fluorescence. Taq-Man PCR was performed with DNA extracted from 42 isolates of *R. solanacearum* and genetically or serologically related strains to demonstrate the specificity of the assay. In pure culture, detection of *R. solanacearum* to $\geq 10^2$ cells ml^{-1} was achieved. Sensitivity decreased when Taq Man PCR was performed with inoculated potato tissue extracts. A third fluorogenic probe (COX), designed with the potato cytochrome oxidase gene sequence, was also developed for use an internal PCR control and was shown to detect potato DNA in an RS-COX multiplex Taq-Man PCR with infected potato tissue. The specificity and sensitivity of the assay, combined with high speed, robustness, reliability and the possibility of automating the technique, are potential advantages in routine indexing of potato tuber and other plant material for the presence of *R. solanacearum*.

Pastrik *et al.* (2002) used polymerase chain reaction (PCR) techniques to identify *R. solanacearum* strains the causal agent of potato brown rot disease.

Poussier *et al.* (2002) reported that DNA amplification for pathogen offers many advantages over traditional techniques; neither purification nor cultivation of the pathogen is required. The specificity, sensitivity and response time of tests are improved.

Kumar *et al.* (2004) isolated thirty-three strains of *Ralstonia solanacearum* from ginger, paprika, chilli, tomato, *Chromolaena* and potato. Phenotypic characterization for biovar revealed the predominance of biovar 3 in India. Molecular analysis by REP–PCR, ITS– PCR and RFLP–PCR classified the strains into three clusters at 70% similarity, where ginger strains are grouped in Clusters I and II. Strains from potato (biovar 2) clustered in the III cluster. Molecular analysis also revealed that ginger strains isolated from different locations during different years had 100% similarity according to Dice's coefficient. The analysis further revealed that the genetic diversity of *Ralstonia* is very low within ginger, confirming that the pathogen population is of clonal lineage and is distributed through 'rhizome transmission' of the inoculum between locations and also between seasons within the locality.

Fikre *et al.* (2010) assessed the genetic diversity among the Ethiopian strains of *Ralstonia solanacearum*, the causal agent of bacterial wilt, by repetitive sequence-based polymerase chain reaction (rep- PCR) method with BOX and ERIC primer sets. The study comprised 62 strains collected from potato, tomato and pepper grown in Ethiopia (43 strains were identified as biovar 2 race 3, and 19 as biovar 1 race 1) and five reference strains obtained from different countries. The rep-PCR defined two major groups (1 and 2) among Ethiopian strains at 55% similarity level, each matching to a single biovar. Group 1 comprised biovar 2 and group 2 biovar 1 strains. At 90% similarity level, biovar 2 strains were grouped into group five and biovar 1 into group one.

Bader (2012) identified five bacterial isolates among the ten isolates of those isolated from Beheira and Qalubiya governorates during season 2009 which previously identified as *R solanacearum* using the traditional and IFAS techniques using PCR technique visualized the specific DNA band with molecular weight 718bp in the five tested bacterial isolates and the positive control one under UV light. The results revealed also that there were very close similarity without any variation among the five tested isolates and the positive control one under investigation, confirming that these five tested bacterial isolates are *R solanacearum.*

2.6 Virulence of brown rot pathogen:

Kelman (1954) stated that the shift from high virulence to avirulence in *R. solanacearum* can be routinely detected on the basis of mucoid (highly virulent) and non mucoid (avirulent or less virulent) colony morphologies on Kelman's agar medium. This phenomenon is now known as phenotype conversion.

Morales and Sequeira (1985) studied the plasmid content of virulent and avirulent colony types of *Pseudomonas solanacearum* and found no detectable difference in the plasmids of highly virulent and avirulent colonies; however, **Negishi *et al.* (1990)** found that the mini plasmid (pJTPS1) was present in avirulent colonies but was absent in virulent types. **Brumbley and Denny (1990)** found that non expression of the regulatory gene PhcA is responsible for phenotype conversion to avirulence.

Habashy *et al.* (1991) reported that culture filtrate of virulent *R. solanacearum* showed low polygalacturonase (PG), high pectin methylestrase (PME) and high cellulase (Cx) activities as compared

with avirulent ones. Incorporation of calcium in the propagation medium resulted in a sharp decline in activity of PG and Cx secreted by the isolates under investigation. The virulent *R. solanacearum* showed low dehydrogenase activity as compared with avirulent ones. Viable cells of both forms of the pathogen did not show any β- glucosidase activity.

Huang *et al.* (1993) discovered a new *Ps. solanacearum* gene, vsrB, using TnphoA mutagenesis. When inactivated, the gene caused a major reduction in the virulence and production of an extracellular polysaccharide (EPS).

Aley *et al.* (1994) reported that the purified culture of *R. solanacearum* on King's medium can be inoculated in tomato or potato seedlings to confirm its pathogenicity. Young tomato or potato seedlings (third to fifth true leaves) are inoculated by injecting the stems with 20 μl of a bacterial suspension of *R. solanacearum* at 10^8 cells/ml with a 1 ml-propylene syringe and a hypodermic needle, just above the cotyledons. Greenhouse conditions that favor disease development are 28 ± 4°C, 80 - 90% R.H., and natural daylight. Plants are irrigated normally, except one day before inoculation. If the isolate is a pathogenic strain of *R. solanacearum*, the wilting of tomato or potato seedlings may begin in less than a week, but will certainly appear within 4 weeks.

Grimault *et al.* (1994) studied the colonization of resistant and susceptible tomato cultivars by *Ps. solanacearum* by light and electron microscopy to investigate the nature of the barriers involved in the limitation of bacterial spread in resistant cultivars. In resistant cultivars,

tyloses occluded the colonized vessels and the contiguous ones, limiting bacterial spread. In the wilting susceptible cultivars, no tyloses were observed in colonized vessels and bacterial spread was not limited. Tylose production in the susceptible cultivar seemed delayed and less focused compared with the resistant cultivar, because numerous non-colonized vessels were occluded by tyloses. Vascular colonization seemed generalized in the vascular bundle of the susceptible cultivar in contrast to the resistant one. Other reactions involved in resistance or susceptibility were observed, such as gums, cell wall breakdown and modifications of the primary cell wall. The limitation of bacterial spread associated with the resistance of tomato to bacterial wilt was thus mainly attributed to an induced, non-specific, physical barrier.

Elphinstone *et al.* (1996) reported that the bioassay test using tomato seedlings is reliable to detect as few as 10^4 cells per ml of suspension of infected potato extract. Tomato seedlings are widely used for both pathogenicity testing and typical wilting symptoms are usually apparent within a week of inoculation, depending on the inoculum potential of the bacterium and the availability of optimum environmental conditions.

Tans-Kersten *et al.* (1998) reported that *R. solanacearum* produces several extracellular plant cell wall-degrading enzymes, including polygalacturonases (PGs) and pectin methylesterase (PME). PME removes methyl groups from pectin, thereby facilitating subsequent breakdown of this cell wall component by PGs, which are known bacterial wilt virulence factors. *R. solanacearum* PGs could not degrade 93% methylated pectin unless the substrate has first been

demethylated by PME, but as the degree of methylation of the pectin substrate decreased, PG activity increased.

Huang and Allen (1998) reported that polygalacturonases (PGs) are significant virulence factors for this pathogen. *R. solanacearum* mutants lacking PehA (an endo-PG), or PehB (an exo-PG), or both PehA and PehB, are all less virulent than the wild-type strain on wounded eggplants. To more closely mimic the natural infection process, unwounded tomato plants were soil-soak inoculated with wild-type and PG mutant strains. All three PG mutants were significantly reduced in virulence on intact tomato plants, despite surviving well in potting mix. Over the course of disease development, populations of PG mutant and wild-type bacteria in plants were significantly different. The PG mutants, especially the two PehA mutants, colonized fewer tomato stems, colonized stems more slowly, and had lower mean bacterial populations in stems than the wild-type strain. These results suggest that PehA and PehB are necessary for rapid host colonization, and that production of these enzymes, contributes quantitatively to the ability of *R. solanacearum* to colonize host vascular tissue and wilt plants. A threshold bacterial population of around 1×10^8cfu in the centimeter of tomato stem directly above the cotyledons correlated with the appearance of wilt symptoms.

Gonzalez and Allen (2003) reported that *Ralstonia solanacearum* produces three extracellular polygalacturonases (PGs): PehA, PehB, and PehC. All three PGs hydrolyze pectin's polygalacturonic acid backbone, but each releases different reactions products. PehA and PehB contribute significantly to pathogen

virulence, probably by facilitating root invasion and colonization. To determine the collective contribution of PGs to virulence and saprophytic survival, they cloned, characterized, and mutated the *R. solanacearum* PehC gene, which encodes a distinctive monogalacturonate-releasing exo-PG. The virulence of a PehC mutant on tomato was indistinguishable from that of its wild-type parent; thus, this exo-PG alone does not contribute significantly to wilt pathogenesis. Unexpectedly, a completely PG-deficient triple PehA/B/C mutant was slightly more virulent than a PehA/B mutant. PehC may degrade galacturonide elicitors of host defense, thereby protecting the pathogen from plant antimicrobial responses. A galacturonate transporter gene, exuT, is immediately down stream of PehC and the two genes are co-transcribed. It has been hypothesized that galacturonic acid released by PGs from plant cell walls nourishes bacteria during pathogenesis. To separate the pectolytic and nutrient-generating role of the PGs, an exuT mutant was made which still produces all three isozymes of PG but cannot uptake PG degradation products. This exuT mutant had wild-type virulence on tomato, demonstrating that metabolism of galacturonic acid does not contribute significantly to bacterial success inside the plant.

El-Ariqi *et al.* (2005) showed that isolates of *R. solanacearum* displayed varying levels of virulence on potato Spunta and Diamont cultivars.

Siri *et al.* (2011) found that inoculation of *R. solanacearum* strains on tomato and potato plants showed different levels of aggressiveness for the first time among *R. solanacearum* strains belonging to phylotype IIB, sequevar 1.

Aggressiveness assays were also performed on accessions of *Solanum commersonii*, a wild species native to Uruguay that is a source of resistance for potato breeding. No significant interactions were found between bacterial strains and potato and *S. commersonii* genotypes, and differences in aggressiveness among *R. solanacearum* strains were consistent with previously identified groups based on tomato and potato inoculations. Moreover, variation in responses to *R. solanacearum* was observed among the *S. commersonii* accessions tested.

Mikhail *et al.* (2012) characterized the genetic variation between virulent and avirulent isolates of *Ralstonia solanacearum* race 3 (biovar II), the causal agent of potato brown rot disease. Nine isolates of *R. solanacearum* recovered from the natural habitats (potato tubers, weeds, soil and water) were used. Six virulent and three avirulent isolates were tested for their pathogenicity. Both virulent and avirulent forms of *R. solanacearum* were pathogenic to potato plants causing different symptoms.

Bader (2012) stated that regarding the virulence of *R. solanacearum*, all tested *R. solanacearum* isolates caused bacterial wilt disease symptoms on potato plants compared with the un-inoculated control in sterilized and un-sterilized soils. *R. solanacearum* (R6) recorded the highest infection percent and disease severity percent at 35 day post inoculation of potato plants (cv. Spunta) followed by *R. solanacearum* isolates (R1, R3 & R4). While the least infection percent was recorded by *R solanacearum* (R8) and (R5).

2.7 Host range

Tusiime *et al.* (1998) mentioned that no other bacterial pathogen attacks different plant species as *R. solanacearum.* Several hundred species representing more than 50 plant families have been identified as hosts of this pathogen as well as the number of new host species still increasing. The bacterium naturally infected solanaceous weed hosts like *Solanum dulcamara, S. nigrum, S. cinerum***.** Also, they reported a number of latently infected non – solanaceous weeds such as *Amaranthus* spp., *Bidens pilosa, Galinsoga perviflora, Oxalis latifolia, Spergula arvensis* , *Rumex abyssinicum, Tagates minuta* , *Stellaria sennii* and *Pelargonium zonale* in Uganda.

Hayward (2000) suggested that, the recorded host range of *R. solanacearum* is widely diverse. Representatives of more than 50 families of plants include one or more hosts; most are dicotyledons but banana (*Musa* spp.) and ginger (*Zingibar officinale*) and their relatives are major hosts in some parts of the world. Hosts of solanaceae include tomato, potato, tobacco, eggplant and pepper while, leguminous hosts include groundnut (peanut). Also, there are many solanaceous and composite weed hosts and some of these might be latently infected. Bacterial wilt has been recorded on various woody, perennial plants of economic importance in horticulture and in forestry.

Matter (2008) stated that using indicator plant such as tomato still had high accuracy for detecting the pathogen, therefore tomato seedlings were selected to detect pathogenicity for bacterial isolates caused wilt symptoms. No wilt symptoms were induced by any bacterial isolate against bean, maize and onion plants.

3- Compost collection and its microorganisms:

Hoitink and Fahy (1986) reported that specific physical, chemical, and biological properties of composts may have a major effect on their suppressiveness.

Beffa *et al.* (1996 a,b) reported that the incorporation of the compost microbial diversity into soil increases its fertility (e.g. nitrogen fixers, nitrifies, sulphur oxidizers), structure (e.g. exopolysaccharide producers), and can have other effects as a result of high activity and population levels, as well as through specific biochemical traits of the microorganisms.

Whitney and Lynch (1996) reported that during composting, the microbial community follows a predictable successional pattern resulting in the re-colonization of compost with metabolically active mesophilic populations that can be suppressive towards plant pathogens.

Hoitink *et al.* (1997) reported that increased numbers of microorganisms in compost also have an indirect role in improving plant health where microorganisms form symbiotic associations with plant roots and synthesize and excrete nutrients (amino acids, vitamins), plant growth hormones and chelators, alter physical conditions to optimize plant growth, and decompose or neutralize toxic substances. Otherwise, **Whipps (2001)** reported that microorganisms may also negatively affect each other through competition for nutrients, oxygen or space, or by excreting inhibitory metabolic products.

Chen *et al.* (1998) found that suppressive compost possess a higher microbial activity causes a depletion in essential nutrients for the

survival and multiplication of the pathogen, then preventing infection of the host. Also, the composts have the potential to provide a conductive environment for the proliferation of rhizobacteria antagonistic to certain soil borne root pathogens.

Tuitert *et al.* (1998) reported that additions of sources of organic matter such as compost or manure enhance the suppressiveness of soil towards a range of, mainly fungal, plant pathogens. However, antagonism from compost against soil-borne pathogens, e.g. *Rhizoctonia solani*, was recently shown to be dependent on the degree of compost maturation.

Van Bruggen and Semenov (2000) defined soil suppressiveness as the capacity of soils to restrict the survival and activity of plant pathogens also, soil suppressiveness might be enhanced due to an increased abundance and activity of antagonistic microbial populations.

Lazarovits (2001) reported that organic amendment containing high nitrogen, such as poultry manure, swine manure, meat and bone meal, and soy meal significantly reduced the population of the pathogens causing Verticillium wilt and common scab of the potato and plant parasitic nematodes.

Leslie (2002) defined composting as the intense microbial activity that leads to the decomposition of most biodegradable materials into biologically stable, humic substances that make excellent soil amendments.

Megan (2002) stated that compost is dark and earthy, humus-like material that is rich in variety of nutrients, minerals and beneficial soil organisms. In many cases, compost can be used in place of commercial fertilizer and is much more efficient in making nutrients steadily

available to plant life. It also improves the physical and biological properties of soil in ways that commercial fertilizer cannot. For these reasons, gardeners often refer to compost as "black gold".

Schonfeld *et al.* (2003) suppressed the incidence of tomato bacterial wilt disease caused by *Ralstonia solanacearum* in the soils amended with cow manure and household compost.

De Clerq *et al.* (2004) suggested that compost may provide substrates for organisms that enhance general antagonism through competition, or for specific agents that involve antibiosis or induced resistance against plant pathogens.

Postma *et al.* (2005) mentioned that, in multiple cases suppression of soil borne plant pathogens have been shown to be correlated with the microbial composition of the compost/peat or compost/soil mix.

Venglovsky *et al.* (2005) declined the population and survival of *R. solanacearum* at the early stage of composting, the compost pH during the early periods of composting process was decreased due to production of organic acids causing further acidification as a result of microbial activity. The effect of compost on *R. solanacearum* is biotic and related to shift in soil microbial community structure towards a community with enhanced antagonism.

Yousef (2007) used compost to increase soil fertility of reclaimed lands, improve existing crop land, stimulate growth and suppress diseases caused by soil-borne plant pathogens.

Bader (2012) found that adding the different compost types to the infected soil with brown rot pathogen reduced significantly the

disease incidence compared with the control treatment (without compost).

4- Antagonistic effect of isolated bacteria and fungi against *R. solanacearum in vitro:*

Papavizas *et al.* (1968) stated that antagonism is an important factor affecting the population density and survival of the pathogen in the rhizosphere.

Kempe and Sequeira (1983) used some antagonistic bacteria to induce resistance against bacterial wilt disease by treating potato tubers with the antagonistic bacteria and found that, strain 70 of avirulent *Pseudomonas solanacearum* inhibited the growth of virulent strain 276 whereas strain B 82 of avirulent *P. solanacearum* and the strains of *P. syringae lachrymans* and *P. syringae glycinea* did not. The two *P. fluorescens* strains (w 163 and w p 95) were inhibitory to strain 276 of highly virulent *P. solanacearum.* On the other hand, strain 276 did not inhibit the growth of any of the other strains used.

Anuratha and Gananamanickam (1990) stated that from among 125 strains of fluorescent and 52 strains of non-fluorescent bacteria initially screened in the laboratory for their antibiosis towards the bacterial wilt pathogen, *Pseudomonas solanacearum*, strain Pfcp of *Pseudomonas fluorescens* and strains B 33 and B 36 of *Bacillus* spp., were chosen and evaluated further in greenhouse and field tests. Pfcp, treated banana, eggplant and tomato plants were protected from wilt up to 50, 61 and 95% in greenhouse and up to 50, 49 and 36% respectively in field. Also, they mentioned that protection afforded by bacillus strains was lower, and that the plant height and biomass increased in the infected plants with *R. solanacearum* and treated with the

antagonistic bacteria and were close to those of non-infected and non-treated control plants.

Stindt (1990) found that 33 bacterial antagonists, belonging to the genera Pseudomonas, Bacillus and Enterobacter isolated from cattle manure compost extracts, inhibited the conidial germination of *Botrytis cinerea.*

Ketterer (1990) isolated two bacterial strains with antagonistic properties against *Phytophthora infestans* on detached potato leaves from extracts of composted horse manure.

Phae *et al.* (1992) isolated some strains of *Bacillus subtilis* from compost that showed suppressive effect against several phytopathogenic fungi such as *Verticillium dahliae* and *Fusarium oxysporum f.sp. lycoperisici.*

Shekhawat *et al.* (1993) mentioned that several microorganisms have been tried out with variable success for biological control of bacterial wilt.

Holloway (1995) mentioned that antibiosis, competition and parasitism are three types of antagonism activity. Also, there is no effective chemical control against potato bacterial wilt. Therefore, the disease incidence can only be decreased if more than one control components are combined such as the planting of healthy seeds in clean soil, planting of tolerant potato varieties, rotation with non-susceptible crops and the application of various sanitation and cultivation practices. He added that some strains were antagonistic in vitro but had no control effect in greenhouse and filed. On the other hand, some strains not only

antagonistic in vitro but also had a good effect in the greenhouse and field.

Karuna *et al.* (1997) used three bioagents i.e., *Pseudomonas fluorescence, P. aeruginosa* and *Bacillus subtilis* in inhibiting the growth of *Ralstonia solanacearum.* They found that *P. fluorescence* produced the highest inhibition zone followed by *B. subtilis.*

Abd Alla *et al.* (1999) reported that *Bacillus subtilis* (B1), *Pseudomonas* spp (106, 115) and *Pseudomonas cepacia* had high inhibitory effect against *R. solanacearum* race 1 bv. 4 isolated from wilted tomato plants in Al-Qassim region. Central Saudi Arabia.

Toyota and Kimura (2000) studied the suppressive effect of some antagonistic bacteria on *R. solanacearum* and they found that *P. fluorescens* and *B. subtilis* cause a great suppressive effect when introduced to the soil before *R. Solanacearum.*

Tawfik *et al.* (2001) evaluated the pre-plant treatment of seed tubers (*cv.* Spunta) with potato antagonistic rhizospheric isolates belonging to *Bacillus subtilis, P. fluorescens* and *Pseudomonas putida* for their effect on brown rot, soft rot and black leg development during their successive summer seasons. Isolates of fluorescent pseudomonas as well as *Bacillus subtilis* were the most efficient in bio-controlling the aforementioned diseases in soil. However, the commercial formulation of rhizo-N (*B. subtilis*) showed efficiency. Also, significant increases in yield of potato plantation were achieved by these treatments.

Zayed (2004) reported that *Pseudomonas fluorescens, P. aeruginosa* and *P. putida* were more effective in inhibiting the growth of *R. solanacearum* on king's B medium than on nutrient agar medium. However, *Streptomyces* spp. was less effective on NA medium, where

inhibition length was 2 mm. Meanwhile, this isolate was not able to inhibit growth of the pathogen on KB medium.

Lotta (2008) evaluated *Pseudomonas fluorescens* SBW25 as a bio-agent towards the phytopathogenic bacterium *Ralstonia solanacearum* on tomato and potato plants. By using gfp-tagged *R. solanacearum* and red fluorescent protein (rfp) -tagged SBW25 it was possible to distinguish these two strains on plant surfaces using microscopic techniques. Some bio-control effects of SBW25 were detected on tomato plants. Very specific interactions were found and also synergistic bio-control effects suggesting that a consortium of microorganisms might be a better choice when applied as bio-control agents.

Naser *et al.* (2008) screened one hundred-twenty rhizosphere bacterial isolates as plant growth-promoting rhizobacteria (PGPR) in potato against virulent strain of *R. solanacearum* PPRC-Rs. After in vitro screening, they detected six antagonistic strains i.e., *P. fluorescens* MRI, *B. subtilis*-DFS, *P. fluorescens* 9, *P. fluorescens* 20, *B. circulans*, and *B. subtilis*-wly were selected and studied further in the greenhouse.

Seafelyazel (2008) reported that among the bacterial, isolates recovered from the rhizosphere of potato plants, only six bacterial isolates showed antagonistic effect against *R. solanacearum* in vitro. The bacterial isolates were *Pseudomonas tolaasii;* two isolates of *P. syringae;* three isolates of *P. fluorescens*.

