EFFECT OF COMBINATION OF SOME BIO- AND ABIOTIC AGENTS WITH COMPOST ON POTATO BROWN ROT DISEASE CAUSED BY *RALSTONIA SOLANACEARUM*

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ABSTRACT

Ten isolates of Ralstonia solanacearum (Rs) were isolated and identified with the traditional and modern techniques from the diseased potato tubers and soil collected from the tested fields in Qalubia and Behara governorates, Egypt. All tested R. solanacearum isolates induced bacterial wilt disease symptoms on all inoculated potato plants compared with the non-inoculated control. The highest increase in infection % and disease severity were induced by the isolate of R. solanacearum (No.6) compared with other isolates. Treatment of infected soil by bio-agent (B. subtilis and Ps. fluorescents) with 10% mixed compost and spraying potato shoots (Cara cv.) by abiotic factors (salicylic acid and K_2HPO_4 at rate of 4.5 mM) reduced the brown rot disease symptoms expression on plants even after 45 days from infection of soil by pathogenic bacterium. Most treated treatments induced a marked increase in activities of peroxidase (PO), catalase (CL) and polyphenoloxidase (PPO) enzymes in the leaves of treated plants comparing to the untreated control plants. Total phenolic compounds content in the treated plants was higher than in the untreated plants. On the other hand, protein analysis of the potato plant leaves treated with combination between of bioagents and abiotic factors show induced positive changes in protein against to brown rot disease. Some new proteins bands appeared when compared with the control. The appearance of new bands may be due to effect of the used treatments and the active defense of potato plants under the effects of the aforementioned treatments.

Key words: *Potato, Brown rot, R. solanacearum, Disease control, Bioagents, Abiotic factors, Induced resistance, Oxidative enzymes, Phenolic compounds, Protein analysis.*

الملخص

في هذه الدراسة تم عزل وتعريف 10 عزلات من بكتيريا *Ralstonia solanacearum* المسببة لمرض العفن البني (الذبول) على البطاطس من عينات الدرنات والتربة المجموعة من محافظتي القليوبية والبحيرة وقد اظهر نتائج اختبار القدرة المرضية قدرة العزلات علي انتاج المرض وكانت العزلة R_6 اكثر فعالية من العزلات الأخرى. وقد أدى معاملة التربة ببعض العوامل الحيوية (*B. subtilis* و *P. fluorescens*) والعوامل غير حيوية (حمض السليسليك ، K_2HPO_4 في تركيز 4.5 مل مولر) في وجود 10% كومبوست الى خفض اعراض مرض العفن البني(الذبول) على نباتات البطاطس حتى بعد 45 يوما من المعاملة، كما أظهرت معظم المعاملات المختبرة زيادة ملحوظة في نشاط الانزيمات المؤكسدة (بيروكسيداز ، بولي فينول اكسيداز، كتاليز) والمركبات الفينولية الكلية في اوراق النباتات المعاملة مقارنة بغير المعاملة. كما اظهر نتائج تحليل البروتينات باستخدام (SDS-PAGE) ملاحظة تكون بندات جديدة (412.86، 87.72، 85.08،79.63، 63.09 , 44,24، 38.2 , و 36.6 KDa) من البروتين في مستخلصات اوراق نباتات البطاطس المعاملة كرد فعل من النباتات المعاملة لمقاومة المرض حيث لوحظ ظهور البند الجديد 412.86 كيلو دالتون مع المعاملة (حامض السليسليك+ K_2HPO_4 في تركيز 45 مل مولر) حيث انخفضت نسبة الإصابة المرض الى 20% بينما لم يظهر في معاملة الكنترول في وجود المسبب المرض (نسبة الإصابة 80 %).

INTRODUCTION

Brown rot disease caused by *Ralstonia solanacearum* **(Yabuuchi *et al.*1995)** is the second major constraint to potato production in tropical, subtropical and warm temperate regions worldwide after late blight (**Hayward, 1994).** The brown rot is a serious economic problem in several countries where potatoes are cultivated over large areas and economical exportation crop like Egypt. *R. solanacearum* bacterium was isolated on tetrazolium chloride medium (TZC) from infected plants, symptom less plants, seeds extracted from fruits of wilted and stem inoculated plants developed with symptoms **(Sumithra *et al.*, 2000)**. Characterization of strains of *Ralstonia solanacearum*, the causal agent of potato bacterial wilt disease from Nepal and Thailand, was performed based on their pathogenicity, biochemical, physiological and serological tests **(Shambhu *et al.*, 2001)**. **Al-Ani *et al.* (2004)** isolated three *Ralstonia solanacearum* isolates (R_1, R_2 and R_3) from roots and stems of tomato wilting plants collected from fields of Iraq. White, raised and shiny colonies with rose center were developed on TZCA medium. The genus *Ralstonia* was identified according to the visible characteristics of colonies on NA and PDA media, cell shape, and its ability to grow on KB selective medium and to produce oxidase, catalase and acids was reported. The species *solancearum* was identified on the basis of its ability to reduce nitrate, produce H_2S, produce ammonia from peptone and on its ability to assimilate certain carbohydrates. Assimilation results of mannitol, lactose, cellobiose, sorbitol and dulcitol reveal that the R_2 isolate belong to biotype 3, whereas $R_1$and R_3 isolates belong to biotype 5. **Balabel *et al* (2006)** suggested that plating bacterial suspensions of *R. solanacearum* from different sources revealed virulent and a virulent 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.