Amara *et al.* (2009) revealed that fluorescent pseudomonad's is a group of bacteria used for biological control against soil-borne plant pathogens. Thirty two species were isolated from the rhizosphere of

different plants then tested for their activity as phosphate dissolvers and producers of antibiotic-like substance. The most potent four isolates were species *P. aeruginosa, P. fluorescens* biovar I, *P. fluorescens* biovar II and *P. putida* biovar B. The highest salicylic acid and siderophores were produced by *P. aeruginosa* and *P. fluorescens* biovar I respectively. All bacterial treatments caused considerable increase of both total microbial or pseudomonad's counts in the rhizosphere resulted in a significant increase in all growth parameters especially total tan in content, which was higher in pseudomonad's-treated plants than untreated ones. It might be concluded that some fluorescent Pseudomonas species could be used successfully to promote plant growth and to suppress plant disease.

Bader (2012) studied the effect of some bioagents on brown rot infection and some growth characters of potato plants, all tested bioagents inhibited the growth of *R. solanacearum* compared with control. The highest inhibition zone of *R. solanacearum* growth was recorded with *Ps. fluorescens* (*Pf*-5) on KBA medium, followed *Ps. fluorescens* (*Pf*-4 & *Pf*-6) on KBA medium, *Bacillus subtilis* (*Bs*-3) on NA medium, *B. subtilis* (*Bs*-1 & *Bs*-2) on GNA medium, *Ps. fluorescens (Pf-4)* on GNA medium, *B. subtilis (Bs-1 & Bs-2),* respectively compared with control. Also, all tested bioagents reduced effectively the incidence of potato brown rot disease *in vivo* except isolate of *B. circulans* which was the least effective one.

5. Identification of antagonistic bacterial isolates:

Selenska-Pobell (1994) stated that traditional assignments of bacterial isolates into identity groups via physiological traits can be misleading due to differential expression patterns according to cell

growth stage or growth media so development of species and strains specific molecular markers would overcome such barriers.

Versalovic *et al.* (1994) stated that traditional identification based on Gram stain, colony morphology, motility, and biochemical testing is time-consuming, somewhat subjective and labour intensive procedures.

Stenbro-Olsen (1998) reported that a variety of methods have been used so far to investigate the microorganisms during composting. These include the use of traditional plating and identification of culturable microorganisms for determining microbial diversity during composting.

El-Ariqi (2001) isolated nine isolates of *Bacillus* spp. from potato rhizosphere soil. These isolates were spore former, growth in 7% NaCl was negative, starch hydrolysis was positive and acid production from sucrose, D (+) mannose, mannitol and D (-) fructose was also positive.

Tiquia and Michel (2002) mentioned that there are more recent methods that give an indication of the microbial community composition in compost without culturing of organisms on agar media, such as the direct analysis of phospholipid fatty acid (PLFA) patterns and of extractable DNAs or rRNAs.

Horwood *et al.* (2004) mentioned that biochemical characteristics are useful in distinguishing two different bacterial genera or species. Further it can also identify different compounds produced by different bacterial strains.

Liu *et al.* (2008) isolated four effective antagonistic *Bacillus* strains from the rhizosphere soil of cucumber and found them producers of the antifungal volatiles. Interactions mediated by microbial volatiles could be widespread in soils, and volatiles may play an important role in reducing disease levels. Through headspace sampling and Gas chromatography (GC) and mass spectrometry (MS) analysis, a rich profile was found from *B. subtilis* and overlapping volatile patterns could be found among the different species. Studies are under the way to find the possible action mechanisms and to seek the effective application of bacterial volatiles in greenhouse.

Bader (2012) found that all *Bacillus* spp isolates (No. 1, 2 and 3) and the fluorescent pseudomonads isolates (4, 5 and 6) are non-pathogenic and were identified as *Bacillus subtilis* and *Pseudomonas fluorescens* according to their morphological and physiological characters.

5.1 Polymerase chain reaction:

Claus and Berkeley (1986) reported that over than 60 species of genus *Bacillus* are recognized which display an enormous range of phenotypes including for example: strict aerobes, facultative anaerobes and even acidophiles, alcalophiles, chemolithotrophs, halophiles, psychrophiles and thermophiles. In addition to this very considerable phenotypic heterogeneity, the chromosomal DNA base composition varies from 33 (*B. anthracis*) to 64 (*B. schlegelii*) mol% G+C, which is too great to be encompassed by a single genus.

Welsh *et al.* (1990) stated that PCR is a method for the rapid amplification of specific sequence of DNA which uses two oligonucleotides primers complementary to opposite strands of the

sequence to amplify the DNA in a sample that denaturated and cooled to allow annealing of the primers to the single strand template.

Ash *et al.* (1991) stated that over the past decade great advances have been made in elucidating the 'natural' interrelationships of bacilli. In particular, 16S rRNA oligonucleotides cataloguing and more recently comprehensive 16S rRNA (or gene) sequence analyses that finally shown that the genus *Bacillus* is phylogenetically very heterogeneous.

Versalovic *et al.* (1991) reported that the finger printing of gram negative bacteria has been facilitated through the use of the (PCR) technique using oligonucleotides complementary to repetitive extragenic palindromic (REP) or enterobacterial repetitive intergenic consensus (ERIC) elements, however, the rarity of these elements in gram positive bacteria such as *Bacillus* precludes their utilization. Consequently alternative primers have employed to distinguish closely related bacillus species.

Petersen *et al.* (1995) designed a specific PCR test based on the 23S rRNA gene that could identify *Bacillus polymyxa* and *Bacillus megaterium.*

Zhou *et al.* (1996) reported that PCR analysis provides a sensitive and specific means to detect and monitor microorganisms in complex environmental samples.

Wattiau *et al.* (2001) designed a PCR test based on the 16S rRNA gene that could identify *Bacillus*. The test was directly applicable to single colonies and showed excellent specificity.

6. Ability of antagonistic isolates to produce siderophores:

Neilands (1995) mentioned that the name siderophore is a Greek word means 'iron carrier'. Siderophores are defined as low molecular-weight organic compounds with high binding affinity to ferric ions (Fe3+).

Cendrowski *et al.* (2004) showed that some bacterium and fungi can produce more than one type of siderophore.

Butler and Martin (2005) showed that the mechanism of Fe uptake may involve reduction of Fe_3 to Fe2, however, the efficiency of uptake will depend on the Fe-siderophore complex. Also, the efficiency of siderophore excretion and Fe-siderophore uptake can be affected by physics of diffusion, chemical kinetics of coordination reaction and by kinetics of uptake by the cell.

Das *et al.* (2007) reported that siderophores are produced as free ligands in cell cultures or the natural environment, where they preferably bind to Fe3+. However, they also exhibit affinity to other metals.

Diaz de Villegas (2007) reported that siderophores produced by bacteria, fungi and some monocotyledonous plants also, they solubilize Fe3+ and transport it into the cell through siderophore-specific receptors.

Mawji *et al.* (2008) mentioned that in bacteria the organisms typically obtained Fe from the Fe-siderophore complexes. The Fe-siderophore compounds are transported through their outer membranes using a receptor, which recognize the Fe-complex compounds while, in the periplastic space a transporter is used to internalize Fe.

Hopkinson and Morel (2009) reported that in organisms which lack the outer membrane receptors, the likely Fe uptake mechanisms involved reduction of Fe3+ to Fe2+. The Fe-siderophore becomes available through cell surface reduction or by photoreduction of the photolabile Fe-siderophore complex. The reducible forms (Fe, FeY and FeSid) diffuse to the cell, which are reduced by an enzyme (cell surface reductase). This weakens the complex and aids Fe transport.

Vraspir and Butler (2009) stated that iron is essential for many life processes including photosynthesis, respiration, and nitrogen fixation also, many microorganisms that grow under aerobic condition need at least 1 μM of Fe to grow.

Butler and Theisen (2010) reported that siderophores are classified based on different criteria (e.g. linear or cyclic; or according to the organisms that produce the siderophores), however, most studies base their classification on the chemical nature of the functional groups donating the oxygen ligands for Fe3+. The three common functional groups that coordinate to Fe3+ in siderophores are hydroxamic acids, catechols, and α-hydroxy-carboxylic.

7. Ability of the antagonistic isolates to produce antibiotics:

Cane *et al.* (1998) stated that majority of the antibiotics from *Bacillus* sp., are low molecular weight peptides, produced *via* the nonribosomal biosynthetic pathway, which involves specific enzymes called peptide synthetases. These peptides possess a varied range of remarkable biological activities, including antimicrobial, antiviral and antitumoral activities.

Ming and Epperson (2002) reported that most of the peptide antibiotics produced by *Bacillus* are active against Gram positive bacteria. However, compounds such as polymyxin, colistin, and circulin exhibit activity almost exclusively upon Gram-negative forms, whereas bacillomycin, mycobacillin, and fungistatin are effective agents against molds and yeasts.

Schallmey *et al.* (2004) reported that there are many species of the genus *Bacillus* which can produce a wide variety of antibiotics including bacitracin, polymyxin, colistin etc.

Sharga *et al.* (2004) stated that antibiotic production is a feature of several kinds of soil bacteria and fungi and may represent a survival mechanism whereby organisms can eliminate competition and colonize a niche also; Members of the genera *Bacillus*, *Streptomyces,* and *Pseudomonas* are soil bacteria that produce a high proportion of agriculturally and medically important antibiotics.

Jamil *et al.* (2007) assumed that soil microbes in nature produces antibiotics in their natural habitat and use them to gain advantage over their competitors; that is, antibiotics are presumed to be involved in naturally occurring amensal relationship in the soil.

Awais *et al.* (2010) defined antibiotics as low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. Also, he reported that the amount of antibiotics produced by bacilli was approaching 167, being 66 derived from *B. subtilis*, 23 from *B. brevis* and the remaining peptide antibiotics are produced by other species of genus *Bacillus.* He reported also that using synthetic media containing all essential minerals, carbon and nitrogen sources as production media is required because certain

metal ions are required for the activity and proper functioning of polypeptide antibiotics, such as bleomycin, streptonigrin, and bacitracin.

8. Biological control of brown rot disease:

Kempe and Sequeira (1983) treated tuber seed pieces in the greenhouse experiment, by dipping them in suspension of different bacteria or by introducing the bacteria directly into the basis of the emerging buds, they found that, treatment with the *Pseudomonas solanacearum* and *P. fluorescens* strains caused a significant reduction in disease severity. Also, there was no correlation between effectiveness of tuber treatment with different bacterial strains and their ability to inhibit growth of *P. solanacearum* in vitro.

Akiew *et al.* (1993) reported that biological control not only suppresses the disease and increases the crop yield but also it will be important in preventing the environmental pollution due to pesticides.

Sunaina *et al.* (1997) reported that three *Bacillus* spp., *i.e., B. subtilis* (S1, B5), *B. cereus* (B4) and an avirulent strain of *P. solanacearum* (BCA) exhibited antagonism against the bacterial wilt pathogen under *in vitro* and greenhouse conditions. Also they tested their antagonistic activities in field and reported that healthy seed potatoes bacterized with B4, B5 and BCA brought down the incidence of wilt to nearly one third in case of B4, and B5 and one fourth in case of BCA compared to control. Reduction in brown rot infection in harvested potato tubers was brought down to be nearly one third by B4, one fourth by B5 and one sixth by BCA from that of control. Substantial increases in percent yields of 22.72, 46.47 and 62.27 were

obtained compared to the control, when healthy tubers were treated before planting with B4, B5 and BCA, respectively. Also, they found that S1 of *B. subtilis* did not prove effective in controlling bacterial wilt when tried in the field even though it did inhibit *P. solanacearum* under culture and greenhouse conditions.

Abd Alla *et al.* (1999) found that in greenhouse studies, a significant reduction in the number of wilted plants could be achieved by the application of antagonistic bacteria such as *Bacillus subtilis*, *Pseudomonas* spp. and *Pseudomonas cepacia* to tomato seedlings which were then infested with *R. solanacearum.*

Ramamoorthy *et al.* (2001) reported that biological control of bacterial wilt disease could be achieved using antagonistic bacteria. Moreover, **Reiter *et al.* (2002)** used some antagonistic microbes i.e. *Alcaligenes* spp. and *Kluyvera* spp., *Pseudomonas fluorescens*, *P. alcaligenes*, *P. putida*, *Flavobacterium* spp., *B. pumilus*, *Microbacterium* spp., *Clavibacter michiganensis, Curtobacterium* spp. *B. subtilis* and *Bacillus megaterium* in the control of *R. solanacearum* under field conditions.

Zayed (2004) found that the application of *B. subtilis, P. aeruginosa, P. fluorescens, P. putida* and *Streptomyces* sp. isolates as soil treatments were more effective than tuber treatments in decreasing severity of potato bacterial wilt and increasing the yield. However, *P. aeruginosa* isolate was the most effective in reducing disease severity and increasing the yield as soil and tuber treatments. Meantime, *Streptomyces sp.* isolate was less effective in disease reduction as soil and tuber treatments respectively.

Naser *et al.* (2008) evaluated six antagonistic strains i.e., *P. fluorescens* MRI, *B. subtilis*-DFS, *P. fluorescens* 9, *P. fluorescens* 20, *B. circulans*, and *B. subtilis*-wly in the greenhouse for their effect in suppressing disease development in terms of area under disease progress curve (AUDPC) and increasing biomass (plant height and dry weight) of potato. Accordingly, PF-MRI, BS-DFS, and PF9, significantly reduced AUDPC by 78.6, 66, and 64.3%, and wilt incidence by 82.7, 66.2, and 65.7%, respectively, compared to the control. During the sole application, the strains significantly ($P < 0.0001$) increased plant height by 35.6, 45.9, and 45%, and dry matter by 111, 130.4, and 129%, respectively compared to non-bacterized control. In the presence of the pathogen strain PF-MRI, BS-DFS, and PF9 increased plant height by 66, 50, and 48.2%, and dry matter by 153.8, 96.8, and 92.5%, respectively compared to the pathogen treated control.

Bader (2012) found that the highest effective bioagents in reducing the potato brown rot disease incidence were *B. subtilis* (*Bs*1), *Ps. fluorescens* (*Pf*-4 & 5) followed by *B. subtilis* (*Bs*-2 & *Bs*-3), *Ps. fluorescens* (*Pf* -6), *B. megaterium* (*Bm-7*) and *P. polymyxa* (*Pp-8*), respectively compared with *R. solanacearum* treatment. On the other hand, all tested bioagents increased the fresh and dry weights of shoots and roots of potato plants (cv. Cara) except *B. circulans* treatment. The highest increases in fresh and dry weights of potato shoots and roots were recorded with using *P. polymyxa* (*Pp*-8) followed by *B. subtilis* (*Bs-1*, *Bs*-2 & *Bs*-3), *Ps. fluorescens* (*Pf*-4, *Pf*-5 & *Pf-6*) and *B. megaterium* (*Bm*-7), respectively.

MATERIAL
AND METHODS

MATERIALS & METHODS

1.Isolation of *Ralstonia solanacearum* from naturally infected potato tubers:

Naturally infected samples of potato tubers cvs. Spunta, Nicola, Draga showing external and internal symptoms of potato brown rot disease were collected from the production of potato cultivations of Talia village, Menoufya governorate for investigation by the Potato Brown Rot Project (PBRP), Agric. Res. Center, Giza – Egypt. These infected samples were used in isolation trials of *Ralstonia solanacearum* the causal agent of potato brown rot disease.

Infected potato tubers were washed in running tap water, surface sterilized with 90% alcohol by flaming and the stolon ends were aseptically removed. Cores of 5-10 mm diameter and 5 mm length, containing mainly vascular and cortical tissues were macerated in 1.0 mL of sterile phosphate buffer; the suspension then was transferred to sterile 1 mL eppendorf. The macerate was allowed to stand for 30 minutes. Plating was made on modified Selective Medium South Africa (SMSA) which considered the selective medium for isolation of *R. solanacearum* as described by **Engelbrecht, (1994)** and modified by **Elphinstone, (1996).** Purification was made by streaking and plating for typical colonies on basal and nutrient agar (NA) medium. Incubation was held at 28°C and daily observed for developing fluidal, slightly raised, irregular white or white with pink center colonies, typical for virulent colonies of *Ralstonia solanacearum*. Colonies were selected, picked up and streaked on glucose nutrient agar medium, incubated for 48hr at 28°C **(Dowson, 1957)** for further studies.

2. Identification of isolated bacteria:

Cultural and morphological characteristics, physiological and biochemical tests, immunofluorescence (IFAS), polymerase chain reaction (PCR), bioassay test (pathogenicity test), were used for identification of the isolated bacteria as follows:

2.1. Cultural and morphological characteristics:

Cultural and morphological characteristics of the isolated bacterium were studied by re-inoculating them on nutrient agar (NA), King's B (KB) and SMSA media and were compared by description of **Engelbrecht (1994) and Elphinstone *et al.* (1996).** Shape of bacterial cells, sporulation and reaction to gram stain were recorded.

2.2. Physiological and biochemical tests:

Physiological and biochemical tests were studied according the methods described by **McCarter (1991) and Bergy and Holt, (1994).**

Biovar identification was determined by the ability of isolates to oxidize lactose, maltose, sorbitol, mannitol, cellobios and dulcitol. Where tests were performed at 28 – 30°C **(Hayward, 1964).**

2.3 Identification by fatty acid profiling:

For many years analysis of short chain fatty acids or volatile fatty acids has been routinely used for identification of bacteria. The fatty acids between 9 and 20 carbon atoms in length have been used to characterize genera and species of bacteria. The use of gas chromatography of whole cell fatty acid methyl esters to identify a wide range of bacteria was practically adapted. Fatty acid profiles were prepared according to standard protocols **(Stead, 1992)** using the Microbial Identification System based on software available

commercially (MIDI) or self-generated using MIDI library generation analysis **(Roy, 1988).**

This test was carried out using Gas chromatography system at central search laboratory, York, UK. The method is briefly described in the following: The bacterial isolates were all grown on trypticase soy broth agar (TSBA) at 28°C for either 24 or 48 hours to minimize the effect of different growth conditions, and harvested in glass tubes then saponification (reagent 1), methylation (reagent 2), extraction in organic solvent (reagent 3) and washing of the extract (reagent 4) was carried out as follows:

2.3.1 Saponification:

One millilitre of reagent 1 was added to each tube containing cells. Tubes were heated in a boiling water bath for 5 min, vigorously shaken for 5-10 second and returned to the water bath to complete 30 min heating, then cooled under tap water and kept at room temperature.

2.3.2 Methylation:

Two mL of reagent 2 were added into each cooled tube. Tubes were heated and were shaken for 10 min at 80°C, then cooled under tap water and kept at room temperature.

2.3.2. Extraction:

Reagent 3 was added to each cooled tube at the rate of 1.6 mL and gently tumbling on a clinical rotator for about 10 min. The aqueous phase was then pipetted out and discarded.

2.3.3 Washing:

Three mL of (reagent 3) were added to the organic phases remaining in test tubes. Tubes were tumbled for 5 min. about 2/3 of the

organic phase was pipetted into a gas-chromatography vial for analysis. The Microbial Identification System (MIDI) uses an external calibration mixture. The standard is a mixture of the straight chain saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and has five hydroxy acids in the mixture. All compounds are added quantitatively so the gas chromatographic performance would be evaluated by the software each time the calibration mixture is analysed. The databases consisted of more than 60,000 analyses of strains obtained from experts and from culture collections. Analysis of an unknown culture results in an automatic comparison of the composition of the unknown strain to a stored database was done using a covariance matrix, principal component analysis and pattern recognition software.

2.4. Immunofluorescence antibody stain (IFAS):

IFAS is a serological method for rapid detection and presumptive identification of bacteria. The polyclonal antibodies were produced in rabbits against living whole cells **(Janse, 1988)** heat killed **(Coleno *et al.*, 1976)** formalized **(Harrison and Freeman, 1961)** or glutaraldehyde-fixed **(Robinson, 1993).** The anti-rabbit antiserum is conjugated with fluorescin isothyocyanate (FITC) and used in IFAS testing.

All isolates of *R. solanacearum* were grown on SMSA medium then subcultured on nutrient agar medium **(Jacobs and Gerstein, 1960),** A suspension containing 10^6 cells per mL from the culture and reference was prepared from a 48 hr culture and adjusted to a standard optical density at 590nm to 10^6 colony forming units (cfu/mL), then a standard volume of 20 µL was pipetted on five successive windows of a 10-window test slide (multi-well slides 6 mm window diameter, ICN,

USA). Slides were air dried at room temperature (or on a hot-plate at 40°C) and gently heat-fixed by flaming **(PBRP protocol, Egypt)**. All windows were covered with 25 µL of the antiserum (anti *R. solanacearum* polyclonal) in 4 dilutions (1:800, 1:1600, 1:3200 and 1:6400). Slides were incubated for 30 min at room temperature in a humid chamber, washed with tween buffer and 0.01 M phosphate buffer (PB), and then incubated with 25 µL of anti-rabbit Nordic SW/AR fluorescein isothiocyanate conjugate in a 100 fold dilution (prepared in phosphate buffer saline) for 30 min in a humid chamber. Slides were then washed with tween buffer and 0.01 M PB, and then the excess moisture was removed carefully by blotting with filter paper. One droplet of 0.1 mol l^{-1} phosphate buffer glycerin (pH 7.6) was added to each window and the slides were covered with long cover glasses. Slides were examined with a microscope (tube factor 1.25) with an epifluerescent light source and suitable filters with FITC, using a 100 (600X) oil immersion objective and a 10 X eyepiece. At least 20 microscope fields per window were scanned for the presence of morphologically typical fluorescing cells **(Janse, 1988).** This test was carried out potato brown rot project (PBRP)-Dokki-Egypt.

2.5 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is based on the techniques described by **Pastrik *et al.* (2002)** without the internal control (no potato primers were used) but using *R. solanacearum* specific oligonucleotide primers i.e. forward primer Rs-1-F (5`-ACT AAC GAA GCA GAG ATG CAT TA -3`) and reverse primer Rs-1-R (5`-CCC AGT CAC GGC AGA GAC T- 3`). The expected amplicon size

from *R. solanacearum* template DNA = 718bp (Rs-primer set). The primers were provided by Applied Biosystems company-Egypt.

2.5.1. Extraction of DNA

Crude DNA was extracted in 150 μL eppendorf by heating of 100 μL aliquots of each cell suspension (10^6 CFU/mL) to 100°C for 5 min. Boiled samples were placed on ice for 5 min or stored at (-20°C) until use.

2.5.2. DNA amplification

Two μL of extracted DNA was added to 23 μL reaction mixtures [(14.25 μL, Sterile Ultra-Pure Water; 2.5 μL of 10X PCR buffer; 1.5 μL $MgCl_2$ (25mM) Promega; (d-nTP mix 10mM) 0.0625 μL of each d-ATP, d-CTP, d-GTP and d-TTP; 2 μL Rs-1-F primer 10μM; 2 μL Rs-1-R primer 10μM and 0.2 μL Taq polymerase (5U/μL))] in 0.2 mL micro tubes DNA-RNA free. Different PCR cycles were performed in PCR machine (Biometra, T-personal, Germany) according to **(Pastrik *et al.*, 2002)** **(I)** 1 cycle of 5 min at 95°C to denaturate template DNA; **(II)** 35 cycles of 30 seconds each at 95°C for denaturation. **(III)** 1 cycle of 30 seconds at 58°C for annealing of primers. **(IV)** 1 cycle of 45 seconds each at 72°C for extension of copy. **(V)** final extension cycle of 5 min at 72°C. **(VI)** holding at 4°C.

2.5.3. Analysis of the PCR product:

PCR fragments were detected by agarose gel 3% electrophoresis horizontal system and stained using eithidium bromide solution (0.5 μg mL^{-1}) (3μL for each prepared 60mL gel). Agarose gel was prepared using 1X Tris Acetate EDTA (TAE) buffer **(Pastrik *et al.*, 2002).** Agarose in TAE buffer was boiled for 5 min then cooled to 50-60°C. Lanes on gel were made using specific combs at 10-15 mm from the

edge of the comb, the tray was placed in a large electrophoresis tank containing (1X) TAE buffer (the same buffer used for gel preparation) to at a depth of at least 5 mm buffer above the gel. Three μL droplets of loading buffer were loaded on parafilm and mixed to 12 μL of the PCR product (amplicons) for each sample. A reference positive control (PBRP, Egypt) and negative control of distilled water were separately mixed with the loading buffer then loaded into the wells of the gel. An appropriate DNA marker was included as reference in at least one well. Gel was run on 80 V at 400 mA (8 v/1cm) until the front of tracking indicator being within 1 cm from the end then the power supply was switched off. Gel was removed carefully. A specific PCR product of 718 bp was visualized under UV trans-illumination (at 355 nm).

2.6 Real-time, Fluoro genic PCR (Taq-Man) Assay

2.6.1. Bacterial suspension preparation and DNA extraction

Strains were grown (24 h at 27°C) on Casamino Acids Peptone Glucose (CPG) Agar **(Kelman, 1954).** DNA extraction was done according to **Weller *et al.* (2000),** where a single colony was transferred to sterile DNA- RNA free eppendorf containing 100 μl of sterile nucleic acid-free water, vortexed, heated to 100°C for 5 min and cooled rapidly on ice. Samples were finally diluted in 900 μl of the sterile DNA- RNA free water and stored at (-20°C) until required. Automated DNA extraction was carried out using Biosprint 15, Qiagen company as cleared in **Table (2).**

Table (2): Reagents used for DNA extraction in automated machine:

Well number	Reagent Name	Volume of reagent (µL)
1	Isopropanol	200
1	Magnetic attract suspension G	20
1	Sample	200
2	Buffer RPW	500
3	Ethanol 96 %	500
4	Ethanol 96 %	500
5	Elution buffer	80

2.6.2. DNA amplification

DNA amplification was performed in 25-mL volumes using MicroAmp Optical 96-well reaction plates and MicroAmp Optical Caps (Applied biosystems) for each well. All reagents were obtained from (Applied Biosystems).

The PCR mixture was prepared according to **(Weller *et al.*,2000)** but optimized for real-time (Applied biosystems,7500) by manufacture company (Applied biosystems) which consists of 12.5µ from ready universal PCR master mix,1µl of forward primer RS-I-F GCA TGC CTT ACA CAT GCA AGT C, 1 µl of reverse primer RS-II-R GGC

ACG TTC CGA TGT ATT ACT CA, 1 μl of probe RS-P AGC TTG CTA CCT GCC GGC GAG TG, 7 μl of DNase free water and 2.5 μl from DNA(sample).

Real-time 7500 detection system (Applied biosystems) was used for amplification and fluorescence measurement. All cycles began with 2 min at 50°C and then went to 10 min at 95°C, followed by 40 two-step cycles of 10 sec at 95°C and then 1 min at 60°C.

2.7. Virulence of brown rot pathogen

Pure culture of the three isolated bacteria that obtained from infected plant materials were used. This isolates were grown on casmino acid peptone glucose (CPG) agar medium at 28°C for 48hr (**Kelman 1954**). Bacterial growth was suspended in sterile phosphate buffer 0.05M pH 7.2 and adjusted to a standard optical density at 590nm to 10^7 colony forming units (cfu/mL). Potato tubers (Nicola cv.) and tomato plants (Castle rock cv.) were examined to be free from *R. solanacearum*. Four weeks old, tomato seedlings were transplanted in 10cm diameter pots, containing sterilized sand-clay soil mixture (1:1,v/v) while, potato tubers previously stored at 4°C,were placed in trays at room temperature in dark to stimulate germination. Germinated tubers were planted in pots (30 cm diameter) containing sterilized sand-clay soil (1:1, v: v), where one germinated tuber was planted in each pot; five pots were used as replicates for each host. Tomato plants (three-weeks after transplanting) were injected at the leaf axis with a sharp needle laden with the bacterial growth of the pathogen (**Janse, 1988**). Potato plants were inoculated into the axel of the second and third leaf from the apical meristem by injection of bacterial suspension (10μL) using needle (**Martin and El-Nashaar, 1992**). Five plants of

tomato and potatoes were injected with sterilized water as the control treatment, the inoculated plants were covered with polyethylene bags for three days, at 30°C and 28% relative humidity (RH) in an automated quarantine green house, then bags were removed and pots were irrigated daily.

Wilt symptoms severity were recorded daily according to the scale of **Kempe and Sequeira (1983)** where, (0 = no symptoms, 1= up to 25 % wilt, 2 = 26-50 % wilt, 3 = 51-75 % wilt, 4 = 76-100% wilt and 5 = dead plants).

2.8. Host range

Tomato (*Solanum lycopersicum* (*Lycopersicon esculentum* L. cvs.GS 12), eggplant (*Solanum melongena* L.), pepper (*Capsicum annum* L.). bean (*Phaseolus vulgaris* L.), onion (*Allium cepa* L.), maize (*Zea mays* L.), faba been (*Vicia faba* L.), datura (*Datura inoxia* P.), little hogweed (*Portulaca oleracea* L.), and Mallow (*Malva aegyptia* L.) were used as a test plants to determine the host range of *Ralstonia solanacearum.*

Seeds and/or seedlings of plants tested as host range were planted in pots (15cm in diameter, containing about 0.5 kg of clay-sandy soil each) under greenhouse conditions. The stem puncture technique injection described by **Janse, (1988)** was made for tomato, potato, pepper, bean, mallow datura and little hogweed at the leaf axis by a needle laden with a 1 mL bacterial suspension of *Ralstonia solanacearum* (Draga isolate) with inoculum density of 10^8 cfu/mL). While, maize, onion and faba bean were inoculated as described by **Matter, (2008)** where roots were cut using sterilized knife then the pots were inoculated with a rate of 200 mL/pot (10^8 cfu/mL). The inoculated

plants were covered with polyethylene bags for three days, at 30°C, then bags were removed and pots were irrigated daily. Five replicates were made for each host and another five replicates was treated with sterilized water as control.

Wilt symptoms were recorded daily according to the scale of **Kempe and Sequeira (1983)** as mentioned above.

3. Compost collection:

Twelve compost samples were collected from different Agriculture companies, or farms and arranged according to kind of waste material (5 types of compost were of vegetarian source, one type was of animal source and 6 were mixture of animal and vegetarian source). All the collected compost samples were tested to be free from *R. solanacearum* using IFAS, SMSA and PCR. Samples were prepared as follow:

Sample of 100 gram each on dry basis of homogenized compost was added to each flask containing 900 mL sterilized tap water. Each flask was shaken at 100 rpm for 2h then centrifuged at 10000 rpm for 10 min at 15°C, the pellet was collected in 2 mL sterilized eppendorf using 2 mL sterilized phosphate buffer 0.01 M. For SMSA test one mL of the prepared sample inoculated to sterilized Petri dishes containing SMSA medium then incubated at 28 °C for 3 days. As for the IFAS test, the sample was prepared as mentioned before. For the PCR test DNA extraction was carried out using automated DNA extraction (Biosprint 15, Qiagen) using reagents approved by Qiagen, DNA amplification and analysis of PCR products was carried out as been mentioned before.

4. Isolation of antagonistic bacteria and fungi from different compost types:

Sample of 100 grams on dry bases of homogenized compost was added to flask containing 900 mL sterilized tap water. Each flask was shaken at 100 rpm for 2h. A series of 10-fold dilutions up to 10^6 were prepared. Nutrient agar medium **(Lelliott and Stead, 1987)** and Martin's rose Bengal medium **(Martin, 1950)** were used for the isolation of bacteria and fungi, respectively. One mL of the prepared dilutions added to sterilized Petri dishes and about 20 mL of molten specific agar medium at around 50°C was poured onto it. Three plates were used as replicates for each particular treatment. After gently rotating, the plates were incubated at 28-30°C and checked periodically. Selected colonies of bacteria and fungi (2-7 day old) were transferred from mixed culture of the plates onto respective agar plates for purification by streak plate technique for bacterial colonies and fungal discs for fungi then the plates were incubated at 28-30°C for 2-7 days.

5. Evaluation of the antagonistic effect of isolated bacteria and fungi against *R. solanacearum* in vitro:

The degree of antagonism of these antagonists was determined by both cross-culture method and filter-paper disk method **(Dhingra & Sinclair, 1995).** The effective antagonists were selected based on the degree of inhibition of pathogen and growth rate of antagonist for *in vitro* evaluation studies.

5.1. Antagonistic bacteria:

5.1.1. Streak plate method

Two types of media *i.e.* nutrient agar (NA) and King's B were used to evaluate the antagonistic effect of bacteria against *R.*

solanacearum where plates were prepared and inoculated with the tested bacteria cultures by a single streak of inoculum at certain distance (3 cm) from the center of the Petri dish and incubated at 37°C for 48 hrs. for the tested bacteria. Later, the plates were seeded with *R. solanacearum* by a single streak starting from the center of the plate and directed to the tested bacteria at a 90° angle to the tested organism. Antagonism was measured by determination the width of the inhibition zones (mm).

5.1.2. Filter disc method

300 µL of 48-h-old-grown *R. solanacearum* isolate (approximately 10^{10} cfu/mL) was seeded into 500 mL of KB agar medium and poured into 9 cm Petri dishes. Sterilized filter discs was immersed in 48-h-old-grown antagonistic bacterial suspension (approximately 3.5×10^{10} cfu/mL) and plated on the plates. Inhibition zone around the filter discs of the antagonistic bacteria were measured after 48 h. All the experiments were conducted at 28°C ± 2.

5.2. Antagonistic fungi:

The antagonistic effects of the fungal isolates were determined by the technique described by **Tolba (1998).** The procedure used was as follows:

Plate of potato dextrose agar (PDA) medium was flooded with 4 mm of NA medium seeded with *R. solanacearum* for each tested fungal isolates. Mycelium discs (0.5 cm in diameter) were inoculated at the edge of previously prepared plates with *R. solanacearum*. Duplicate plates were prepared, and incubated at 30°C for one week. The fungal isolates having antagonistic potentialities against *R. solanacearum* were selected for further studies.

6. Identification of antagonistic bacterial isolates:

In this experiment, among 101 isolates of isolated bacteria from the different compost types, only the bacteria having antagonistic effect against *R. solanacearum in vitro* were identified based on their morphological characteristics, physiological and biochemical tests.

6.1 Identification of antagonistic bacterial using the traditional technique:

The three antagonistic bacterial isolates were grown on nutrient agar (NA), medium and King's B (KB) medium for studying their morphological characteristics including shape of bacterial cells, sporulation and their reaction to Gram stain according to the method described by **Fahy and Persley (1983).** Physiological and biochemical tests were studied according the methods described by **McCarter, (1991)** and **Bergy and Holt, (1994).** In this trial, the three antagonistic bacterial isolates were identified as *Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis.*

6.2. Identification of antagonistic bacterial isolates using PCR technique:

In this trail, the three bacterial isolates *i.e., Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* were identified using PCR technique to confirm their identification.

6.2.1. DNA extraction:

Bacterial isolates were grown in nutrient broth medium and incubated at 28-30°C for overnight under shaking. About 1.5 mL of culture was taken in micro centrifuge tube, centrifuged at 12,000 rpm for 5 minutes. This procedure was repeated for three times. The supernatant was discarded and the pellet was collected. To the pellet

650 µL of extraction buffer was added then it was vortexed, tube was kept in water bath at 65°C for 30 min. After that, 100 µL of 5 mM potassium acetate solution was added. It was kept in ice for 10 min, centrifuged at 12,000 rpm for 5 minutes. Supernatant was collected and pellet was discarded. To that tube equal volume of Phenol: Chloroform: IAA (25: 24: 1) was added mixed well and centrifuged at 12,000 rpm for 5 min. Supernatant was collected and pellet was discarded. To that tube, equal volume of Chloroform: IAA (24:1) was added, mixed well and centrifuged at 12,000 rpm for 5 min. Supernatant was collected and pellet was discarded. To the supernatant equal volume of chilled isopropanal was added on the walls of the tube and it was kept in ice for 10 min. Again centrifuged at 12,000 rpm for 5min. Pellets of the DNA that remain attached to walls of the tube was found. Tube was washed with 100 µL of 70 % ethanol and centrifuged at 12,000 rpm for 2 minutes. The supernatant was discarded and pellet was collected. The pellet was dissolved and stored in 40 µL of TE buffer at 4°C in a medical refrigerator. **(Shiva *et al.*, 2010)**

6.2.2 Amplification:

For *Bacillus polymyxa*:

Forward primer BP-F (5′- GATAGGCCTTTCCTTCGGGAC AGA-3′)

Reverse primer BP-R (5′- TGCGGGTTACCCCACCGACT-3′)

Expected amplicon size = 440 bp, primers were designed and provided by Applied biosystems company-Egypt.

For *Bacillus megaterium*:

Forward primer BM-F (5′- CCGCACAAGCGGTGGAGCAT-3′)

Reverse primer BM-R (5′- GCCACCGGTAGTGTAGGCCAG-3′)

Expected amplicon size = 288 bp, primers were designed and provided by Applied biosystems company-Egypt.

For *Bacillus subtilis*: (Wattiau, 2001)

Forward primer BS-F (5′-AAGTCGAGCGGACAGATGG-3′)

Reverse primer BS-R (5′-CCAGTTTCCAATGACCCTCCCC-3′)

Expected amplicon size = 595 bp, primers were provided by Applied biosystems company-Egypt.

PCR reactions were performed in a final volume of 25 µL containing 30 ηg of extracted DNA, 0.75 µL of 2mM dNTPs each, 2.5µL of 10 X Taq buffers, 0.36µL of 1 unit of Taq DNA polymerase, 3 mL of 10 pico mole primer.

Amplifications were achieved in Biometra thermocycler with the program consisting 1 cycle 96 C then 30 cycles of (95 ^{0}C for 30 sec, 65 ^{0}C for 2 min and 72 ^{0}C for 2 min) and a final extension step 72 for 5 min.

6.2.3 Analysis of the PCR product

PCR fragments were detected by agarose gel 3% electrophoresis horizontal system and stained using eithidium bromide as mentioned before.

7. Ability of the antagonistic isolates to produce siderophores:

Eight – hydroxyl quinolone (50 mg/L) was added to 500mL conical flasks containing 250 mL of trypton soya agar (TSA) medium, sterilized at 121°C for 15 min. Plates of TSA medium were inoculated by loop full of tested isolates then incubated at 28°C for 3 days. The ability of grown antagonistic bacteria on TSA medium for producing siderophores were measured as positive or negative **(Alexander and Zuberer, 1991).**

7.1 . Determination the amount of siderophores production:

The ability of the antagonistic isolates to produce siderophores and the amount of siderophore was determined according to the method of **Meyer and Abdallah (1978),** where antagonistic isolates were seeded in succinate broth medium for 3 days at 30°C, then bacterial cells were removed by centrifugation at 10000 rpm and measuring the absorbance of the supernatant at 400 nm.

% Siderophore = (Ar-As)/Ar x 100

Where:

Ar = absorbance of reference at 400 nm (succinate media)

As = absorbance of sample at 400 nm.

8. Ability of the antagonistic bacterial isolates to produce antibiotics:

8.1 . Inoculum preparation:

The inocula of the antagonistic isolates were prepared by culturing in nutrient broth medium then incubating at 30°C for 48 hours in an orbital shaker at 120 rpm.

8.2 Production of antimicrobial compounds:

About 10 mL inoculum of each one of the tested antagonistic bacteria was added to 100 mL of sterilized synthetic medium (SSM) in 250 mL flask. Shake flask fermentation method (37°C at 150 rpm for 96 hour) **(Jamil *et al.*, 2007)** was used for antibiotic production. After 96 hour of culturing, the cultivated medium was centrifuged at 10000rpm for 20 minutes at 4°C to get cell free supernatant. The pellet was discarded and supernatant was sterilized through 0.2 μm filter paper to be used for agar well diffusion assay.

8.3 Agar diffusion assay:

Agar well diffusion method was used to check the cultures for the production of antibiotics **(Sen *et al.*, 1995).** In this respect, 300 mL of 48 hr old grown *Ralstonia solanacearum* was inoculated into 500 mL KB agar and poured into 9 cm Petri dishes. Wells were made in the inoculated plates using sterile cork borer. About 80 µL cell free supernatants were added into the wells and the plates were incubated at 28°C+2 for 24 hours.

After 24-48 hours, the zones of inhibition were observed and the diameter of the zone of inhibition was measured in mm; boiled supernatant was used as control.

9. Greenhouse experiment:

This experiment was carried in Potato Brown Rot Project – Dokki- Giza to evaluate the antagonistic bacterial isolates against *R. solanacearum* the causal of potato brown rot disease under quarantine greenhouse conditions where, temperature was adjusted to 25°C during the day and 20°C during the night, with a RH of 75 to 80% and a 14 h light d^{-1} in both sterilized and non-sterilized soil through the growing season 2011.

9.1. Pots preparation:

Plastic pots 40 cm in diameter were sterilized by soaking in formalin solution 2% for 5 min and left in air for two weeks to get rid of formalin residues.

9.2. Soil preparation:

Clay soil was collected from Talia village-Monofiya governertae while sandy soil was collected from Sadat village-Menoufya governerate. The mixture of used soil (sandy/clay soil at rate 1:1 v/v)

was divided into two portions, one portion of the soil was autoclaved (120°C/1.5 bar for 20 min.) for 3 days while, another portion was left without autoclaving. Also, the used soil was chemically analyzed where its chemical characteristics as showen in **Table (3)**.

Table (3): Chemical characteristics of used soil under greenhouse conditions

Sample	pH	EC (dS/m)	N	P	K	Fe	Mn	Zn	NH_4	NO_2	NO_3	DHA	Nitrog-enase
			mg/kg										
Sandy/ Clay soil	7.7	1.0	56	4	97	1.5	Trace	0.004	0.0	0.04	0.02	0.20	0.00

9.3. Preparation of *R. solanacearum* inoculum:

Pathogenic *Ralstonia solanacearum* isolate was grown on sucrose peptone agar medium at 28°C for 48 hr. Bacterial colonies was suspended in buffer solution (pH, 7.2) and the population densities of the bacterium was adjusted to give 10^8 colony forming units (CFU/mL) using a spectrophotometer (OD_{600}) according to **Michel and Mew (1998).**

9.4. Preparation of bacterial antagonists inoculum:

The three antagonistic bacterial isolates *i.e., Bacillus polymexa, Bacillus megaterium* and *Bacillus subtilis* were grown on NA medium for 48 h. A concentration of 10^8 CFU/mL was prepared for the three tested antagonistic bacteria using a spectrophotometer (OD_{600}) according to **Ciampi-Panno *et al.* (1980).**

9.5 Preparation of potato tubers:

Surface sterilized potato tubers of cvs. Spunta and Draga were socked in the prepared suspension of the tested antagonistic bacteria *i.e., Bacillus polymexa, Bacillus megaterium* and *Bacillus subtilis* containing 0.1% of carboxymethyl cellulose (1: 1 v/v) plus 0.1 M magnesium sulphate for 15 min and left to dry 2 hr approximately before sowing in the prepared pots.

9.6. Soil infestation:

Five days before planting of potato tubers, infestation of sterilized and non-sterilized sandy clay soil was carried out by adding 100 mL of previously prepared *Ralstonia solanacearum* suspension per kg soil. Then the mixture of sandy clay soil (v/v) was putted into the previously prepared pots (6 kg soil/pot) according to **Michel and Mew (1998)**. The proposed treatments were as follows: (1) *Ralstonia solanacearum* + sterilized soil + tested antagonists + two potato cvs. (2) *Ralstonia solanacearum* + sterilized soil+ two potato cvs as control (1) treatment (infested soil). (3) Sterilized soil + two potato cvs as control (2) treatment (uninfested soil). (4) *Ralstonia solanacearum* + non-sterilized soil + tested antagonists + two potato cvs. (5) *Ralstonia solanacearum* + non-sterilized soil + two potato cvs as control (1) treatment (infested soil). (6) Non-sterilized soil+ two potato cvs as control (2) treatment (uninfested soil). Five replicates established for each treatment where each one contains 5 seed pieces.

9.7. Disease assessment:

The disease was assessed at 21, 31, 41 and 46 days after planting. Infection of potato bacterial wilt was determined as percentage of wilted plants (W) which showing wilt symptoms in relation to total

plants and disease severity (disease index %) was calculated from disease rating for individual plant **(Kempe and Sequeira, 1983)** as mentioned before.

Disease Severity (DS) was calculated by the following formula:

$$DS = \sum R.T / 5 \times N \times 100$$

Where:

T=Total number of plants with each category.

R = Disease ratting scale R (R = 0, 1, 2, 3, 4 and 5).

N = Total number of tested plants.

Also, percentage of disease reduction (PDR) was calculated from percentage of wilted plants (W) as the following formula:

$$PDR = (Wck - Wtr) / Wck \times 100$$

Where:

Wck = Percentage of wilted plants in check treatment.

Wtr = Percentage of wilted plants in treated treatment.