Some bioagents and abiotic factors could be used to control the soil borne disease including potato brown rot disease (**Zayed 2004).** Resistance of plants to pathogens can be enhanced by application of various bioagenta and abiotic factors called induce systemic resistance in plants and the most effective treatments in increasing PO activity were salicylic acid (SA) 5.0 mM, K_2HPO_4 100 mM and ethephon 300 ppm (**Abd-El-Kareem 2008**). Treatment of cucumber leaves with K_2HPO_4 induced systemic accumulation of peroxidase in the leaves (**Irving and Kuc (1990)**. Treatment with inducers led to an increase in activities of peroxidase, polyphenoloxidase, phenylalanine ammonia lyase in cucumber and tobacco leaf tissue and additionally in tobacco to an increase in lysozyme activity. However, the extent and the time-course of the increase varied according to the inducer and host plant. The time-course of resistance and increased enzyme activities in cucumber also correlated with the increased formation of papillae in epidermal cells **(Schneider and Ullrich1994)**. The most effective treatments were K_2HPO_4 and K_3PO_4. Powdery mildew infection was significantly reduced by 92% when the plants were treated with 50mM K_2HPO_4, 3 days after inoculation. K_2HPO_4 treatment caused a remarkable increase bf peroxidase activity in 60^{th} infected on non infection control plants **(Mosa 1997)**.

The activation of peroxdase (PO), catalase (CL) and polyphenoloxidase (PPO) plays were a crucial role in the biological control and resistance of plants to pathogenic attack (**Kortekamp and Zyprian, 2003)** and polyphenoloxidase enzymes **(Agrios 1997)**. Peroxidase enzyme is also related d, phenolypropanoid metabolism in plant leads to

formation of numerous phenolic compound that are bto plant defense acting in strengthening the cell wall (**De Ascensao and Dubery, 2003**). It is indicated that faba been seed treatment or foliar treatment with biotic agents inducers i.e. *Pseudomonas* fluorescens and *P. aeruginosa* increased greatly the activities of peroxidase after 24-h and polyphenoloxidase after 12-h compared with the untreated control with superiority of *P. aeruginosa* than *P. fluorescens*. On the other hand, the highest increase in β-1-3-glucanase activity was recorded after 24-h in foliar and seed treatments with *P. fluorescens compared with P. aeruginosa* and control (**Mazen, 2004**). On the other hanelieved to have important function in plant defense responses to wounding and pathogen infection **(Hahlbrock and scheel 1989; Nicholoson and Hammershmid, 1992).** As well as the protein bands that disappeared or generations in the potatoes plants were discussed with many investigators such as **Gregory *et al.* (2003)** stated that, the mechanism of plant defense against the pathogen might back for these protein bands. Recently, **Hajhamed (2011)** revealed that, number of protein bands detected in infected shoots of all cultivars was less than those in healthy shoots and the number of absent band due to infection in resistance cultivar is less than the number of absent band in susceptible or moderately susceptible cultivars. Meanwhile, found that, infected plants of susceptible cultivar (Lady rosetta) comparing with healthy plant gave only 14 bands with absence of bands number 11, 13, 15 and 16 with molecular weights 39.506, 32.675, 26.331 and 24.355 KDa, respectively. While, resistance cultivar (Lady balfor) comparing with healthy plant gave 15 bands with absence of band number 13, 15 and 16 with molecular weights 32.675, 26.331 and 24.355 KDa, respectively.

This work aimed to evaluate the effect of combination of some bioagent and abiotic factors with 10 % compost as resistance induced against bacterial brown rot disease on potato under greenhouse conditions in Egypt. Also, to study the effects of tested treatments on disease severity, infection %, activity of oxidative enzymes, phenol compounds and protein analysis.