Area under disease progress curve AUDPC was estimated to compare different response of the tested bacterial inoculation (**Pandey *et al.*, 1989**) using the following equation:

$$AUDPC = D [1/2 (Y_1+Y_k) + Y_2 + Y_3+ ..... Y (K-1)]$$

Where:

D = days between readings

Y_1= first disease recording

Y_k= last disease recording

9.8 Plant analysis:

An extra pot was added to each treatment to study the effect of treating potato tubers with tested antagonists on some plant growth characters where samples was collected at 21, 31, 41 and 46 days after planting, washed by distilled water then dried between two filter papers. Shoots and roots were separated, root depth and shoot heights

of the samples were measured and expressed in cm. Then, samples of shoot and roots were dried in aerated oven at 50 °C till constant weight. After the end of the experiment, samples of each treatment were analyzed for NPK content in laboratories of the Soil and Water Institute, Agriculture Research Center, Giza, Egypt.

10. The used media, buffers and reagents:

10.1. Media used:

10.1.1. Casamino-peptone glucose agar (CPGA) (Kelman, 1954):

This medium is used for propagation of *R. solanacearum* isolates for Taq-Man assay, incubation at 28°C for 3 days. The components of the medium are 5.0 g Dextrose, 10.0 g Peptone, 1.0 g Casamino acid, 20.0 g Agar and 1000 mL Distilled water (pH 7.2).

10.1.2. King's medium B. (KB medium) (King *et al.*, 1954):

This medium is used for propagation of *R. solanacearum* isolates, incubation at 28°C for 3 days. The components of the medium are 20.0 g Proteose peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 15 mL glycerol, 20.0 g agar and 1000 mL distilled water (pH 7.2).

10.1.3. Glucose nutrient agar medium (Dowson, 1957):

This medium is used for purification of *R. solanacearum* isolates, incubation at 28°C for 3 days. The components of the medium are 5.0 g peptone, 3.0 g beef extract, 3.0 g glucose, 20.0 g agar and 1000 mL distilled water

10.1.4. Nutrient agar (Jacobs and Gerstein, 1960):

This medium is used for propagation of *R. solanacearum* isolates, incubation at 28°C for 3 days. The components of the medium are 3.0 g

beef extract, 5.0 g peptone, 20.0 g agar and 1000 mL of distilled water (pH 7.2).

10.1.5. Trypticase soy broth agar (TSBA):

This medium is used for propagation of bacteria for fatty acid profiling studies, incubation at 28°C for 3 days. The components of the medium are 30.0 g Trypticase soy broth, 20.0 g agar and 1000 mL distilled water (pH 7.2).

10.1.6. Oxidation/Fermentation (OF) medium (Fahy and Persley, 1983):

The components of the medium are 1.0 g Peptone, 1.0 g $NH_4H_2PO_4$, 0.20 g KCl, 0.20 g. $MgSO_4.7H_2O$, 0.08 g. Bromothymol blue, 1% Sterile (filtered) glucose, 3.0 g Agar and 1000 mL of Distilled water (pH 7.0-7.1).

The medium should be brought to dark olivaceous green colour by addition of 20% NaOH solution.

10.1.7. Gelatin medium (Collins and Patricia, 1984):

This medium was used for detection the gelatin liquefaction, incubation at 28°C for 3 days. The components of the medium are 3 g Yeast extract, 5 g Peptone, 120 g gelatin and 1000 mL of distilled water (pH 7.0).

10.1.8 Peptone water sugar medium (Collins and Patricia, 1984):

The components of the medium are 3.0 g beef extract, 5.0 g peptone, 1.0 mL Bromothymol blue (0.4%) and 0.50 % Sterile sugar solution (pH 6.8).

Agar is melted by steaming and mixed well by stirring. Then the final pH is adjusted to 7.0-7.1 before autoclaving (the medium should be dark olive green in color) at 121°C for 15 minutes. Sterile sugar

solution 0.5% is prepared by filtration. The hot basal medium is dispensed into sterile tubes and cooled to 60°C in a water bath. Each carbohydrate solution is added to the different tubes at rate 1:10 to give a final concentration of 1% carbohydrate.

10.1.9. Starch agar (Collins and Patricia, 1984):

This medium is used for detection the starch hydrolysis, incubation at 28°C for 3 days. The components of the medium are 3.0 g beef extract, 5.0 g peptone, 2.0 g Soluble starch, 20.0 g Agar and 1000 mL Distilled water (pH 7.2).

10.1.10. Arginine medium (Thornley's medium 2A) (Lelliot and Stead, 1987):

This medium is used for detection the arginine dihydrolase, incubation at 28°C for 3 days. The components of the medium are 1.0 g. peptone, 5.0 g NaCl, 0.3 g K_2HPO_4, 0.01g phenol red, 10.0 g L-arginine HCl, 3.0 g Agar and 1000 mL. distilled water (pH 7.2).

10.1.11. Glucose phosphate medium (Collins and Patricia, 1984):

This medium is used for detection the Vogus Proskeur (VP), incubation at 28°C for 3 days. For (VP) test, 0.5% of glucose and K_2HPO_4 was added to peptone broth.

10.1.12. Semi Selective Medium of South Africa (SMSA) (Elphinstone *et al.*, 1996):

This medium is used for isolation of *R. solanacearum* from different habitats, incubation at 28°C for 3 days. The basal medium consisted of: 1.0 g casamino acid, 10.0 g bactopeptone, 5.0 mL glycerol, 20.0 g agar and 1000.0 mL distilled water (pH 6.9).

After dissolving the ingredients and sterilization by autoclaving at 121°C for 15 min, the medium was cooled to 50°C. The following ingredients as cleared in **Table (4)** were dissolved in water, then sterilized by filtration and added to the basal medium at specified.

Table (4): Added antibiotics to the Semi Selective Medium of South Africa basal medium

Name of the Antibiotic	Weight/powder for 100 mL	Quantity/ liter basal media
Crystal violet	1 g	0.5 mL
Polymixin-B-sulphate	741 mg	10 mL
Bacitracin	1.022 g	7.5 mL
Chloromphenicol	0.2 g	2.5 mL
Penicillin-G	50 mg	1.0 mL
2,3,5 triphenyltetrazolium chloride salts	1 g	5.0 mL

10.1.13. Succinate medium:

This medium consists of: 6 g KH_2PO_4, 3 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 4 g succinic acid and 1000 mL distilled water.

10.1.14 Rose Bengal agar (Martin 1950):

This medium consists of: 10 g glucose, 0.5 g peptone, 0.1 g KH_2PO_4, 0.50g $MgSO_4$, 30 milligram streptomycin, 20 g agar, 0.035 g Rose Bengal and 1000 mL distilled water.

The antibiotic Streptomycin was filter sterilized separately and added to the sterile medium.

10.1.15 PDA medium (potato dextrose agar medium):

This medium consists of: 200 g diced potatoes, 20 g dextrose, 15-20 g agar and 1000 mL distilled water (pH 5.6 ± 0.2).

Diced potatoes is boiled in 500 mL of water until thoroughly cooked then filtered through cheesecloth and water is added to the filtrate to complete one litre.

10.1.16 Sterilized synthetic medium (Jamil *et al.*, 2007):

This medium consists of: 5.0 g L-glutamic acid, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.01g $MnSO_4.H_2O$, 0.01g NaCl, 0.01g $FeSO_4.7H_2O$, 0.01g $CuSO_4.7H_2O$, 0.015g $CaCl_2.2H_2O$, 10g glucose and 1000 mL distilled water (pH 7.0).

10.1.17. Trypton soy agar medium (TSA) (Alexander and Zuberer, 1991):

This medium consists of: 15g Bacto trypton, 5g Bacto soytone, 5g sodium chloride and 15g Bacto agar (pH 7.3)

10.2. Buffers used:

10.2.1. Buffers used for Immunofluorescenc Antibody Stain (IFAS) test (Lelliot and Stead, 1987):

10.2.1.1. Phosphate buffer (0.05 M)

This buffer consists of: 10.75 g Na_2HPO_4. $12H_2O$, 2.72 g KH_2PO_4 and 1000 mL distilled water (pH 7.2).

10.2.1.2. Phosphate buffer (0.01 M)

This buffer consists of: 2.7 g Na_2HPO_4. $12H_2O$, 0.4 g NaH_2PO_4. $2H_2O$ and 1000 mL distilled water (pH 7.2).

10.2.1.3. Phosphate buffer saline (0.01 M)

This buffer consists of: 2.7 g Na_2HPO_4. 12 H_2O, 0.4 g NaH_2PO_4. $2H_2O$, 8.0 g NaCl and 1000 mL distilled water (pH 7.2). Used for preparation of antiserum and conjugate in IFAS test.

10.2.1.4. Phosphate glycerin buffer (0.1 M)

This buffer consists of: 3.2 g Na_2HPO_4. $12H_2O$, 0.15 g NaH_2PO_4. $2H_2O$, 50 mL glycerol and 50 mL distilled water pH 7.6).

10.2.2. Buffers used in PCR technique:

10.2.2.1. 10X Tris Acetate EDTA (TAE) buffer for PCR:

This buffer consists of: 48.4 g Tris buffer, 11.42 mL glacial acetic acid, 3.72 g EDTA (disodium salt) and 1000 mL distilled water (pH 8.0).

10.2.2.2. Preparation of the loading buffers:

Fristly, bromophenol blue (10% stock solution) was prepared by dissolving 5 g bromphenol blue in 50 mL distilled water. Then the loading buffer components are 3.5 mL glycerol (86%), 300 µl bromophenol blue (10%) and 6.2 mL distilled water.

10.3. Reagents used for Fatty Acids Analysis (FAA) (Stead, 1988)

10.3.1 Reagent 1 (Saponification)

This reagent consists of: 45 g sodium hydroxide, 150 mL methanol and 150 mL distilled water.

10.3.2. Reagent 2 (Methylation)

This reagent consists of: 325 mL of 6.0 N HCl and 275 mL Methanol. The pH below 1.5 and causes methylation. The fatty acid methyl ester is poorly soluble in aqueous phase at this point.

10.3.3. Reagent 3 (Extraction)

This reagent consists of: 200 mL Hexane and 200 mL Methyl tetra-butyl ether. This will extract the fatty acid methyl esters into the organic phase for use with the gas chromatograph.

10.3.4. Reagent 4 (Sample cleanup)

This reagent consists of: 10.8 g Sodium hydroxide and 9000 mL distilled water; this procedure reduces the contamination of the injection port liner, the column, and the detector.

EXPERIMENTAL RESULTS

Experimental Results

1. Isolation of *Ralstonia solanacearum* from naturally infected potato tubers

As clear in **Fig. (1&2),** naturally infected potato tubers of three different cvs (Spunta, Draga and Nicola) showing brown rot diseases were used for isolation of *R. solanacearum* pathogen. All isolates of *R. solanacearum* showed typical morphological growth on SMSA medium as showed in **Fig. (3).** Where, colonies were fluidal white with red center. Results showed that these isolated were *R. solanacearum* which confirmed by giving positive result using IFAS test (Immunoflurescence Microscope Antibody Staining) as clear in **Fig. (4).**

Fig (1) ***R. solanacearum*** **wilt symptoms on potato plants**

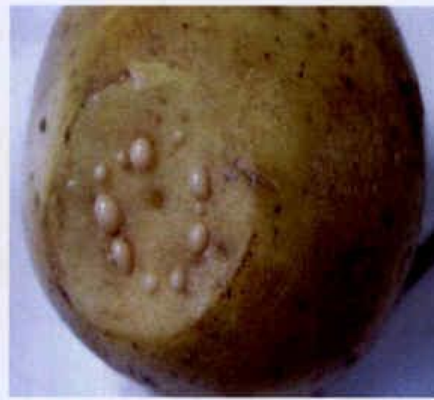
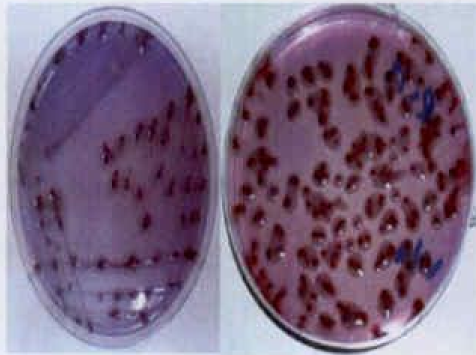


Fig (2) Internal brown rot symptoms in potato tubers showing bacterial ooze



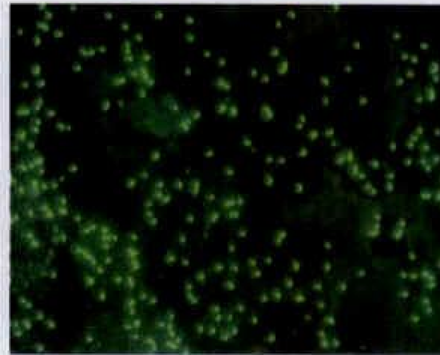


Fig.(3) Typical colonies of *R. solanacearum* on SMSA medium

Fig. (4) IFAS test showing positive reaction of *R. solanacearum*

2. Identification of isolated bacteria:

The isolates in concern were subjected to complete their identification up to species level. The results showed that all isolates recovered from different sources were similar in their morphological and physiological reactions. No strain variation could be detected.

2.1 Cultural and morphological characteristics:

Data in **Table (5)** exhibit the cultural and morphological characteristics of the three tested pathogenic bacterial isolates which isolated from naturally infected potato tubers of three different cvs (Spunta, Draga and Nicola) exhibiting brown rot disease where, stained smears of the bacteria showed non-sporulating short rods with weak Gram negative reaction. Colonies developed on nutrient agar (NA) medium were irregularly / round, convex, smooth surface, entire margin, translucent and yellowish brown in colour. Meantime, these colonies were whitish – gray in colour on King's B (KB) medium forming brown pigments in most cases as clear in **Fig (5).** Colonies on tetrazolium chloride (TTC) medium and semi – selective medium of South Africa (SMSA) were fluidal white with red center.

Table (5): Morphological and cultural characteristics of isolates identified primary as *Ralstonia solanacearum* from different potato cultivars.

Identification Test	***R.s* Spunta**	***R.s* Draga**	***R.s* Nicola**
Gram reaction	-	-	-
Cell shape	Short Rods		
Spore formation	-	-	-
Motility	+	+	+
Colony shape	Irregular / Round		
Colony elevation	Convex		
Colony surface	Smooth		
Colony margin	Entire		
Colony density	Translucent		
Colour on NA media	Yellowish brown		
Colour on KB	Whitish grey		
Colour on TTC and basal SMSA	Fluidal white with red centre		

NA= nutrient agar medium,
KB= King's B medium,
TTC= Triphenyl tetrazolium chloride agar medium,
SMSA= semi selective medium of South Africa

Fig. (5) Brown diffusible pigment of *R. solanacearum* on king's B medium

2.2.1 Physiological and biochemical tests:

Data in **Table (6)** show the physiological and biochemical tests of the three tested pathogenic bacterial isolates which isolated from naturally infected potato tubers of three different potato cvs (Spunta, Draga and Nicola) exhibiting brown rot disease where, all the three tested bacterial isolates showed oxidative metabolism of glucose and positive results with oxidase reaction, catalase reduction, H_2S production, nitrate reduction. However, the three isolates were negative to Indole production, gelatin liquefaction, Arginine dihydrolase, starch hydrolysis, Voges proskauer test and Levan formation. Fluorescent pigment was not produced into king's medium but showed diffusible brown non fluorescent pigment. Also, the three pathogenic bacterial isolates were not able to grow on 4 or 41°C mean while they grow on 1 and 2 % NaCl.

Table (6): Physiological and Biochemical characteristics of isolates identified as *Ralstonia solanacearum* from different potato cultivars.

Identification Tests	*R.s* Spunta	*R.s* Draga	*R.s* Nicola
Starch hydrolysis	-	-	-
Production of fluorescent on KB	-	-	-
Diffusible non fluorescent pigment	Brown	Brown	Brown
O/F	O	O	O
Oxidase reaction	+	+	+
Arginine dihydrolase	-	-	-
Catalase reduction	+	+	+
Voges Proskauer (VP)	-	-	-
Gelatine liqufication	-	-	-
Reduction of nitrate	+	+	+
Levan production	-	-	-
Indole formation	-	-	-
H_2S production	-	-	-
Growth at 41°C	-	-	-
Growth at 4°C	-	-	-
Growth on 1% NaCl	+	+	+
Growth on 2% NaCl	+	+	+

2.2.2 Determination of races and biovars:

Data in **Table (7)** indicate that all three tested isolates of *R. solanacearum* were virulent to potato and tomato plants. On the other side, these isolates were avirulent to eggplant, pepper, tobacco and banana plants under artificial inoculation conditions which reveal that theses isolates belong to race 3. Also, data in **Table (8)** reveal that the three tested isolates utilized maltose, lactose, cellobiose and glucose but

not oxidized mannitol, sorbitol and dulcitol (other characteristics are shown). These tests confirm that these isolates belong to biovar 2.

Table (7): Race determination of the three tested *Ralstonia solanacearum* isolates based on pathogenicity test to different host plants.

Host	**Tested isolates of *R. solanacearum***		
	Cv. Spunta	**Cv. Nicola**	**Cv. Draga**
Pepper	-	-	-
Eggplant	-	-	-
Tobacco	-	-	-
Potato	+	+	+
Tomato	+	+	+
Banana	-	-	-
Proposed Race	**3**	**3**	**3**

Table (8): Ability of isolates identified as *Ralstonia solanacearum* from different potato cultivars to utilize some carbohydrates and biovar determination.

Identification Test	Tested isolates of *R. solanacearum*		
	Cv. Spunta	Cv. Draga	Cv. Nicola
Maltose	+	+	+
Lactose	+	+	+
Cellobiose	+	+	+
Mannitol	-	-	-
Sorbitol	-	-	-
Dulcitol	-	-	-
Salicin	-	-	-
Glucose	+	+	+
Galactose	+	+	+
Glycerol	+	+	+
Mannose	+	+	+
Fructose	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
Inositol	-	-	-
Arabinose	-	-	-
Raffinose	-	-	-
Ribose	-	-	-
L-arginine	-	-	-
Xylose	-	-	-
Proposed Bivoar	**2**	**2**	**2**

2.3 Identification of *R. solanacearum* isolates using fatty acid profiles:

Data in **Table (9)** show the fatty acid (FA) profiles of three *R. solanacearum* isolates which isolated from naturally infected potato tubers of three different cvs (Spunta, Draga and Nicola) exhibiting brown rot disease. It is clear from the obtained results that the extracted material contained 18 fatty acids in addition to two acids unknown

where one of them might be in aldehydic form with C12:0 chain. The majority of profiled fatty acids were un-branched along with two cyclo-FA.

It is established that (FA) profiles denote much useful taxonomic information. In general, the Gram (-) genera have a unique pattern of hydroxy fatty acids and rarely have large quantities of branched (FA). Gram (+) genera rarely have hydroxy (FA) but most have large quantities of branched (FA).

The extracts showed reasonable quantities of C16:0 and C16:1 ω7C/C15 ISO 2OH and the percentages ranged from 21.97 to 24.44% and 23.03 to 25.17% for the fatty acids respectively. Those of longer chains, however, showed smaller quantities as indicated by the recorded percentage(s). The C17:0 cyclo ranged from 5.4 to 9.52 %, however, the C18:1 ω7C/ω9t/ω12t ranged from 19.55 to 20.26 %.

Cellular fatty acid analysis of the three tested *R. solanacearum* isolates showed various degree of homology with different bacterial reference as clear in **Table (10)**. In this respect, all tested isolates gave a highest percentage of homology with the reference bacteria *R. solanacearum* (Rev. 3.90). The Draga isolate has 92.5% similarity followed by the Nicola isolate 78.1%, followed by Spunta isolate 66.2% similarity with the reference isolates.

Table (9): Fatty acids (FA) profiles and their percentages of the three tested *R. solanacearum* isolates

Fatty acid analysis	*R. solanacearum* isolated from potato tubers		
	cv. Spunta	cv. Draga	cv. Nicola
Unknown	10.93	10.93	10.93
C12:0	0.15	0.10	0.10
C14:0	4.65	4.52	4.35
C15:0 ISO	0.17	0.12	0.14
C15:0 ISO 2OH/C16:1ω7C	23.03	22.21	25.17
C15:1ω 6C	0.27	0.23	0.25
C15:0	0.42	0.25	0.30
C14:0 3OH/C16:1 ISO 1	6.72	7.32	7.38
C16:1 ω7C/C15 ISO 2OH	23.03	22.21	25.17
C16:1 ω5C	0.30	0.37	0.26
C16:0	21.97	24.92	24.44
C17:0 Cyclo	9.52	7.63	5.40
C17:0	0.45	0.17	0.29
C16:1 2OH	4.75	6.33	5.73
C16:0 2OH	0.74	0.99	0.84
C18: 1ω7C/ω9t/ω12t	19.92	19.55	20.26
C18: 0	0.30	0.28	0.42
C19: 0 Cyclo ω8C	0.66	0.51	0.32
C18: 1 2OH	4.77	4.28	4.37
C12: 0 ALDE?	7.94	7.42	7.49

Table (10): Percentage of homology of cellular fatty acid of three isolates of *R. solanacearum* with bacterial reference.

Bacterial reference	Tested *R. solanacearum* isolates		
	cv. Spunta	cv. Nicola	cv. Draga
TSBA (Rev.3.90)			
Ralstonia solanacearum	66.2	78.1	92.5
Ralstonia pickettii	63.4	76.6	82.2
PD (Rev 1.90)			
Ralstonia solanacearum	51.2	58.7	75.4
Ralstonia B1,3,4/R1 48H	51.2	55.0	75.2
Ralstonia B2R3 48H	29.4	58.7	75.4
Ralstonia pickettii 48 H	38.4	39.5	46.3
BLDB	59.1	61.4	64.2
CLIN (Rev 3.90)			
Ralstonia pickettii	37.5	55.4	69.8

- TSBA, PD and CLIN: represent three different libraries using the Microbial Identification System-based on software available from MIDI DOS system (Newark, DE,USA).
- TSBA, PD and CLIN: different reference labs

2.4 Immunofluorescence antibody stain:

Results in **Table (11)** reveal the reaction of the three tested *R. solanacearum* isolates which isolated from tubers of different potato cultivars (Spunta, Draga and Nicola) to IFAS test where ten colonies of each isolate of those developed on SMSA medium were tested by IFAS. It is clear from the obtained results that the cells morphology of the tested bacteria had short rod shape stained evenly as bright green fluorescent as clear in **Fig. (4)**.

Table (11): IFAS test results for developed colonies of the three tested *R. solanacearum* isolates on SMSA media.

Identification test	Tested *R. solanacearum* isolates		
	cv. Spunta	cv. Nicola	cv. Draga
SMSA	+	+	+
IFAS	+	+	+

2.5. Polymerase Chain Reaction (PCR)

The results of identification of the three tested *R. solanacearum* isolates which isolated from different potato cultivars i.e., Spunta, Draga and Nicola are shown in **Fig. (6)**. In this respect, the visualized specific 718bp PCR product under UV light showed very close similarity of the three isolates under investigation.

Fig. (6) Shows a single band in the agarose gel electrophoresis where no variation between the 3 isolates of *R. solanacearum* isolated from Spunta (1), Nicola (2) and Draga (3) cultivars reference isolate (4).

2.6. Real time, Fluorogenic PCR (Taq-Man) Assay:

Three isolates of *R. solanacearum* were tested using two primer/probe sets. The RS primers and probe detect are specific for detection of the race 3 biovar 2 strain of *R. solanacearum*. Positive results were obtained for all three isolates, indicating that the all three tested isolates were *R. solanacearum* race 3 biovar 2 (**Fig. 7**).

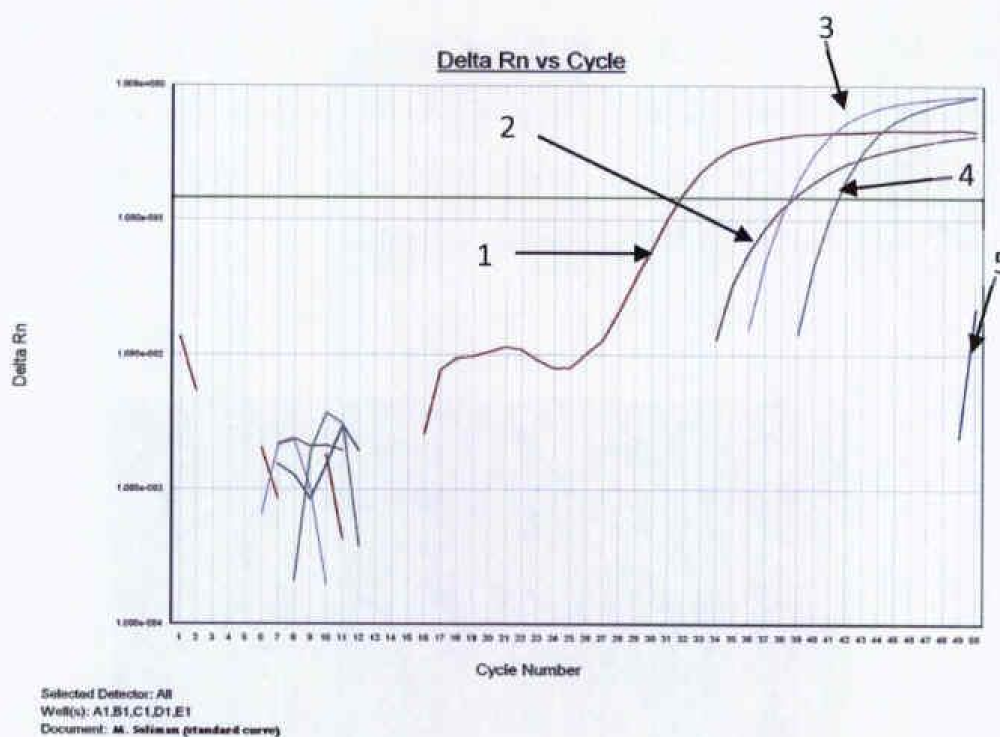


Fig (7): Real time PCR for the 3 isolates of *R. solanacearum* where, (1) Reference isolate, (2) Spunta isolate, (3) Nicola isolate, (4) Draga isolate, (5) Negative control

2.7. Virulence of the three tested *R. solanacearum* isolates:

Data in **Table (12)** indicate that the three tested *R. solanacearum* exhibited different percentages of disease severity ranging from 76.59% to 98.4 % infection when tested on potato plants (cv.Nicola) at 15 days of incubation period. Meanwhile, the determined disease severity of the three tested *R. solanacearum* reached 100 % on tomato plants (cv. Castle rock) to reveal that Draga- isolate was the fastest one among the three tested isolates of *R. solanacearum* where, it recorded disease severity 100% at 4 days of incubation period followed by Nicola –isolate.

Table (12): Pathogenicity test on potato and tomato plants using three isolates of causal organism under artificial inoculation conditions.

Tested isolates	Potato (cv. Nicola)		Tomato (cv. Castle rock)	
	DS %	IP (days)	DS %	IP (days)
R. solanacearum **(cv.Spunta)**	76.59	15	100	7
R. solanacearum **(cv.Nicola)**	87.0	15	100	5
R. solanacearum **(cv.Draga)**	98.4	15	100	4
Control	0.0	15	0	7

DS %= Disease Severity%
IP= Incubation period (days)

2.8. Host range:

It is clear from the obtained previously results of pathogenicity test trial that cv Draga- isolate of *R. solanacearum* was the more aggressive one among the three tested isolates. Thus, this isolate used in the host range trail of *R. solanacearum*.

In this respect, data in **Table (13)** show that Draga-isolate of *R. solanacearum* could infect many hosts with different degrees of wilt severity% where it was virulent to tomato, mallow, datura and little hogweed. The highest recorded wilt severity% was 80% on mallow plants followed by 60% on tomato and little hogweed plants while they were 40% only on datura. Also, positive results were obtained with SMSA, IF and PCR techniques to confirm the infection with Draga-isolate. On the other hand, Draga-isolate was not able to exhibit any wilt symptoms on pepper, eggplant, bean, maize, faba bean and onion plant hosts with no visual latent infection on the last 4 hosts except pepper and eggplant where the detection methods using SMSA, IF and PCR techniques exhibited latent infection with Draga-isolate on it.

Table (13): Host range of *R. solanacearum* (Draga-isolate), determined as wilt severity % and latent infection which confirmed by SMSA, IF and PCR techniques.

Tested hosts	Wilt severity %		Detection method		
	Draga isolate	Control	SMSA	IF	PCR
Tomato	60	0	+	+	+
Pepper	0	0	+	+	+
Eggplant	0	0	+	+	+
Bean	0	0	-	-	-
Maize	0	0	-	-	-
Faba bean	0	0	-	-	-
Onion	0	0	-	-	-
Mallow	80	0	+	+	+
Datura	40	0	+	+	+
Little hogweed	60	0	+	+	+

3- Detection of *R. solanacearum* in different compost collections:

In this trail, twelve compost samples collected from different companies and farms were investigated for presence of *Ralstonia solanacearum* the causal of potato brown rot disease. Data in **Table (14)** verify that all tested compost samples were completely free from *Ralstonia solanacearum* using the different inspection methods where SMSA, IF and PCR techniques gave negative results confirming absence of potato brown rot pathogen in the tested composts.

Table (14): Detection of *R. solanacearum* in different compost collections using SMSA, IF and PCR techniques.

Producing Company	Compost type	SMSA	IF Test	PCR
Kaha	Vegetarian	-	-	-
El-Nile	Vegetarian	-	-	-
EL-Wadi	Vegetarian	-		-
Sekeem	Vegetarian	-	-	-
Pico	Vegetarian	-	-	-
EL-Shafee a	Vegetarian	-	-	-
El-Waha	Animal	-	-	-
El-Shafee b	Vegetarian + animal	-	-	-
Delta mix	Vegetarian + animal	-	-	-
Agrofood	Vegetarian + animal	-	-	-
El-Gharbiya	Vegetarian + animal	-	-	-
El-Dakahliya	Vegetarian + animal	-	-	-

4. Isolation of antagonistic fungal and bacterial microorganisms from composts:

Data in **Table (15)** reveal that the highest number of bacterial and fungal groups were detected in El-Dakahliya compost sample (V+A) where the recorded average of bacterial groups was 22 groups/plate while, the recorded average of fungal groups was 7 groups/plate followed by El-Gharbiya compost sample (15 and 4 group/plate) respectively. Meanwhile, the least average of bacterial and fungal groups were recorded in Sekeem company compost sample (3 and 3 groups/plate) followed by El-Shafee- A company compost sample (4 and 1 groups/plate). On the other hand, the highest average of bacterial

colonies were recorded in Agrofood and Pico company compost samples where they were 116 and 88 colony/plate respectively while the least average of bacterial colonies were recorded in El-Wadi and El-Shafee-A company compost samples. Moreover, the highest average of fungal colonies were recorded in El-Dakahliya and El-Gharbiya company compost samples where the recorded averages of fungal colonies were 88 and 87 colony/plate while, the least averages of fungal colonies were recorded in El-Shafee-A and El-Waha company compost samples.

Table (15): The estimated averages of isolated bacteria or fungi from compost collections

Compost name	Compost Type	Average No. of isolated bacteria		Average No. of isolated fungi	
		Group /plate	Colony /plate (10^{-5})	Group /plate	Colony /plate (10^{-1})
Kaha	V	5	12	3	5
El-Nile	V	7	13	2	5
El-Wadi	V	6	7	4	6
Sekeem	V	3	10	3	4
Pico	V	8	88	4	19
El-Shafee-A	V	4	7	1	3
El-Waha	A	11	20	2	4
El-Shafee-B	V + A	6	14	4	7
Delta mix	V + A	8	21	3	7
Agrofood	V + A	6	116	3	52
El-Gharbiya	V + A	15	37	4	87
El-Dakahliya	V + A	22	60	7	88

V= vegetarian
A= animal
V+A = vegetarian +animal

5. Evaluation of the antagonistic effect of isolated bacteria and fungi against *R. solanacearum in vitro*:

A total of 101 different bacterial isolates as listed in **Table (16 a, b, c)** and 40 different fungal isolates as listed in **Table (17)** were evaluated for their antagonistic effect against the three tested *Ralstonia solanacearum* isolates. In this respect, only three bacterial isolates among the 101 tested isolates inhibited the growth of *R. solanacearum* where 2 isolates were isolated from El-Gharbiya company compost (vegetarian +animal) while the other one was isolated from El-Dakahliya company compost (vegetarian +animal).

Table (16a): Antagonistic effect of isolated bacteria against *R. solanacearum in vitro.*

Compost source of bacterial groups	Isolate No.	Tested *R. solanacearum* isolate		
		Spunta-isolate	Draga-isolate	Nicola-isolate
Group (1) Kaha	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
Group (2) El-Nile	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	
	7	-	-	-
Group (3) El-Wadi	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
Group (4) Sekeem	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
Group (5) Pico	1	-	-	-
	2	-	-	-
	3	-	-	-

Table (16b): Antagonistic effect of isolated bacteria against *R. solanacearum in vitro.*

Compost source of bacterial groups	Isolate No.	Tested *R. solanacearum* isolate		
		Spunta-isolate	Draga-isolate	Nicola-isolate
Group (6) El-Shafee-a	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
	7	-	-	-
	8	-	-	-
Group (7) El-Waha	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
	7	-	-	-
	8	-	-	-
	9	-	-	-
	10	-	-	-
	11	-	-	-
Group (8) El-Shafee-b	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
Group (9) Delta mix	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
	7	-	-	-
	8	-	-	-
Group (10) Agrofood	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-

Table (16c): Antagonistic effect of isolated bacteria against *R. solanacearum in vitro*

Compost source of bacterial groups	Isolate No.	Tested *R. solanacearum* isolate		
		Spunta-isolate	Draga-isolate	Nicola-isolate
Group (11) El-Gharbiya	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
	7	-	-	-
	8	-	-	-
	9	-	-	-
	10	(+)	(+)	(+)
	11	-	-	-
	12	-	-	-
	13	-	-	-
	14	(+)	(+)	(+)
	15	-	-	-
Group (12) El-Dakahliya	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
	7	-	-	-
	8	-	-	-
	9	-	-	-
	10	-	-	-
	11	-	-	-
	12	-	-	-
	13	-	-	-
	14	-	-	-
	15	-	-	-
	16	-	-	-
	17	-	-	-
	18	(+)	(+)	(+)
	19	-	-	-
	20	-	-	-
	21	-	-	-
	22	-	-	-

Table (17): Antagonistic effect of isolated fungi against *Ralstonia solanacearum*

Compost source of fungal groups	Isolate No.	Tested *R. solanacearum* isolate		
		Spunta-isolate	Draga-isolate	Nicola-isolate
Group (1) Kaha	1	-	-	-
	2	-	-	-
	3	-	-	-
Group (2) El-Nile	1	-	-	-
	2	-	-	-
Group (3) El-Wadi	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
Group (4) Sekeem	1	-	-	-
	2	-	-	-
	3	-	-	-
Group (5) Pico	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
Group (6) El-Shafee-a	1	-		
Group (7) El-Waha	1	-	-	-
	2	-	-	-
Group (8) El-Shafee-b	1	-	-	-
	2	-	-	-
	3	-	-	-
	4			
Group (9) Delta mix	1	-	-	-
	2	-	-	-
	3	-	-	-
Group (10) Agrofood	1	-	-	-
	2	-	-	-
	3	-	-	-
Group (11) El-Gharbiya	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
Group (12) El-Dakhliya	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	6	-	-	-
	7	-	-	-

Also, it is clear from the obtained results in **Table (16)** that no tested fungal isolate from the different sources of compost exhibited any antagonistic effect against the three tested *R. solanacearum*. The antagonistic bacterial isolates were designed as: 1Gh, 2Gh and 3D respectively.

5.1. Evaluation of the antagonistic effect of the three selected bacteria against *R. solanacearum (Draga isolate) in vitro*:

In this trail, the length of the inhibition zone of *R. solanacearum* growth was used as a parameter to evaluate the effect of different antagonists on two different media namely, nutrient agar (NA) using filter paper disc method and King's B medium using streaking method respectively. Data in **Table (18)** and **Fig. (8)** reveal that all three tested antagonistic bacterial 1Gh, 2Gh and 3D reduced the growth of the pathogenic bacterium compared with control treatment.

Table (18): Determined inhibition zone (mm) of tested antagonistic isolates against *R. solanacearum in vitro*.

Antagonistic isolates	Inhibition zone (mm)	
	NA medium (Filter paper disc)	King's B medium (Streaking)
Isolate-1Gh	5	9
Isolate-2Gh	4	6
Isolate-3D	5	8

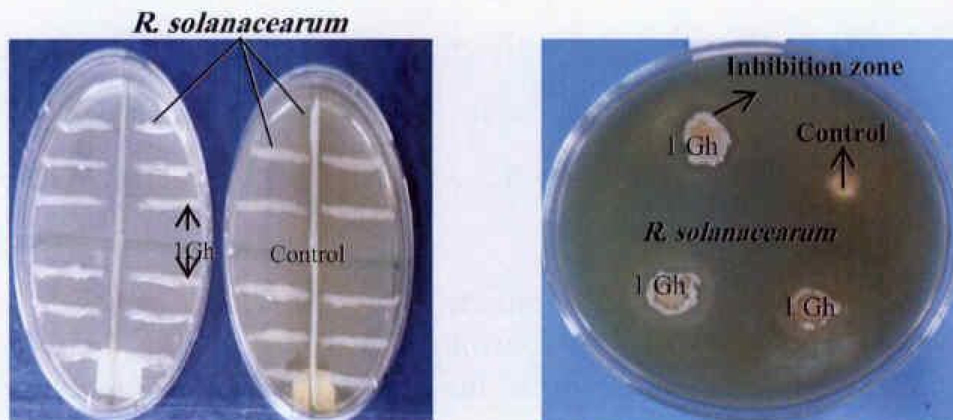


Fig (8): Antagonism of 1 Gh isolated bacteria against *R. solanacearum* on King's B medium (Streaking) at left and on NA medium (Filter paper disc) at right.

6. Identification of antagonistic isolates:

In this experiment, three bacterial isolates among 101 previously isolated from the different compost samples were found to possess the abilities to inhibit the growth of *R. solanacearum in vitro*. Identification of the three antagonistic bacteria was done according to their morphological, physiological as well as biochemical characteristics.

6.1. Morphological, physiological and biochemical characteristics of antagonistic bacterial isolates:

Data in **Table (19 and 20)** show that the three tested bacterial isolates were bacilli shaped, gram positive, aerobic, starch hydrolysis and spore forming. Both isolate-1Gh and isolate-2Gh were (+) ve for VP test, nitrate reduction. But isolate-1Gh could utilize glucose and lactose oxidatively in oxidation- fermentation producing gas. While isolate 2Gh and 3D could utilize citrate. So the three isolates could be

BM-R resulted in amplified DNA at 288 bp to confirm that this isolate-3D is *Bacillus megaterium*.

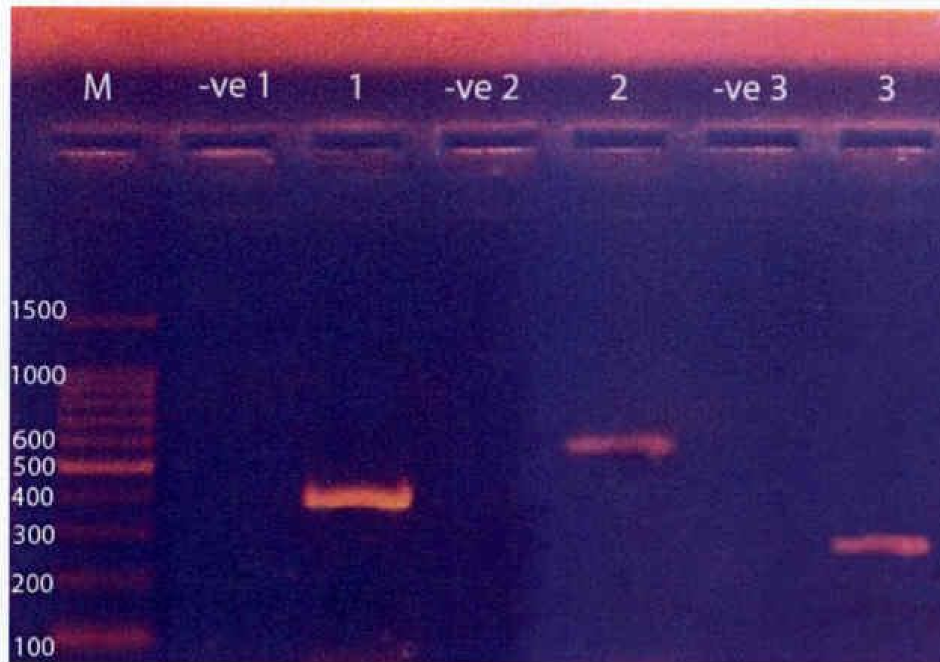


Fig (9): PCR amplicons of DNA on the agarose gel electrophoresis confirming the identification of the three antagonistic bacteria where (1) *B. polymexa*, (2) *B. subtilis*, (3) *B. megaterium*

7. Ability of the antagonistic bacterial isolates to produce siderophores:

Data in **Table (21)** show that all the three tested antagonistic isolates were positive producers of siderophores on TSA medium. Meanwhile, measuring the amount of produced siderophores revealed that *Bacillus megaterium* produced the highest amount of siderophores followed by *Bacillus subtilis* and *Bacillus polymyxa*.

Table (21): Ability of tested antagonistic isolates on producing siderophores

Antagonistic isolates	Siderophore production on TSA*	Siderophore measuring at OD 400 nm
Bacillus polymyxa	+	0.250
Bacillus megaterium	+	0.606
Bacillus subtilis	+	0.340

*TSA= tryptone soy agar

8. Ability of the antagonistic bacterial isolates to produce antibiotics:

Data in **Table (22)** show that all three tested antagonistic isolates are positive producers of antibiotics. Also, measuring the length of inhibition zone revealed that *Bacillus polymyxa* and *Bacillus subtilis* were more producers of antibiotics than *Bacillus megaterium* which came in the second rank when tested against *R. solanacearum*.

Table (22): Ability of tested antagonistic isolates on antagonism and producing antibiotics against *Ralstonia solanacearum.*

Isolate	Antibiotic production	Inhibition zone (mm)
Bacillus polymyxa	+	8
Bacillus megaterium	+	6
Bacillus subtilis	+	8

identified as isolate-1Gh = *Bacillus polymyxa,* isolate-2Gh = *Bacillus subtilis* and isolate-3D = *Bacillus megaterium.*

Table (19): morphological, physiological and biochemical characteristics used for identification of antagonistic isolates at level of genus:

Identification test	**Antagonistic Isolates**		
	1Gh	**2Gh**	**3D**
Gram reaction	+	+	+
Shape	Bacilli	Bacilli	Bacilli
KOH 3%	-	-	-
Growth on common media	+	+	+
Color	Creamy	Creamy	Creamy
Spore production	+	+	+
Pigment K.B	-	-	-
Starch hydrolysis	+	+	+
Gelatin liquefaction	+	+	+
Yeast extract dextrose $CaCO_3$	+	+	+
Levan formation	-	-	-
Catalase activity	+	+	+
Growth on peptone yeast extract	+	+	+
Glucose fermentation	+	-	-
Lactose fermentation	+	-	-
H_2S production	-	-	-
Arginin dihydrolase	-	-	-
Yellow pigment	-	-	-
Relation to O_2	A.	A.	A.
Motility	+	+	+
Bacterial genus	Bacillus	Bacillus	Bacillus

Table (20): Biochemical tests used for identification of antagonistic isolates at level of species:

Identification test	Antagonistic isolates		
	1GH	2GH	3D
Shape	rods	rods	Large rods
Voges Proskauer (VP)	+	+	-
Glucose fermentation	+,gas	-	-
Lactose fermentation	+,gas	-	-
Nitrate reduction to nitrite	+	+	-
Nitrate reduction to N_2	-	-	-
Indol	-	-	-
Mannitol	+	+	+
Citrate	-	+	+
Bacterial species	*B. polymyxa*	*B. subtilis*	*B. megaterium*

6.2. Identification of antagonistic bacterial isolates using PCR technique:

The traditional identification of the three antagonistic bacteria which previously isolated from compost samples of El-Gharbiya (1&2 GH) and El-Dakhliya (3D) based on their morphological, physiological and biochemical characteristics verified that these isolates belongs to *Bacillus polymyxa*-1, *Bacillus subtilis-2* and *Bacillus megaterium*-3. Thus, this trial using PCR technique with specific primers was done to confirm the traditional identification of the three antagonistic isolates. In this respect, as clear in **Fig (9)** using the forward primer BP-F with the reverse primer BP-R resulted in amplified DNA at 440 bp to confirm that this isolate-1GH is *Bacillus polymyxa.* While, using the forward primer BS-F with the reverse primer 5BS-R resulted in amplified DNA at 595 bp to confirm that this isolate-2GH is *Bacillus subtilis*. Also, using the forward primer BM-F with the reverse primer

9. Greenhouse experiment:

9.1. Evaluation of tested antagonists on *R. solanacearum* infection in sterilized and non-sterilized soils cultivated with different potato cvs.

In this trail, three antagonistic bacterial isolates were evaluated for their abilities on reducing potato brown rot or wilt infection caused by *R. solanacearum* on two potato cultivars (cvs. Spunta and Draga) in sterilized or non-sterilized soil under greenhouse conditions. Data in **Table (23-a, b, c and d)** reveal that treating potato tubers of cvs. Spunta and Draga by soaking in the prepared suspension of the tested antagonistic bacteria *i.e., Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* before sowing in the sandy clay soil either sterilized or non-sterilized soils decreased the wilt disease incidence (DI) and wilt disease severity (WDS) of inoculated plants with *R. solanacearum* compared with the control treatment (un-inoculated with *R. solanacearum*).

Moreover, area under disease progress curve (AUDPC) was reduced in all treated plants with the tested antagonists compared with control treatment. Also, it is clear from the obtained results in **Table (23-a)** that *Bacillus megaterium* in sterilized soil treatment (cultivated with cv. Spunta) recorded the highest disease reduction percentage (72%), while, the recorded disease incidence was 28% and the recorded wilt disease severity was 15.2 %. Meanwhile, *Bacillus subtilis* recorded the lowest disease reduction percentage (44%) while, the recorded DI was 56% and the recorded WDS was 29.6 %. On the other hand, Data in **Table (23-b)** show that *Bacillus polymyxa* in non-sterilized soil treatment (cv. Spunta) recorded the highest disease reduction percentage (80%), while, the recorded DI was 20% and the recorded

WDS was 8.8%. Meanwhile, *Bacillus subtilis* and *Bacillus megaterium* recorded equal disease reduction percentage (68%) and equal disease incidence (32%), while they recorded different (WDS and AUDPC). Also, data in **Table (23-c)** show that *Bacillus subtilis* in sterilized soil treatment cultivated with cv. Draga recorded the highest disease reduction percentage (72%), while, the recorded DI was 28% and the recorded WDS was 9.6%. Meanwhile, *Bacillus polymyxa* recorded the lowest disease reduction percentage (60%) while, the recorded (DI) was 40% and the recorded WDS was 12.8%. It is clear also, from the obtained results in **Table (23-d)** show that *Bacillus polymyxa* and *Bacillus subtilis* in non-sterilized soil treatment (cv. Draga) recorded equal diseases reduction percentage (80%) and equal disease incidence (20%), while they recorded different WDS and AUDPC. Meanwhile, *Bacillus megaterium* recorded the lowest disease reduction percentage (64%) while, the recorded DI was 36% and the recorded WDS was 12%.

Moreover, it was clear from the obtained results in **Tables (23-a, b, c &d)** that individual treating of potato tubers (cvs. Spunta and Draga) with the three antagonists before cultivation in the sterilized and non-sterilized inoculated soil with *R. solanacearum* prevented the appearance of wilt infection at 21 and 31 days of incubation except *Bacillus subtilis* + *R. solanacearum* treatment in sterilized soil cultivated with tubers of cv. Spunta which was not able to avoid the appearance of potato wilt infection at 31 day. Also, results cleared that the determined disease index measurements (DI, WDS and AUDPC) increased gradually by increasing the incubation periods from 21-46 days where they reached their maximum values at 41 and 46 days of

incubation especially with infected soil only with *R. solanacearum* treatment.

Table (23-a): Evaluation of tested antagonists on *R. solanacearum* infection in sterilized soil cultivated with potato cv. Spunta

Treatment	21 days			31 days			41 days			End of experiment (46 days)			
	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DRP
Bacillus polymyxa* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	12.0	4.8	12.0	36.0	16.0	60.8	64.0
Bacillus megaterium* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	12.0	5.6	13.2	28.0	15.2	63.6	72.0
Bacillus subtilis* + *R.s	0.0	0.0	0.0	4.0	0.8	1.2	28.0	13.6	58.8	56.0	29.6	161.2	44.0
Infested soil with *R.s*	12.0	2.4	0.0	88.0	43.2	192.4	100.0	98.4	938.6	100.0	100.0	1436	-
Un-infested soil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-

DI= disease incidence, WDS= wilt disease severity, AUDPC= area under disease progress curve, DRP= disease reduction percentage

Table (23-b): Evaluation of tested antagonists on *R. solanacearum* infection in non-sterilized soil cultivated with potato cv. Spunta

Treatment	21 days			31 days			41 days			End of experiment (46 days)			
	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DRP
Bacillus polymyxa + R.s	0.0	0.0	0.0	0.0	0.0	0.0	4.0	2.4	5.2	20.0	8.8	29.2	80.0
Bacillus megaterium + R.s	0.0	0.0	0.0	0.0	0.0	0.0	16.0	6.4	25.6	32.0	25.6	83.6	68.0
Bacillus subtilis + R.s	0.0	0.0	0.0	0.0	0.0	0.0	12.0	5.6	23.6	32.0	15.2	77.2	68.0
Infested soil with *R.s*	4.0	0.8	0.0	84.0	40.8	158.4	100.0	87.2	880.2	100.0	100.0	1370.6	-
Un-infested soil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-

DI= disease incidence, WDS= wilt disease severity, AUDPC= area under disease progress curve, DRP= disease reduction percentage

Table (23-c): Evaluation of tested antagonists on *R. solanacearum* infection in sterilized soil cultivated with potato cv. Draga

Treatment	21 days			31 days			41 days			End of experiment (46 days)			
	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DRP
Bacillus polymyxa* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	16.0	4.8	12.0	40.0	12.8	52.0	60.0
Bacillus megaterium* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	12.0	3.2	5.6	32.0	12	41.2	68.0
Bacillus subtilis* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	12.0	3.2	5.6	28.0	9.6	36.8	72.0
Infested soil with *R.s*	0.0	0.0	0.0	88.0	27.2	121.6	100.0	88.0	538.6	100.0	100.0	1017.4	-
Un-infested soil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-

DI= disease incidence, WDS= wilt disease severity, AUDPC= area under disease progress curve, DRP= disease reduction percentage

Table (23-d): of tested antagonists on *R. solanacearum* infection in non-sterilized soil cultivated with potato cv. Draga

Treatment	21 days			31 days			41 days			End of experiment (46 days)			
	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DI%	WDS%	AUDPC	DRP
Bacillus polymyxa* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	8.0	0.8	1.2	20.0	6.4	12.8	80.0
Bacillus megaterium* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	12.0	3.2	5.6	36.0	12.0	36.4	64.0
Bacillus subtilis* + *R.s	0.0	0.0	0.0	0.0	0.0	0.0	8.0	2.4	5.2	20.0	7.2	26.8	80.0
Infested soil with *R.s*	0.0	0.0	0.0	92.0	26.4	117.2	100.0	88.0	513.8	100.0	100.0	1003	-
Un-infested soil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-

DI= disease incidence, WDS= wilt disease severity, AUDPC= area under disease progress curve, DRP= disease reduction percentage

9.2. Effect of treating potato tubers with tested antagonists on some plant growth characters in sterilized and non-sterilized soil inoculated with *R. solanacearum*

9.2.1. Shoot length

Data in **Tables (24a, b, c & d)** indicate generally that individual treating of potato tubers (cvs. Spunta and Draga) with the three tested bacterial antagonists i.e., *Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen improved the resulted growth characters where they increased shoot length of grown potato plants. In this respect, data in **Table (24a)** show that treating potato tubers cv. Spunta with *Bacillus subtilis* before sowing in sterilized infested soil with *R. solanacearum* recorded the highest shoot length comparing with the sterilized infested soil treatment with *R. solanacearum* at all potato shoot ages which ranged between 21-46 days. Meanwhile, *Bacillus megaterium* followed by *Bacillus polymyxa* came in the second rank where they recorded 48.0 and 47.5cm of shoot length at 46 days of cultivation. Also, data in **Table (24b)** show that *Bacillus megaterium* and *Bacillus subtilis* were the most effective treatments in increasing potato shoot length in non-sterilized infested soil with *R. solanacearum* followed by *Bacillus polymyxa* which came in the second rank in this respect.

Table (24-a): Shoot length (cm) of potato cv. Spunta in infested sterilized soil

Treatment	Potato shoot age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	25.0	32.5	44.0	47.5
Bacillus megaterium* + *R.s	25.0	31.0	45.0	48.0
Bacillus subtilis* + *R.s	24.0	33.0	46.0	49.5
Infested soil with *R.s*	23.0	28.0	30.0	30.0
Un-infested soil	26.0	34.0	43.0	54.0

Table (24-b): Shoot length (cm) of potato cv. Spunta in infested non-sterilized soil

Treatment	Potato shoot age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	25.0	32.0	45.0	50.0
Bacillus megaterium* + *R.s	25.0	32.0	46.0	52.0
Bacillus subtilis* + *R.s	25.0	33.0	45.0	52.0
Infested soil with *R.s*	20.0	23.0	28.0	28.0
Un-infested soil	25.0	35.0	45.0	56.0

On the other hand, it was pronounced from the obtained data in **Table (24c&d)** that treating potato tubers with *Bacillus subtilis* followed by *Bacillus polymyxa* then *Bacillus megaterium* before sowing in sterilized and non-sterilized soil infested with *R. solanacearum* were effective in increasing the emerged potato shoot lengths comparing with the sterilized

and non- sterilized infested soil treatment with *R. solanacearum* at all potato shoot growth stages which ranged between 21-46 days.

Table (24-c): Shoot length (cm) of potato cv. Draga in infested sterilized soil

Treatment	Potato shoot age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	26.0	33.0	45.0	52.0
Bacillus megaterium* + *R.s	28.0	40.0	45.0	50.0
Bacillus subtilis* + *R.s	33.0	45.0	53.0	60.0
Infested soil with *R.s*	24.0	28.0	31.0	31.0
Un-infested soil	30.0	45.0	55.0	64.0

Table (24-d): Shoot length (cm) of potato cv. Draga in infested non-sterilized soil

Treatments	Potato shoot age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	28.0	45.0	50.0	55.0
Bacillus megaterium* + *R.s	23.0	35.0	40.0	45.0
Bacillus subtilis* + *R.s	38.0	50.0	55.0	60.0
Infested soil with *R.s*	25.0	30.0	33.0	33.0
Un-infested soil	30.0	47.0	58.0	65.0

9.2.2. Root length

Data in **Tables (25a, b, c & d)** reveal that individual treating of potato tubers (cvs. Spunta and Draga) with the three tested bacterial antagonists i.e., *Bacillus polymyxa*, *Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen increased root length of grown potato plants. In this respect, data in **Table (25a)** show that treating potato tubers cv. Spunta with *Bacillus subtilis* before sowing in sterilized infested soil with *R. solanacearum* recorded the highest root length comparing with the sterilized infested soil treatment with *R. solanacearum* at all potato growth stages which ranged between 21-46 days. Meanwhile, *Bacillus megaterium* and *Bacillus subtilis* treatments came in the second rank where they recorded equal root length being 12cm.

Also, data in **Table (25b)** show that *Bacillus polymyxa* was the most effective treatment in increasing potato root length in non-sterilized infested soil with *R. solanacearum* followed by *Bacillus megaterium* and *Bacillus subtilis* which came in the second rank in this respect.

Table (25-a): Root length (cm) of potato cv. Spunta in infested sterilized soil

Treatment	Potato root age (days)			
	21	31	41	46
Bacillus polymyxa + *R.s*	3.0	7.0	10.0	13.0
Bacillus megaterium + *R.s*	3.0	6.0	9.0	12.0
Bacillus subtilis + *R.s*	2.4	7.0	10.1	13.4
Infested soil with *R.s*	2.2	2.8	4.1	4.1
Uninfested soil	4.7	8.7	13.0	16.4

Table (25-b): Root length (cm) of potato cv. Spunta in infested non- sterilized soil

Treatment	Potato root age (days)			
	21	31	41	46
Bacillus polymyxa + *R.s*	3.0	8.0	10.0	14.0
Bacillus megaterium + *R.s*	3.0	7.0	10.0	13.0
Bacillus subtilis + *R.s*	2.5	6.0	10.0	13.0
Infested soil with *R.s*	2.1	3.7	4.0	4.0
Uninfested soil	4.1	8.2	13.3	16.2

On the other hand, it was pronounced from the obtained results in **Table (24c&d)** that treating potato tubers before sowing in sterilized and non-sterilized soil infested with *R. solanacearum* with *Bacillus polymyxa* and *Bacillus megaterium* were effective more than *Bacillus subtilis* in increasing the emerged potato root lengths comparing with the sterilized and non- sterilized infested soil treatment with *R. solanacearum* at all potato root growth stages which ranged between 21-46 days.

Table (25-c): Root length (cm) of potato cv. Draga in infested sterilized soil

Treatment	Potato root age (days)			
	21	31	41	46
Bacillus polymyxa + *R.s*	4.0	9.0	11.0	15.0
Bacillus megaterium + *R.s*	3.0	9.3	12.0	15.0
Bacillus subtilis + *R.s*	6.0	10.3	13.0	16.0
Infested soil with *R.s*	2.8	3.2	4.3	4.3
Uninfested soil	4.0	9.0	12.0	16.0

Table (25-d): Root length (cm) of potato cv. Draga in infested non-sterilized soil

Treatment	Potato root age (days)			
	21	31	41	46
Bacillus polymyxa + *R.s*	3.0	7.0	10.0	13.0
Bacillus megaterium + *R.s*	2.0	7.0	10.0	13.0
Bacillus subtilis + *R.s*	6.0	10.1	13.2	16.0
Infested soil with *R.s*	2.8	3.9	4.5	4.5
Uninfested soil	4.0	9.0	12.5	15.5

9.2.3. Shoot dry weight

Results in **Tables (26a, b, c & d)** indicate that individual treating of potato tubers (cvs. Spunta and Draga) with the three tested bacterial antagonists *i.e., Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen increased shoot dry weight of grown potato plants comparing with the infested soil only with *R. solanacearum* pathogen without any one of tested antagonists. In this respect, data in **Tables (26a&b)** exhibit that treating potato tubers cv. Spunta with the three tested antagonists before sowing in sterilized or non-sterilized infested soil with *R. solanacearum* increased shoot dry weight comparing with the infested soil only with *R. solanacearum* pathogen (control treatment) without any one of tested antagonists at all potato shoot ages which ranged between 21-46 days. It is clear from the obtained results that the highest determined dry weights of shoots were recorded with free soil of antagonists and pathogen (un-infested sterilized or non-sterilized soil treatments). It is clear also that *Bacillus polymyxa* and *Bacillus megaterium* treatments were better in their effects on shoot dry weight than *Bacillus subtilis* treatment in both sterilized and non-sterilized soils cultivated with cv. Spunta.

Table (26-a): Shoot dry weight (g) of potato cv. Spunta in infested sterilized soil

Treatment	Shoot dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	0.35	0.77	1.22	1.35
Bacillus megaterium* + *R.s	0.34	0.79	1.25	1.35
Bacillus subtilis* + *R.s	0.35	0.65	1.20	1.30
Infested soil with *R.s*	0.30	0.34	0.35	0.35
Un-infested soil	0.35	0.8	1.35	1.60

Table (26-b): Shoot dry weight (g) of potato cv. Spunta in infested non-sterilized soil

Treatment	Shoot dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	0.35	0.72	1.23	1.37
Bacillus megaterium* + *R.s	0.36	0.73	1.27	1.37
Bacillus subtilis* + *R.s	0.35	0.64	1.21	1.35
Infested soil with *R.s*	0.28	0.30	0.31	0.31
Un-infested soil	0.35	0.85	1.40	1.65

On the other hand, data in **Tables (26c&d)** exhibit that the highest determined dry weights of potato shoots among the tested treatments were recorded with free soil of antagonists and pathogen (un-infested sterilized or non-sterilized soil treatments) cultivated with potato cv. Draga. It is clear also from the obtained results that all tested bacterial

antagonists increased the dry weights of potato shoots (cv. Draga) in sterilized or non-sterilized soils comparing with the infested soil only with *R. solanacearum* pathogen without any one of tested antagonists. In this respect, *Bacillus polymyxa* recorded the highest shoot dry weight of potato cv. Draga followed by *Bacillus megaterium* then *Bacillus subtilis* in either sterilized or non-sterilized soil infested with *R. solanacearum* pathogen.

Table (26-c): Shoot dry weight (g) of potato cv. Draga in infested sterilized soil

Treatment	Shoot dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	0.37	0.75	1.25	1.4
Bacillus megaterium* + *R.s	0.35	0.75	1.26	1.38
Bacillus subtilis* + *R.s	0.35	0.7	1.22	1.34
Infested soil with *R.s*	0.33	0.73	0.78	0.78
Un-infested soil	0.36	0.85	1.45	1.80

Table (26-d): Shoot dry weight (g) of potato cv. Draga in infested non-sterilized soil

Treatment	Shoot dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	0.37	0.76	1.27	1.42
Bacillus megaterium* + *R.s	0.36	0.76	1.27	1.40
Bacillus subtilis* + *R.s	0.36	0.75	1.25	1.36
Infested soil with *R.s*	0.34	0.75	0.80	0.80
Un-infested soil	0.37	0.88	1.5	1.85

9.2.4. Root dry weight

Data in **Tables (27-a, b, c & d)** exhibit that individual treating of potato tubers (cvs. Spunta or Draga) with the three tested bacterial antagonists *i.e., Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen increased root dry weight of grown potato plants comparing with the infested soil only with *R. solanacearum* pathogen without any one of tested antagonists. In this respect, results in **Tables (27-a&b)** exhibit that treating potato tubers cv. Spunta with the three tested antagonists before sowing in sterilized or non-sterilized infested soil with *R. solanacearum* increased root dry weight comparing with the infested soil only with *R. solanacearum* pathogen (control treatment) without any one of tested antagonists at all potato root ages which ranged between 21-46 days. It is clear from the obtained results that the highest determined dry weights of potato roots were recorded with free soil of antagonists and pathogen (un-infested sterilized or non-sterilized soil treatments). It is clear also that *Bacillus subtilis* treatment was the best on increasing the dry weight of potato root in

sterilized infested soil with *R. solanacearum* comparing with *Bacillus polymyxa* and *Bacillus megaterium* treatments. While, *Bacillus polymyxa* and *Bacillus megaterium* treatments were better in their effects on root dry weight than *Bacillus subtilis* treatment in non-sterilized soils cultivated with cv. Spunta.

Table (27-a): Root dry weight (g) of potato cv. Spunta in infested sterilized soil

Treatment	Root dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa + R.s	0.11	0.13	0.17	0.25
Bacillus megaterium + R.s	0.11	0.14	0.18	0.25
Bacillus subtilis + R.s	0.10	0.15	0.18	0.26
Infested soil with *R.s*	0.06	0.09	0.11	0.11
Un-infested soil	0.15	0.2	0.25	0.35

Table (27-b): Root dry weight (g) of potato cv. Spunta in infested non-sterilized soil

Treatment	Root dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa + R.s	0.10	0.14	0.19	0.29
Bacillus megaterium + R.s	0.11	0.15	0.19	0.27
Bacillus subtilis + R.s	0.10	0.13	0.17	0.26
Infested soil with *R.s*	0.05	0.08	0.10	0.10
Un-infested soil	0.17	0.22	0.29	0.37

On the other hand, data in **Tables (27- c&d)** exhibit that the highest determined dry weights of potato roots among the tested treatments were recorded with free soil of antagonists and pathogen (un-infested sterilized or non-sterilized soil treatments) cultivated with potato cv. Draga. It is clear also that all tested bacterial antagonists increased the dry weights of potato roots (cv. Draga) in sterilized or non-sterilized soils comparing with the infested soil only with *R. solanacearum* pathogen without any one of tested antagonists. In this respect, *Bacillus polymyxa* recorded the highest root dry weight of potato cv. Draga followed by *Bacillus subtilis* then *Bacillus megaterium* in sterilized soil. While, *Bacillus megaterium* treatment recorded the highest root dry weight followed *Bacillus polymyxa* and *Bacillus subtilis* in non-sterilized soil infested with *R. solanacearum* pathogen.

Table (27-c): Root dry weight (g) of potato cv. Draga in infested sterilized soil

Treatment	Root dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa + R.s	0.11	0.15	0.2	0.28
Bacillus megaterium + R.s	0.11	0.16	0.2	0.26
Bacillus subtilis + R.s	0.12	0.16	0.21	0.27
Infested soil with *R.s*	0.09	0.10	0.11	0.11
Uninfested soil	0.16	0.21	0.3	0.39

Table (27-d): Root dry weight (g) of potato cv. Draga in infested non-sterilized soil

Treatment	Root dry weight at age (days)			
	21	31	41	46
Bacillus polymyxa* + *R.s	0.11	0.14	0.21	0.30
Bacillus megaterium* + *R.s	0.12	0.17	0.22	0.31
Bacillus subtilis* + *R.s	0.10	0.16	0.21	0.30
Infested soil with *R.s*	0.09	0.11	0.12	0.12
Uninfested soil	0.17	0.22	0.31	0.40

9.3. Effect of treating potato tubers with tested antagonists on shoot and root NPK contents in sterilized and non-sterilized soil infested with *R. solanacearum*

9.3. 1. Shoots NPK content:

Data in **Tables (28a, b, c & d)** exhibit that individual treating of potato tubers (cvs. Spunta or Draga) with the three tested bacterial antagonists *i.e., Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen increased NPK content in shoots of grown potato plants comparing with the infested soil only with *R. solanacearum* pathogen without any one of tested antagonists. In this respect, results in **Tables (28-a&b)** reveal that *Bacillus megaterium* treatment in sterilized or non-sterilized soil cultivated with potato cv. Spunta recorded the highest N content in shoots of grown potato plants. Meanwhile, *Bacillus subtilis* treatment recorded the highest P content in potato shoots grown in sterilized or non-sterilized soil while, *Bacillus polymyxa* treatment recorded the highest K content in emerged potato shoots grown in either of tested soils. Results revealed also that NPK content in emerged potato shoots in sterilized or non-sterilized soil was higher than that recorded with infested soil only with *R. solanacearum* treatment.

Table (28-a): NPK content (ppm) in potato shoots (cv. Spunta) in sterilized soil

Treatment	N	P	K
Bacillus polymyxa* + *R.s	2325	756	987
Bacillus megaterium* + *R.s	2498	721	715
Bacillus subtilis* + *R.s	1532	811	879
Infested soil with *R.s*	1121	678	721
Un-infested soil	2650	745	782

Table (28-b): NPK content (ppm) in potato shoots (cv. Spunta) in non-sterilized soil

Treatment	N	P	K
Bacillus polymyxa + R.s	2335	760	995
Bacillus megaterium + R.s	2510	730	722
Bacillus subtilis + R.s	1540	820	885
Infested soil with *R.s*	1130	680	725
Un-infested soil	2660	753	790

On the other hand, data in **Tables (28-c&d)** shows that the highest N and P contents were recorded with treating potato tubers cv. Draga with *Bacillus megaterium* before sowing in infested sterilized and non-sterilized soil. Meanwhile, *Bacillus polymyxa* treatment recorded the highest K content in either infested sterilized and non-sterilized soils. Also, results cleared that NPK content in emerged potato shoots in sterilized or non-sterilized soil was higher than that recorded with infested soil only with *R. solanacearum* treatment as well as *Bacillus subtilis*.

Table (28-c): NPK content (ppm) in potato shoots (cv. Draga) in sterilized soil

Treatment	N	P	K
Bacillus polymyxa + R.s	2175	632	765
Bacillus megaterium + R.s	2234	750	654
Bacillus subtilis + R.s	1500	611	611
Infested soil with *R.s*	1112	623	676
Un-infested soil	2567	709	775

Table (28-d): NPK content (ppm) in potato shoots (cv. Draga) in non-sterilized soil

Treatment	N	P	K
Bacillus polymyxa + R.s	2183	640	773
Bacillus megaterium + R.s	2240	760	660
Bacillus subtilis + R.s	1515	620	618
Infested soil with *R.s*	1115	628	680
Un-infested soil	2575	720	783

9.3.2. Roots NPK content:

Data in **Tables (29-a, b, c & d)** exhibit that individual treating of potato tubers (cvs. Spunta or Draga) with the three tested bacterial antagonists *i.e., Bacillus polymyxa*, *Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen increased NPK content in roots of grown potato plants comparing with the infested soil only with *R. solanacearum* pathogen. Also, It is clear from the obtained results in T**able (29-a&b)** that *Bacillus polymyxa* treatment in sterilized or non-sterilized soil cultivated with potato cv. Spunta recorded the highest N, P and K content in

roots of grown potato comparing with the other two tested antagonists treatments as well as the treatment of infested soil only with *R. solanacearum* pathogen.

Table (29-a): NPK content (ppm) in potato roots (cv. Spunta) in sterilized soil

Treatment	N	P	K
Bacillus polymyxa* + *R.s	890	345	276
Bacillus megaterium* + *R.s	789	310	220
Bacillus subtilis* + *R.s	876	311	222
Infested soil with *R.s*	734	212	176
Un-infested soil	995	398	300

Table (29-b): NPK content (ppm) in potato roots (cv. Spunta) in non-sterilized soil

Treatment	N	P	K
Bacillus polymyxa* + *R.s	897	340	255
Bacillus megaterium* + *R.s	795	315	225
Bacillus subtilis* + *R.s	885	320	230
Infested soil with *R.s*	744	222	185
Un-infested soil	1010	415	315

On the other hand, data in **Table (29-c&d)** show that *Bacillus polymyxa* treatment in sterilized and non-sterilized soils cultivated with potato cv. Draga recorded the highest N and P contents while, the highest K content was recorded with *Bacillus subtilis* treatment in either sterilized and non-sterilized soils cultivated with potato cv. Draga.

Table (29-c): NPK content (ppm) in potato roots (cv. Draga) in sterilized soil

Treatment	N	P	K
Bacillus polymyxa* + *R.s	886	320	199
Bacillus megaterium* + *R.s	759	295	213
Bacillus subtilis* + *R.s	872	300	219
Infested soil with *R.s*	712	213	167
Un-infested soil	989	376	298

Table (29-d): NPK content (ppm) in potato roots (cv. Draga) in non-sterilized soil

Treatment	N	P	K
Bacillus polymyxa* + *R.s	894	330	210
Bacillus megaterium* + *R.s	770	305	220
Bacillus subtilis* + *R.s	880	310	225
Infested soil with *R.s*	720	220	175
Un-infested soil	995	385	308

DISCUSSION

DISCUSSION

Potato brown rot, caused by *Ralstonia solanacearum* **(Yabuuchi *et al.*, 1995),** has been reported in Egypt many years ago **(Briton-Jones, 1925).** The disease has created a lot of quarantine problems during the course of exportation of table potatoes to Europe **(Farag, 2000).** The disease is known to be favored by warm climates; however, serious outbreaks in Europe have been reported **(Walker, 1992; Stead, 1996 and Grousset *et al.*, 1998).** Therefore, the origin of the disease in Egypt is thought to be the potato seeds imported from Europe **(Balabel, 2006).**

Results of isolation from naturally infected potato tubers of three different cvs. (Spunta, Draga and Nicola) showing brown rot diseases revealed that all isolated bacteria showed typical morphological growth of *R. solanacearum* on SMSA medium where; colonies were fluidal white with red center. Also, IFAS test (Immunoflurescence Microscope Antibody Staining) confirmed that these isolated bacteria are *R. solanacearum* by giving positive result. Repeating the IFAS test of the three tested purified *R. solanacearum* isolates verified that their cells morphology of had short rod shape stained evenly as bright green fluorescent. These results are in agreement with those of **Bader (2012)** who found that identification of tested bacterial isolates using immunofluorescent antibody staining technique (IFAS) gave positive results which mean that these tested isolates are *R solanacearum.* The morphology of bacterial cells appeared as short rod shape and green fluorescent with specific fluorescent-labeled antiserum.

The results showed that all isolates recovered from different sources were similar in their morphological and physiological reactions. In this respect, the traditional identification of the three tested pathogenic bacterial isolates which isolated from naturally infected potato tubers of three different cvs (Spunta, Draga and Nicola) exhibiting brown rot disease using exhibited the similarity of cultural and morphological characteristics of the three tested bacterial isolates where, these isolates were non-sporulating short rods with weak Gram negative reaction. Also, their colonies developed on nutrient agar (NA) medium were irregularly / round, convex, smooth surface, entire margin, translucent and yellowish brown in colour. Meantime, these colonies were whitish – gray in colour on King's B (KB) medium forming brown pigments in most cases. Their colonies on tetrazolium chloride (TTC) medium and semi – selective medium of South Africa (SMSA) were fluidal white with red center. Also, the physiological and biochemical tests of the three bacterial isolates from naturally infected potato tubers of three different cvs. (Spunta, Draga and Nicola) exhibiting brown rot disease revealed that the three tested bacterial isolates showed oxidative metabolism of glucose and positive results with oxidase reaction, catalase reduction, H_2S production, nitrate reduction. However, the three isolates were negative to Indole production, gelatin liquefaction, Arginine dihydrolase, starch hydrolysis, Voges proskauer test and Levan formation. Fluorescent pigment was not produced into king's medium but shows diffusible non fluorescent pigment. The obtained results are in agreement with those obtained by **Kelman, (1954)** who reported that plating the isolates having typical morphological colonies on the SMSA medium showed

that colonies with irregularly round shape and slimy white color with pink centers was considered the typical morphology of *R. solanacearum*. The positive isolates (typical morphological colonies) showed that all isolates were similar in their typical morphological colonies on SMSA medium. The obtained results of identification and race determination of isolates based on pathological and bacteriological tests are similar to those obtained by **Hayward (1964), Cowan & Steel (1974), Schaad (1980), Krieg and Holt (1984), Lelliot & Stead (1987), Klement *et al.* (1990) and McCarter (1991)**. Also, **Atta (2008)** stated that detection methods by plating on the SMSA medium showed that colonies with irregularly round shape and slimy white color with pink centers was considered the typical morphology of bacterial colony. While, **Bader (2012)** isolated ten isolates of *R. solanacearum* (*Rs*) from the diseased potato tubers and soil collected from the tested fields in Qalubiya (Beltan and El-Hadaden) and Beheira (El-Tawfikia and Hosh-Eysa) governorates during growth of 2009-2011 seasons. All testes trails for identification of bacteria based on morphological, physiological and biochemical characteristics confirmed that all ten isolates were *R. solanacearum*.

All three tested isolates of *R. solanacearum* showed virulence against potato plants and tomato plants. On the other side, these isolates were avirulent to eggplant, pepper, tobacco and banana plants under artificial inoculation conditions which reveal that theses isolates belong to race 3. Also, all three tested isolates utilized maltose, lactose, cellobiose and glucose but not oxidized mannitol, sorbitol and dulcitol (other characteristics are shown). These tests confirm that these isolates belong to biovar 2. The physiological and bacteriological characteristics

were found similar to those described for Race 3, biovar 2 of *R. solanacearum* as documented based on host range studies by **Buddenhagen *et al.* (1962), Pegg & Moffett (1971)** and **He *et al.* (1983).** Also, **Farag *et al.* (2004)** confirmed that the dominant race in Egypt of *R. solanacearum* is race 3, biovar 2. Similar results were obtained also by **Shambhu *et al.* (2001)** who performed the characterization of *Ralstonia solanacearum* strains, the causal agent of potato bacterial wilt disease from Nepal and Thailand based on pathogenicity, biochemical, physiological and serological tests. Fifteen *R. solanacearum* strains isolated from wilt infected potato plants and tubers grown in Nepal were characterized as race 3, biovar 2 based on the pathogenicity on different host plants. On the other hand, **Siri *et al.* (2011)** evaluated 28 strains of *R. solanacearum* isolated from major potato-growing areas in Uruguay, including 26 strains isolated from potato tubers and 2 from soil samples. All strains belonged to phylotype IIB, sequevar 1 (race 3, biovar 2). While, **Bader (2012)** stated that infected potato plants with brown rot disease (caused by *Ralstonia solanacearum*) under greenhouse and field conditions exhibited yellow leaves or sudden wilting of leaves then dead plants, whitish exudates seen on the cut surface on tubers, a wet breakdown inhibited at the point of attachment of the stolon and the eyes of tubers. A light-brown breakdown of water-conducting tissues could be seen in tuber crosses. Milky fluid is squeezed from this discolored area in infected potato tubers.

Fatty acid (FA) profiles of the three tested *R. solanacearum* isolates exhibited that the extracted material contained 18 fatty acids in addition to two acids unknown where one of them might be in

aldehydic form with C12:0 chain. The majority of profiled fatty acids were un-branched along with two cyclo-FA. The extracts showed reasonable quantities of longer chain fatty acid C16:0 and C16:1 ω7C/C15 ISO 2OH which consider the distinguishing fatty acid of *R. solanacearum* in addition to the fatty acid C18:1 ω7C/ω9t/ω12t. Cellular fatty acid analysis of the three tested *R. solanacearum* isolates showed various degree of homology comparing with the different bacterial reference. In this respect, all tested isolates gave a highest percentage of homology with the reference bacteria *Ralstonia solanacearum* where the highest homology of reference bacteria was with Draga isolate followed by Nicola isolate then Spunta isolate. Similar results were obtained by **Shehata (2001)** who found that *R. solanacearum* extracted material contained 18 FA in addition to two unknown acids one of which in aldehydic form with C12:0 chain. Also, the obtained results of **Farag (2008)** could be used in interpreting our obtained results where it found that extracts of tubers and weed isolates are similar to large extent in FA content, with only slight variation. The extracts of the weed isolates, however, were poor in C15: ω6C, C15:0 and C17:0 compared to tuber ones. It may be noticed, however, that the extracts of both isolate sources did not show C13:0 and C17: 1ω8C compared to soil and water isolates. The absence of these fatty acids in the extracts may be indicative to the plant origin. While for soil and water isolates, the extract of the first was poor enough in the majority FA detected, compared to the second. The soil isolate extract of FA was lower in 18 FA out of 24 detected. It is important to note that extracts of water and soil isolates did not show C12:0 and C19:0 Iso,

which may be indicative to their origins. The results of **Khakvar, *et al.* (2011)** confirmed our obtained results.

Identification of the three tested *R. solanacearum* isolates using PCR technique visualized distinguishing specific 718bp PCR product under UV light with very close similarity of the three isolates under investigation and indicating that all three tested isolates of *R. solanacearum* were belonged to race 3 biovar 2 where, the primers and probe are specific for detection of the race 3 biovar 2 strain. Results of Real-time PCR technique of the three tested isolates of *R. solanacearum* using two primer/probe sets revealed that the primers and probe are specific for detection of the race 3/biovar 2 strain, giving positive results for all three isolates and indicating that the three tested isolates were *R. solanacearum* biovar 2 race 3. These obtained results could be interpreting in light the findings of these result are in harmony with the obtained result of **Messiha (2006)** who recorded very close similarity of bands related to different isolates and **Pastrik *et al.* (2002)** who used polymerase chain reaction (PCR) techniques to identify *R. solanacearum* strains the causal agent of potato brown rot disease. Also, **Kumar *et al.* (2004)** isolated thirty-three strains of *Ralstonia solanacearum* from ginger, paprika, chilli, tomato, *Chromolaena* and potato. Phenotypic characterization for biovar revealed the predominance of biovar 3 in India. While, **Fikre *et al.* (2010)** assessed the genetic diversity among the Ethiopian strains of *Ralstonia solanacearum*, the causal agent of bacterial wilt, by repetitive sequence-based polymerase chain reaction (rep- PCR) method with BOX and ERIC primer sets. On the other hand, **Bader (2012)** identified five bacterial isolates among the ten isolates of those isolated from

Beheira and Qalubiya governorates during season 2009 which previously identified as *R solanacearum* using the traditional and IFAS techniques using PCR technique visualized the specific DNA band with molecular weight 718bp in the five tested bacterial isolates and the positive control one under UV light. The results revealed also that there were very close similarity without any variation among the five tested isolates and the positive control one under investigation, confirming that these five tested bacterial isolates are *R. solanacearum*.

As for virulence of the three tested *R. solanacearum*, results exhibited different percentages in disease severity on potato plants (cv.Nicola) at 15 days of incubation period. Also, Draga- isolate was the fastest one among the three tested isolates of *R. solanacearum* followed by Nicola -isolate. Draga-isolate of *R. solanacearum* infected many hosts with different degrees of wilt severity where it was virulent to tomato, mallow, datura and little hogweed. Also, Positive results were obtained with SMSA, IF and PCR techniques to confirm the infection with Draga-isolate. Draga-isolate was not able to exhibit any wilt symptoms on pepper, eggplant, bean, maize, faba bean and onion plant hosts with no visual latent infection on the last 4 hosts except, pepper and eggplant where the detection methods using SMSA, IF and PCR techniques exhibited latent infection with Draga-isolate. These results are in agreement with those reported by **Fahy and Persley (1983), Hsu (1993), Adhikari (1993), Abd El-Ghafar *et al.* (1995), Gabr and Saleh (1997), Shehata (2001), Kehil (2002), Zayed (2004), Abd El-Ghafar *et al.* (2004).** Also, such results were confirmed with the obtained results by **Kelman (1953), Buddenhagen and Kelman (1964), Hayward (2000) and Matter (2008)** who found that several

hundred species, representing more than 50 plant families have been identified as hosts of *R. solanacearum*; include tomato, potato, pepper, eggplant, groundnut and bananas as well as a number of ornamental plants, woody perennials and a large group of weed species. **Bader (2012)** stated that all tested *R. solanacearum* isolates caused bacterial wilt disease symptoms on potato plants compared with the un-inoculated control in sterilized and un-sterilized soils. *R. solanacearum* (R6) recorded the highest infection percent and disease severity percent at 35 day post inoculation of potato plants (cv. Spunta) followed by *R. solanacearum* isolates (R1, R3 & R4). While the least infection percent was recorded by *R solanacearum* (R8) and (R5).

Investigation of twelve compost samples collected from different companies, and farms using SMSA, IF and PCR techniques verified that all tested compost samples were completely free from *Ralstonia solanacearum*. On the other hand, there were no one out of 40 fungal isolates isolated from the different sources of compost exhibited any antagonistic effect against the three tested *R. solanacearum*. However, among 101 different bacterial isolates from the different sources of composts, three isolates only having antagonistic effect against the three tested *R. solanacearum*. Among the three antagonistic bacterial isolates, 2 isolates were isolated from El-Gharbyia compost (vegetarian +animal) while the other one was isolated from El-Dakhlyia compost (vegetarian +animal). Evaluating the effect of different antagonists on two different media namely; nutrient agar (NA) using filter paper disc method and King's B medium using streaking method respectively revealed that all three tested antagonistic bacterial isolates from compost samples of El-Gharbyia

and El-Dakhlyia (vegetarian+animal) were found to posses the abilities on inhibiting the growth of *R. solanacearum in vitro* compared with control treatment. Identification of the three tested antagonistic bacteria based on their morphological, physiological as well as biochemical characteristics exhibited that the three isolates could be identified as isolate-1Gh = *Bacillus polymyxa,* isolate-2Gh = *Bacillus subtilis* and isolate-3D = *Bacillus megaterium.* Also, using PCR technique with specific primers confirmed the traditional identification of the three antagonistic isolates. In this respect, the amplified DNA at 440 bp confirmed that isolate-1GH is *Bacillus polymyxa.* While, the amplified DNA at 595 bp confirmed that isolate-2GH is *Bacillus subtilis.* Also, the amplified DNA at 288 bp confirmed that isolate-3D is *Bacillus megaterium.* Identification bases according to **Hayward (1964)** and **Krieg** and **Holt (1984)** confirmed that our three isolated antagonistic bacteria from compost samples were *Bacillus polymyxa* (isolate-1Gh), *Bacillus subtilis* (isolate-2Gh) and *Bacillus megaterium* (isolate-3D)

On the other hand, all three tested antagonistic isolates were positive producers of siderophores on TSA media. Where, measuring the amount of produced siderophores revealed that *Bacillus megaterium* produced the highest amount of siderophores followed by *Bacillus subtilis* and *Bacillus polymyxa.* In this respect, the obtained results of **Hofte *et al.* (1992) and Loper and Henkels (1997)** could be interpreting our obtained results where they explained the role of siderophores in chelating iron and other metals contributing to disease suppression by conferring a competitive advantage to bio-control agents for limiting supply of essential trace minerals in natural habitats. The obtained results are also in harmony with those recorded by **Hu**

(1996); Hewait (2005) and Hu & Xu (2011). Also, all three tested antagonistic bacterial isolates are positive producers of antibiotics where, *Bacillus polymyxa* and *Bacillus subtilis* were more producers of antibiotics than *Bacillus polymyxa* when tested against *R. solanacearum*. These results could be interpreting in light the findings of **Zodor and Anderson (1992) and Sharga *et al.* (2004)** who reported that antibiotics and siderophores may function as stress factors including local and systematic host resistance.

Treating potato tubers of cvs. Spunta and Draga by soaking in the prepared suspension of the tested antagonistic bacteria *i.e., Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* before sowing in the sandy clay soil either sterilized or non-sterilized soils decreased the wilt disease incidence (DI) and wilt disease severity (WDS) of inoculated plants with *R. solanacearum* compared with the control treatment (uninfested with *R. solanacearum*). Moreover, area under disease progress curve (AUDPC) was reduced in all treated plants with the tested antagonists compared with control treatment. Also, individual treating of potato tubers (cvs. Spunta and Draga) with the three tested bacterial antagonists i.e., *Bacillus polymyxa*, *Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen improved the resulted growth characters where they increased shoot length, root length, shoot dry weight and root dry weight of grown potato plants. Meanwhile, individual treating of potato tubers (cvs. Spunta or Draga) with the three tested bacterial antagonists *i.e., Bacillus polymyxa, Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen increased

NPK content in shoots and roots of grown potato plants comparing with the infested soil only with *R. solanacearum* pathogen without any one of tested antagonists. The obtained results could be interpreting in light the findings of **El-Didamony *et al.*, (2003)** who reported that cv. Spunta exhibited the highest percent of infection and disease severity when tested for its resistance to infection with *R. solanacearum* and **Zayed, (2004)** who applied *B. subtilis*, *P. aeruginosa*, *P. fluorescens* and *P. putida* against *R. solanacearum in vitro* and in greenhouse and found that isolates of *B. subtilis, P. fluorescens* and *P. aeruginosa* inhibited the growth of *R. solanacearum.* Also, he showed *B. subtilis isolates* decreased disease severity and increased tubers yield. Also, our results are in agreement with those reported by **Venglovsky *et al.* (2005)** who declined the population and survival of *R. solanacearum* at the early stage of composting, the compost pH during the early periods of composting process was decreased due to production of organic acids causing further acidification as a result of microbial activity. The effect of compost on *R. solanacearum* is biotic and related to shift in soil microbial community structure towards a community with enhanced antagonism. Also, **Yousef (2007)** used compost to increase soil fertility of reclaimed lands, improve existing crop land, stimulate growth and suppress diseases caused by soil-borne plant pathogens. While, **Bader (2012)** found that adding the different compost types to the infected soil with brown rot pathogen reduced significantly the disease incidence compared with the control treatment (without compost).

SUMMARY

ENGLISH SUMMARY

Potato *(Solanum tuberosum L.)* is considered one of the four major and important food crops after wheat, maize and rice around the world. Potato plants are attacked with wide range of fungal, bacterial and viral diseases, where they cause serious losses in crop production and / or tuber quality. One of the most important bacterial diseases of potatoes is brown rot which caused by *Ralstonia solanacearum* where it predominates in tropical, subtropical and temperate regions and this disease is one of the major constraints to production of solanaceous crops in these regions.

The obtained results could be summarized as follows:

1- Three bacterial isolates were isolated from naturally infected potato tubers of three different cvs (Spunta, Draga and Nicola) showing brown rot diseases. All these isolates showed typical morphological growth of *R. solanacearum* on SMSA medium where colonies were fluidal white with red center.

2- Preliminary identification of the three isolates of *R. solanacearum* was confirmed by giving positive result using IFAS test (Immunoflurescence Microscope Antibody Staining).

3- The cultural and morphological characteristics of the three tested pathogenic bacterial isolates confirmed that these

three isolates are *R. solanacearum*. Also, the physiological and biochemical tests of the three tested pathogenic bacterial isolates verified that these three isolates are *R. solanacearum*.

4- Results indicated that all three tested isolates of *R. solanacearum* showed virulence against potato plants and tomato plants. On the other side, these isolates were avirulent to pepper, eggplant, tobacco and banana plants under artificial inoculation conditions which reveal that theses isolates belong to race 3. Also, results confirmed that all three tested isolates belong to biovar 2.

5- It is clear from the obtained results of fatty acid (FA) profiles of the three tested bacterial isolates that these isolates are *R. solanacearum* where cellular fatty acid analysis of the three tested *R. solanacearum* isolates showed various degree of homology with different bacterial reference. In this respect, all tested isolates gave a highest percentage of homology with the reference bacteria *Ralstonia solanacearum* (Rev. 3.90). The Draga isolate gave 92.5% followed by the Nicola isolate 78.1%, followed by spunta isolate 66.2%.

6- Results of IFAS test of the three tested *R. solanacearum* isolates which isolated from tubers of different potato cultivars (Spunta, Draga and Nicola) revealed that the cells morphology of the tested bacteria had short rod shape

stained evenly as bright green fluorescent to confirm that these three isolates are *R. solanacearum.*

7- Results of PCR technique which used for identification and detection of *R. solanacearum* isolates that isolated from different potato cultivars i.e., spunta, Draga and Nicola visualized specific 718bp PCR product under UV light showed very close similarity among the three *R. solanacearum* isolates under investigation.

8- Results of Real-time PCR technique of the three tested isolates of *R. solanacearum* using two primer/probe sets revealed that the primers and probe are specific for detection of the race 3 biovar 2 strain, giving positive results for all three isolates and indicating that the three tested isolates were *R. solanacearum* biovar 2 race 3.

9- Results indicated that the three tested *R. solanacearum* exhibited different percentages in disease severity which ranging from 76.59% infection to 98.4 % when tested on potato plants (cv.Nicola) at 15 days of incubation period. Meanwhile, the determined disease severity of the three tested *R. solanacearum* reached 100 % on tomato plants (cv. Castle rock) to reveal that Draga- isolate was the fastest one among the three tested isolates of *R. solanacearum* where, the recorded disease severity was 100% at 4 days of incubation period followed by Nicola – isolate.

10-Results showed that Draga-isolate of *R. solanacearum* infected many hosts with different degrees of wilt severity% where it was virulent to tomato, mallow, datura and little hogweed. The highest recorded wilt severity% was 80% on mallow plants followed by 60% on tomato and little hogweed plants while they were 40% only on datura. Also, Positive results were obtained with SMSA, IF and PCR techniques to confirm the infection with Draga-isolate. On the other hand, Draga-isolate was not able to exhibit any wilt symptoms on pepper, eggplant, bean, maize, faba bean and onion plant hosts with no visual latent infection on the last 4 hosts except, pepper and eggplant where the detection methods using SMSA, IF and PCR techniques exhibited latent infection with Draga-isolate on it.

11-All twelve tested compost samples were completely free from *Ralstonia solanacearum* using the different inspection methods where SMSA, IF and PCR techniques gave negative results confirming absence of potato brown rot pathogen in the tested composts.

12- Results indicated that the highest number of bacterial and fungal groups were detected in El-Dakahliya compost sample (V+A) where the recorded average of bacterial groups was 22 group/plate while, the recorded average of fungal groups was 7 group/plate followed by El-Gharbiya

compost sample (15 and 4 group/plate) respectively. Also, the highest average of bacterial colonies was recorded in Agrofood and Pico compost samples where they were 116 and 88 colony/plate respectively. Moreover, the highest average of fungal colonies were recorded in El-Dakahliya and El-Gharbiya compost samples where the recorded averages of fungal colonies were 88 and 87 colony/plate while, the least averages of fungal colonies were recorded in El-Shafee-A and El-Waha compost samples.

13- Testing of 101 different bacterial isolates and 40 different fungal isolates for their antagonistic effect against the three tested *Ralstonia solanacearum* isolates revealed that only three bacterial isolates among the 101 tested isolates inhibited the growth of *R. solanacearum* where 2 isolates were isolated from El-Gharbiya compost (vegetarian +animal) while the other one was isolated from El-Dakahliya compost (vegetarian +animal). Also, there are no one of the tested isolated fungal isolates from the different sources of compost exhibited any antagonistic effect against the three tested *R. solanacearum*.

14- Results revealed that all three tested antagonistic bacterial isolated from compost samples of El-Gharbiya and El-Dakahliya (vegetarian+animal) reduced the growth of the pathogenic bacterium compared with control treatment.

15- The traditional identification of the three antagonistic bacteria which previously isolated from compost samples of El-Gharbiya (1&2) and El-Dakahliya (3) based on their morphological, physiological and biochemical characteristics verified that these isolates are belonging to *1-Bacillus polymexa, 2- Bacillus subtilis* and 3- *Bacillus megaterium*. Also, PCR technique with specific primers confirmed the traditional identification of the three antagonistic isolates.

16- Results showed that all the three tested antagonistic isolates were positive producers of siderophores on TSA media. Meanwhile, measuring the amount of produced siderophores revealed that *Bacillus megaterium* produced the highest amount of siderophores followed by *Bacillus subtilis* and *Bacillus polymexa*.

17- Results showed also that all three tested antagonistic isolates are positive producers of antibiotics. In this respect, measuring the length of inhibition zone revealed that *Bacillus polymexa* and *Bacillus subtilis* were more producers of antibiotics than *Bacillus megaterium* which came in the second rank when tested against *R. solanacearum*.

18- Results revealed that treating potato tubers of cvs. Spunta and Draga by socking in the prepared suspension of the tested antagonistic bacteria *i.e., Bacillus polymexa,*

Bacillus megaterium and *Bacillus subtilis* before sowing in the sandy clay soil of both sterilized or non-sterilized soils decreased the wilt disease incidence (DI) and wilt disease severity (WDS) of inoculated plants with *R. solanacearum* compared with the control treatment (un-inoculated with *R. solanacearum*). Moreover, area under disease progress curve (AUDPC) was reduced in all treated plants with the tested antagonists compared with control treatment.

19- Results indicated generally that individual treating of potato tubers (cvs. Spunta and Draga) with the three tested bacterial antagonists i.e., *Bacillus polymexa*, *Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen improved the resulted growth characters where they increased shoot length, root length, shoot dry weight and root dry weight of grown potato plants.

20- Results exhibited also that individual treating of potato tubers (cvs. Spunta or Draga) with the three tested bacterial antagonists *i.e., Bacillus polymexa*, *Bacillus megaterium* and *Bacillus subtilis* before sowing in sterilized or non-sterilized soil infested with *R. solanacearum* pathogen increased NPK content in shoots and roots of grown potato plants comparing with the infested soil only with *R.*

solanacearum pathogen without any one of tested antagonists.

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الملخص العربي

تعتبر البطاطس واحدة من أهم أربع محاصيل غذائية بعد القمح والذرة والأرز علي مستوي العالم. ويهاجم البطاطس عدد كبير من الأمراض الفطرية والبكتيرية والفيروسية وما يتسبب عن ذلك من فقد كبير في إنتاجية وجودة درنات البطاطس الناتجة. ويعتبر مرض العفن البني الناتج عن بكتيريا رالستونيا سولانسيرم هو أكثر الأمراض البكتيرية التي تصيب البطاطس أهمية حيث ينتشر في المناطق الإستوائية وتحت الإستوائية والمناطق الدافئة من العالم كما يعتبر هذا المرض هو العائق الرئيسي للإنتاج لكل المحاصيل الباذنجانية في تلك المناطق.

وقد هدفت هذه الدراسة إلي عزل وتعريف المسبب المرضي للعفن البني في البطاطس باستخدام الطرق التقليدية والحديثة والتي يمكن استخدامها أيضا في كشف المسبب المرضي في الدرنات باستخدام طرق مثل تفاعل البلمرة المتسلسل (PCR) وإختبار الأحماض الدهنية. كما هدفت الدراسة أيضا إلي عزل الكثير من الكائنات الحية الدقيقة الموجودة بالكمبوست النباتي أو الحيواني أو خليط منهما والمجمع من أماكن ومصادر مختلفة ودراسة كفاءة تلك الكائنات الحية الدقيقة في تثبيط مسبب مرض العفن البني في البطاطس. كما تناولت الدراسة أيضا تعريفا لتلك الكائنات الحية الدقيقة المعزولة والتي ثبت امتلاكها القدرة علي تثبيط نمو بكتيريا رالستونيا سولانسيرم تحت ظروف المعمل وكذلك دراسة قدرة تلك الكائنات المضادة المعرفة علي إنتاج مركبات السايدروفورز والمضادات الحيوية التي تضاد بكتيريا رالستونيا سولانسيرم. بالإضافة الي ذلك فقد هدفت الدراسة أيضا إلي دراسة تأثير تلك الكائنات المضادة المعرفة علي مقاومة مرض العفن البني علي البطاطس تحت ظروف الصوبة.

ويمكن تلخيص أهم النتائج المتحصل عليها كاتالي:

١- تم عزل ثلاث عزلات بكتيرية من درنات ثلاث أصناف بطاطس شائعة الزراعة والتداول ويظهر عليها أعراض الإصابة بالعفن البني وهي أصناف سبونتا ودراجا ونيقولا. وقد أظهرت الثلاث عزلات البكتيرية المعزولة تطابقا في النمو الظاهري لبكتيريا رالستونيا سولانسيرم علي

بيئة SMSA حيث ظهرت مستعمراتها مائية بيضاء مع وجود مركز لونه أحمر للمستعمرات.

٢- أعطي إختبار IFAS نتائج إيجابية ليؤكد التعريف الأولي للثلاث عزلات البكتيرية علي أنها بكتيريا رالستونيا سولانسيرم.

٣- أكدت الطرق التقليدية للتعريف بناءا علي الخصائص المورفولوجية والمزرعية والإختبارات البيوكيمائية والفسيولوجية أن الثلاث عزلات البكتيرية المعزولة مسبقا من درنات البطاطس هي بالفعل لبكتيريا رالستونيا سولانسيرم بمقارنتها بوصف البكتريا في المراجع السابقة.

٤- أشارت النتائج أن الثلاث عزلات المختبرة لبكتيريا رالستونيا سولانسيرم كانت ممرضة لنباتات البطاطس والطماطم كما لم تكن تلك العزلات ممرضة لنباتات الباذنجان والفلفل والدخان والموز تحت ظروف العدوي الصناعية بما يشير أن الثلاث عزلات تابعة للسلالة ٣ والطراز الإحيائي ٢.

٥- كان واضحا من نتائج إختبار الأحماض الدهنية للثلاث عزلات البكتيرية المختبرة أن الثلاث عزلات هي لبكتيريا رالستونيا سولانسيرم حيث أوضح تحليل الأحماض الدهنية للبكتيريات الثلاث درجات مختلفة من التشابه مع مختلف البكتيريات المستخدمة للمقارنة والقياس. فقد أعطت الثلاث عزلات المختبرة لبكتيريا رالستونيا سولانسيرم نسبة عالية من التشابه مع بكتيريا بركولدريا سولانسيرم وبكتيريا رالستونيا سولانسيرم المستخدمتين للمقارنة والقياس حيث أعطت عزلة دراجا نسبة تشابه ٩٢.٥% وعزلة نيقولا ٧٨.١% بينما أعطت عزلة سبونتا نسبة تشابه وصلت ٦٦.٢%.

٦- أظهرت نتائج إختبار IFAS للثلاث عزلات المختبرة من بكتيريا رالستونيا سولانسيرم والمعزولة من درنات ثلاث أصناف بطاطس مختلفة (سبونتا، دراجا ، نيقولا) أن موفولوجي خلايا الثلاث عزلات تأخذ الشكل العصوي القصير مع اللون الفلورسنتي الأخضر اللامع لتؤكد أن الثلاث عزلات المختبرة هي لبكتيريا رالستونيا سولانسيرم.

٧- أظهرت نتائج تقنية تفاعل البلمرة المتسلسل (PCR) بإستخدام بادئ متخصص لكشف وتعريف الثلاث عزلات المختبرة من بكتيريا رالستونيا سولانسيرم والمعزولة من درنات ثلاث أصناف بطاطس مختلفة (سبونتا، دراجا ، نيقولا) وجود أمبيلكون مميز من DNA ذو وزن جزيئي (٧١٨bp) مع وجود درجة تشابه عالية بين الثلاث عزلات المختبرة لبكتيريا رالستونيا سولانسيرم في هذا الشأن.

٨- أظهرت نتائج تقنية Real-time PCR technique المستخدمة لكشف وتعريف الثلاث عزلات المختبرة من بكتيريا رالستونيا سولانسيرم والمعزولة من درنات ثلاث أصناف بطاطس مختلفة (سبونتا، دراجا ، نيقولا) باستخدام عدد ٢ بادئ ومجس أن البادئ B_2 ومجسه هما الأنسب تخصصا لكشف السلالة ٣ والطراز الإحيائي ٢ حيث أعطي هذا الإختبار نتائج إيجابية مع الثلاث عزلات المختبرة ليؤكد أن الثلاث عزلات المختبرة هي لبكتيريا رالستونيا سولانسيرم السلالة ٣ والطراز الإحيائي ٢.

٩- أشارت نتائج إختبار القدرة الامراضية أن الثلاث عزلات المختبرة لبكتيريا رالستونيا سولانسيرم أحدثت درجات متباينة من الشدة المرضية ما بين ٧٦.٥٩% - ٩٨.٤% عند فترة تحضين ١٥ يوم عندما أختبرت علي صنف البطاطس نيقولا. وفي نفس الوقت فقد وصلت شدة الإصابة المقدرة للثلاث عزلات المختبرة إلي ١٠٠% علي نباتات الطماطم صنف كاسل روك لتظهر أيضا أن عزلة رالستونيا سولانسيرم المعزولة من درنات البطاطس صنف دراجا هي الأسرع والأشد مرضية بين العزلات المختبرة حيث أعطت شدة إصابة ١٠٠% بعد ٤ يوم تحضين وقد تبعها في ذلك عزلة نيقولا.

١٠- أوضحت النتائج أن عزلة رالستونيا سولانسيرم المعزولة من درنات البطاطس صنف دراجا يمكنها أن تصيب العديد من العوائل النباتية مسببة درجات مختلفة من شدة الإصابة حيث كانت ممرضة لنباتات الطماطم والخبيزة والداتورا وعرف الديك والرجله. فقد كانت أعلي شدة إصابة بالذبول هي ٨٠% علي نباتات الخبيزة متبوعة بـ ٦٠% علي

نباتات الطماطم والرجله بينما كانت شدة الإصابة بالذبول علي الداتورا هي ٤٠%. أكدت النتائج الإيجابية المتحصل عليها من استخدام بيئة SMSA, وإختبار IFAS وتقنية الـ PCR إصابة النباتات المختبرة بعزلة رالستونيا سولانسيرم (صنف دراجا). وعلي الجانب الآخر لم تكن عزلة رالستونيا سولانسيرم (صنف دراجا) قادرة علي إظهار أي أعراض ذبول علي العوائل النباتية المختلفة (فلفل، باذنجان، فاصوليا، ذرة، فول بلدي، بصل) كما لم تكتشف الإصابة الكامنة علي الأربع أصناف الأخيرة بينما أكتشفت الإصابة الكامنة بعزلة صنف دراجا علي الفلفل و الباذنجان بنفس الطرق والإختبارات السابق ذكرها.

١١- أكدت اطرق الفحص المختلفة باستخدام بيئة SMSA وإختبار IFAS وتقنية الـ PCR خلو ١٢ عينة كمبوست مختبرة تماما من بكتيريا رالستونيا سولانسيرم حيث أعطت تلك الإختبارات نتائج سلبية في هذا الشأن.

١٢- أشارت نتائج عزل الكائنات الحية الدقيقة من عينات الكمبوست المختبرة أن أعلي عدد للمجموعات الفطرية والبكتيرية قد سجل في عينة كمبوست الدقهلية (نباتي + حيواني) حيث كانت المجموعات البكتيرية المسجلة ٢٢ مجموعة/طبق بينما كانت المجموعات الفطرية المسجلة ٧ مجموعة/طبق وقد تبعها في ذلك عينة كمبوست الغربية (١٥ ، ٤ مجموعة/طبق) علي التوالي. كما سجل أيضا أعلي عدد للمستعمرات البكتيرية في عينات كمبوست أجروفوود وبيكو (١١٦ ، ٨٨ مستعمرة/طبق) علي التوالي. فضلا عن ذلك، سجل أعلي عدد للمستعمرات الفطرية في عينات كمبوست الدقهلية والغربية (٨٨،٨٧ مستعمرة/طبق) بينما سجل أقل عدد للمستعمرات الفطرية في عينات كمبوست الشفعي-ا والواحة.

١٣- أختبر التأثير التضادي لـ ١٠١ عزلة بكتيرية و ٤٠ عزلة فطرية معزولة من عينات الكمبوست المختبرة ضد ثلاث عزلات من بكتيريا رالستونيا سولانسيرم السابق عزلها من درنات أصناف البطاطس (سبونتا ، دراجا ، نيقولا) ، وقد أظهرت النتائج قدرة ثلاث عزلات

بكتيرية فقط من بين ١٠١ عزلة المختبرة علي تثبيط نمو بكتيريا رالستونيا سولانسيرم حيث كانتا أثنين من العزلات المضادة معزولتان من عينة كمبوست الغربية (نباتي + حيوانى) وواحدة من عينة كمبوست الدقهلية (نباتي + حيوانى). كما أكدت الإختبارات عدم قدرة أي عزلة فطرية من مجموع ٤٠ عزلة معزولة من عينات الكمبوست المختلفة علي تضاد بكتيريا رالستونيا سولانسيرم المختبرة.

١٤- أظهرت النتائج أن الثلاث عزلات البكتيرية المعزولة من عينات كمبوست الغربية والدقهلية (نباتي + حيوانى) قد خفضت من نمو بكتيريا العفن البني الممرضة مقارنة بمعاملة الكنترول عند إعادة تقييم تأثيرها التضادى معمليا مرة أخري.

١٥- بينت طرق التعريف التقليدية للثلاث عزلات البكتيرية المضادة والمعزولة من عينات كمبوست الغربية والدقهلية بناءا علي خصائصه المورفولوجية والفسيولوجية والبيوكيميائية أن تلك العزلات هي لبكتيريا ١- باسيلس بوليمكسا ،٢- باسيلس ستلس (كمبوست الغربية) والثالثة هى لبكتيريا ٣- باسيلس ميجاتيرم (كمبوست الدقهلية). كما أكدت تقنية PCRأيضا باستخدام بادئ متخصص لكل عزلة من العزلات البكتيرية المضادة نتائج التعريف بالطرق التقليدية المتحصل عليها.

١٦- أوضحت النتائج أن الثلاث عزلات البكتيرية المضادة كانت منتجة لمركب السايدروفورز علي بيئة TSA. كما أظهرت النتائج أيضا أن عزلة باسيلس ميجاتيرم هي الأعلي إنتاجا لمركب سايدروفورز متبوعة بعزلتي ، باسيلس ستلس ، باسيلس بوليمكسا علي التوالي.

١٧- أوضحت النتائج أيضا أن الثلاث عزلات البكتيرية المضادة تمتلك القدرة علي إنتاج المضادات الحيوية وكانت عزلات باسيلس بوليمكسا ، باسيلس ستلس هي الأعلي إنتاجا للمضادات الحيوية مقارنة بعزلة عزلة باسيلس ميجاتيرم وذلك عند إختبارها ضد بكتيريا رالستونيا سولانسيرم .

١٨- أظهرت النتائج أن معاملة درنات البطاطس صنفى سبونتا ودراجا بالغمر في معلق معد لبكتيريا باسيلس بوليمكسا ، باسيلس ميجاتيرم ، باسيلس ستلس قبل الزراعة في تربة رملية طينية معقمة وغير معقمة قد خفض بشكل واضح من نسبة وشدة الإصابة بالذبول للنباتات الملقحة ببكتيريا رالستونيا سولانسيرم مقارنة بمعاملة الكونترول (غير ملقحة ببكتيريا رالستونيا سولانسيرم). فضلا عن ذلك، فقد أدى غمر درنات البطاطس صنفى سبونتا ودراجا في معلق البكتيريا المضادة المختبرة إلي تخفيض نسبة منطقة الإصابة تحت منحنى المرض(AUDPC).

١٩- أشارت النتائج بشكل عام أن المعاملة الفردية لدرنات البطاطس صنفي سبونتا ودراجا بالثلاث عزلات البكتيرية المضادة (باسيلس بوليمكسا ، باسيلس ميجاتيرم ، باسيلس ستلس) قبل الزراعة في تربة معقمة وغير معقمة ملقحة ببكتيريا رالستونيا سولانسيرم قد حسنت من صفات النمو المقدرة حيث زادت من طول والوزن الجاف لأفرع وجذور نباتات البطاطس النامية.

٢٠- أظهرت النتائج أيضا أن المعاملة الفردية لدرنات البطاطس صنفي سبونتا ودراجا بالثلاث عزلات البكتيرية المضادة (باسيلس بوليمكسا ، باسيلس ميجاتيرم ، باسيلس ستلس) قبل الزراعة في تربة معقمة وغير معقمة ملقحة ببكتيريا رالستونيا سولانسيرم قد زادت من محتويNPK في أفرع وجذور نباتات البطاطس النامية مقارنة بمعاملة التربة المعدية ببكتيريا رالستونيا سولانسيرم وبدون أي إضافة لأي من البكتيريات المضادة.

صفحــــة الموافقـــــة

دور الكائنات الدقيقة في الكمبوست في مقاومة مرض العفن البني علي البطاطس

رسالة مقدمة من

محمد سليمان محمد يوسف

بكالوريوس العلوم الزراعية – جامعة القاهرة (٢٠٠٣)

وقد تمت مناقشة الرسالة والموافقــــــة عليها من اللجنـــــــة

أ.د/ فاروق محمد بركات أستاذ أمراض النبات – قسم أمراض النبات كلية الزراعة – جامعة القاهرة	....
أ.د/ عبده مهدي محمد مهدي أستاذ أمراض النبات ورئيس قسم النبات الزراعي كلية الزراعة بمشتهر - جامعة بنها	....
أ.د/ جهاد محمد دسوقي الهباء أستاذ أمراض النبات - قسم النبات الزراعي كلية الزراعة بمشتهر - جامعة بنها	....
أ.د/ فتحي جاد محمد عبد الجواد أستاذ أمراض النبات - قسم النبات الزراعي كلية الزراعة بمشتهر - جامعة بنها	....

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لجنـــــة الإشـــــراف

دور الكائنات الدقيقة في الكمبوست في مقاومة مرض العفن البني علي البطاطس

رسالة مقدمة من

محمد سليمان محمد يوسف

بكالوريوس العلوم الزراعية – جامعة القاهرة (٢٠٠٣)

للحصول علي
درجة الماجستير في العلوم الزراعية
(أمراض النبات)

لجنـــــة الإشراف العلمـــــي:

أ.د/ جهاد محمد دسوقي الهباء ....
أستاذ أمراض النبات - قسم النبات الزراعي
كلية الزراعة بمشتهر - جامعة بنها

أ.د/ فتحي جاد محمد عبد الجواد ....
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أ.د/ صفوت عبد الحميد الحداد ....
رئيس بحوث – معهد أمراض النباتات
مركز البحوث الزراعية-الجيزة

قسم النبات الزراعي
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دور الكائنات الدقيقة في الكمبوست في مقاومة مرض العفن البني علي البطاطس

رسالة مقدمة من

محمد سليمان محمد يوسف

بكالوريوس العلوم الزراعية – كلية الزراعة – جامعة القاهرة
(٢٠٠٣)

لاستيفاء متطلبات الحصول على درجة
الماجستير في العلوم الزراعية
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