MATERIALS AND METHODS

I. Isolation and identification of *Ralstonia solanacearum* isolates :

Diseased potato tuber and soil samples collected were from potato cultivations in Qalubiya and Behera governorates of Egypt during growth season 2009. As for isolation from tubers samples of discolored vascular tissues and oozes were placed in 2 ml sterile water whereas soil samples (10 g each) were placed into conical flask (250 ml) containing 90 ml of sterilized distilled water and chicken vigorously for 15 minutes then serial dilutions were made. A loopful of the bacterial suspension obtained from tubers or from diluted soil suspension (10^{-6} and 10^{-7}) was streaked onto glycerol nutrient agar (GNA) medium containing triphenyl tetrazolium chloride (TZC 0.1 g/100ml) (**Kelman,1954).** The cultured plates were incubated at 28 ºC for 48 h and observed daily for any bacterial growth. Colonies of ***R. solanacearum*** were the selective media of South Africa (SMSA) suggested by (**Elphinstone *et al*., 1996**). Traditional identification methods of *Ralstonia solanacearum* isolates based **o**n their morphological, physiological and biochemical characteristics were achieved as described by (**Schaad 1980, Fahy and Persley 1983, Bergy's manual 1984, Lelliott and Stead 1987, Klment *et al*. 1990 and Adhikari 1993**). In addition, the of *Ralstonia solanacearum* isolates was confirmed using Modern identification methods by Immunofluorscence Antibody Stain **(**IFAS) according to **(Robinson 1993)** and Polymerase Chain Reaction (PCR)

according to **(Pastrik *et al.* 2002)**. using the following reaction conditions:

a. Immunofluorescence antibody stains (IFAS):

IFAS was applied as a serological method for rapid detection and presumptive identification of the concerned bacterium. The anti- rabbit antiserum is conjugated with fluorescin isothiocyanate (FITC) and used along with IFAS test. Suspension containing 10 cells per ml from the bacterial culture and reference grown on NA medium was prepared. A standard measured volume (20 µ1 for 6 mm well diameter) was pipetted to five successive wells of a 10- well test slide. Slides were air dried at room temperature or at 40 °C and thin fixed by heating with flame. All wells were covered with 25µl of the antiserum (anti- *R. solanacearum* polyclonal (obtained from the Netherlands) in 4 dilutions (1:1600, 1:3200, 1: 6400 and 1: 12800). Slides were incubated for 30 minutes at room temperature in a humid dark chamber, then washed with Tween 20 buffer and 0.01 M phosphate buffer (PB, pH 7.2) and incubated with 25 µl of anti - rabbit Nordic SW/ AR. Fluorescent isothiocyanate conjugate in a 100 fold dilution for 30 minutes in a humid chamber. Slides were washed with Tween 20 buffer and 0.01M (PB pH 7.2), and blotted carefully with filter paper. One droplet of 0.1M phosphate buffer glycerin (pH 7.6) was added to each well and the slides were then covered with long glass covers. Slides were examined with a microscope (tube factor 1.25) with an epi-fluorescent light source and suitable filters with FITC, using a 100x oil immersion objective (1250x) and a l0x eyepiece. At least 20 microscopic fields per well were scanned for the presence of morphologically typical fluorescing cells (**Robinson 1993**).

b. Polymerase chain reaction (PCR):

Serological techniques can lack specificity due to cross- reactions of polyclonal antibodies with other bacteria and many have limited sensitivity (**Elphinstone *et al.* 1996)**. In contrast, PCR is one of the rapid, highly specific and sensitive tests used for detection and identification of *R. solunacaarum* (*Rs*) from different sources **(Pastrik *et al.* 2002).** In this method, two oligonucleotide primers namely forward primer *Rs*-l-F (5\ ACT AAC GAA GCA GAG ATG CAT TA- 3\) and Reverse primer RS- 1-R (5\ CCC AGT CAC GGC AGA GAG T- 3\). The expected amplicon size from *R. solanacearurn* template DNA is 718 base pair (bp) using the following reaction conditions:

b. 1. Extraction of DNA:

In this study, 6 isolates were propagated on nutrient agar medium for 24 hours at 28 °C. A suspension of 10^6 cm/ml from each isolate in screw capped micro tubes was prepared. Crude DNA was extracted by boiling 100µl from each suspension (10^6 cfu/ml/ isolate) in a micro-vial at 100°C for 5 minutes on a heating block. Cool the micro-vials immediately on ice. Reference strain was provided by Potato Brown Rot Project (PBRP) and used as a positive control.

b.2. DNA amplification:

Two µ1 from each DNA template (isolate) were added to 23 µl reaction mixtures in 0.6 µ l micro tubes DNA & RNAse free [(14.25 µl, Sterile Ultra Pure Water (SUPW); 2.5 µ l of 10X PCR buffer (15mM $MgCl_2$); 1.5 µl $MgCl_2$ Promega (25mM); (d-NTP mix l0µM) 0.0625 µl of each d-ATP, d-CTP, d-GTP and d-TTP; 2 µl *Rs* 1-F primer l0 um; 2 µl Rs 1-R primer l0µM and 0.2 µl Taq polymerase (5 µ /µl))]. Different PCR cycles were performed according to **(Pastrik *et al.* 2002):** (i) 1 cycle of 5 min at 95°C to denature 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 and **(iv)** 1 cycle of 45 seconds each at 72 °C for extension of copy and **(v)** final extension cycle of 5 min at 72 °C. **(vi)** hold at 4 °C.

b. 3. Analysis of the PCR product:

PCR product (amplicons or PCR fragments) were detected by agarose gel electrophoresis and stained with ethidium bromide. 2% (w/v) agarose gel was prepared by slowly mixing 2g agarose in 100 ml of 1x Tris- acetate- EDTA (TAE) buffer and boiling for 5 min. then cool to 50 °c before adding 5 µl of ethidium bromide solution and mixing gently. The gel was poured into the sealed gel tray, avoiding the introduction of bubbles into the gel. 20 wells were made in the gel at 10-15 mm from the edge. The gel was left to solidify for 20 minutes at room temperature then the comp and the sealing tap were removed. The gel tray was placed in a large electrophoresis tank containing 1x TAE buffer to a depth of at least 5mm buffer above the gel. 12 µl of the PCR product from each sample were mixed with 3 µl of loading buffer spotted onto Para film. 6 µ of a 100 bp DNA marker were mixed with 3 µl UPDW and 3 µl loading buffer and loaded into the right well. The positive control (12 µl) and the distilled water as a negative control (12 µl) were mixed with the loading buffer (3µl / each). All samples were loaded into the wells of the gel. Gel was run by adjusting the voltage to 80v at 400 mA (8v/lcm) until the front of tracking indicator being within 1cm from the end. The DNA was run from cathode (black) to Anode (red) by watching the movement of the loading dye. The power supply was switched off and the gel was removed carefully, soaked in the ethidium bromide solution (0.5 µg /ml) for 30- 45 min. A specific PCR products of 718 bp was visualized under short wave UV trans illumination (355nm) and the result was recorded by photograph **(Pastrik *et al.* 2002).**

II. Isolation and Identification of bio-agents bacteria (*Bacillus subtilus* and *Pseudomonas fluorescens*):

Bacillus spp. were isolated from potato rhizosphere soil samples collected from the same localities by streaked on plates of GNA medium. Isolates were identified to species according to morphological and physiological traits recommended by (**Bergy's, 1984; Schaad, 1980**). While, and *Pseudomonas fluorescens* isolates were dilution on King's B (KB) medium (**King *et al.*, 1954 and Lelliot and Stead 1987**). Cells were selected on KB medium and incubated at 30 °C for 24 hr, then, the plates were placed under ultra violet (UV) lamp to detect the presence of fluorescent colonies. A fluorescent pseudomonad was identified to species according to the morphological and physiological traits recommended by (**Schaad, 1980; Fahy and Persley 1983; Bergy's manual, 1984 and Klment *et al.*, 1990**).

III. Pathogenicity test of different *R. solanacearum* isolates:

Potato plants (cv.Cara) were grown in pots (25 cm in diameter), stems were inoculated with prepared bacterial suspension of tested *R. solanacearum* isolates according to (**Kelman 1954)** where the bacterial suspensions of tested RS were prepared and used immediately for inoculating potato plant stems by 23µl of bacterial suspension (10^9 cfu/ml) over the soil level. Plain sterilized water was used instead of bacterial suspension. The inoculated plants were kept under greenhouse conditions and observed for appearance of brown rot (wilt) symptoms. Five weeks after inoculation, the disease severity and infection% were carried out using 1-5 scale according to (**He *et al.* 1983**). The obtained data were statistically analyzed using the analysis variance and the least significant

difference at 0.05 was calculated **(Snedecor and Cochran, 1989).**

IV. Effect of combination between of tested some bio- and abiotic agents with 10 % mixed

compost on:

a. The incidence of potato brown rot disease:

Sterilized soil and amended with 10% mixed compost type (b) (animal + vegetarian) was inoculated by bacterial suspensions ($2x10^9$ cfu/ml) of some bioagents [*B. subtilis* (*Bs*) & *Ps. fluorescents* (*Pf*)] and *R. solanacearum* (No.6) at rate 100 ml/pot to each isolate. Sterilized potato tubers were planting at rate one tuber /pot. The abiotic factors (Salicylic acid and K_2HPO_4 at 4.5 mM conc.) were spraying on potato plants at rate one spray weekly for subsequence five weeks **(Zayed 2004).** The inoculated plants were kept under greenhouse conditions and observed for appearance of brown rot (wilt) symptoms and read was taken weekly after 21 days from planting**.** Disease severity, percentage of infection and efficacy % was recorded according to (**He *et al.* 1983).**

b. On induction the Peroxidase Polyphenoloxidase and Catalase enzymes activity as well as phenolic compounds in treated potato plants:

The four leaves of potato plants (cv. Cara) either treated or untreated (at seedling stage) with combination between of bio- and abiotic agents (as previously described) were cut at the leaf base level after 45 days from soil inoculation by *Rs* and bioagents (*Bs*, *Pf* & *Bs* + *Pf*). Samples were prepared according to **Goldschmidt *et al.* (1968)**. The clear supernatants were collected and used for assaying activities of the oxidative enzymes *i.e.*, Peroxidase (POD) and Polyphenoloxidase (PPO) and Catalase using a Spectrophotometer (SPECTRONIC 20-D) at 27±2°C according to **Allam and Hollis (1972), Matta and Dimond (1963)** and **Maxwell and Bateman 1967,** respectively. Meanwhile, phenolic compounds were assessments according to **Bozarth and Diener (1963)** and **Bray and Thrope (1954).**

c. On protein analysis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) was used according the method of (**Laemmli 1970)**. The samples were randomly chosen from the leaves of each treatment under study and those of the control. Extraction of water soluble proteins and water non-soluble proteins was as the following: One gram from leaves to each treatment were ground with Liquid Nitrogen using a mortar and pestle. Sample powders were transferred to eppendorf tubes with 1 ml of water soluble extraction buffer and left in refrigerator over night, then centrifuged for 10 min at 12,000 rpm under 4° C. The supernatants containing water-soluble protein fraction were transferred to new tubes and stored at -20° C. The residual water soluble proteins in pellets were removed by repeating this step twice, then 1ml water non-soluble extraction buffer was added to each pellet and left in refrigerator overnight, then centrifuged at 12,000 rpm under 4°C for 10 min. The supernatants containing water soluble protein were transferred to new tubes and stored at -20° C until use for electrophoretic analysis.

RESULTS AND DISCUSSION

I. Isolation of *Ralstonia solanacearum* isolates:

As clear in Table (1), Ten ***R. solanacearum* isolates** were isolated from potato tubers and soil samples collected from Qalubiya and Beheira governorates in Egypt. In this respect, five isolates encoded R2, R4, R6, R8 and R10 were isolated from Qalubiya while

the other five isolates encoded R1, R3, R5, R7 and R9 were isolated from Beheira governorate. Meanwhile, three isolates encoded R2, R4 and R6 were isolated from potato tubers and two isolates encoded R8 and R10 were isolated from soil samples which collected from Qalubiya. Whereas, four isolates encoded R1, R3, R7 and R9 were isolated from potato tubers and one isolate encoded R5 was isolated from soil samples collected from Beheira governorate.

Table (1): Isolated bacteria from infected potato tuber and soil samples of Qalubiya and Beheira governorates in Egypt.

Bacterial isolates	Isolation governorates			
	Qalubiya governorate		Beheira governorate	
	Tubers	Soil	Tubers	Soil
R1	-	-	+	-
R2	+	-	-	-
R3	-	-	+	-
R4	+	-	-	-
R5	-	-	-	+
R6	+	-	-	-
R7	-	-	+	-
R8	-	+	-	-
R9	-	-	+	-
R10	-	+	-	-

II.

Identification of *Ralstonia solanacearum* isolates:

Ten bacterial isolates isolated from the diseased potato tubers and field soil samples of potato cultivations in Qolubia and Behara governorates, Egypt during the growing season 2009 were identified as *Ralstonia solanacearum* using traditional identification methods based on their morphological, physiological and biochemical characteristics as cleared in **Tables (2 a &b) and Plate (1).** These results agreed with (**Summithra, *et al.*, 2000, Shambhu, *et al.*, 2001, Al-Ani, *et al.*, 2004 and Balabel, *et al.*, 2006).**

Table (2a): Morphological and physiological characteristics of *R. solanacearum* isolates during the growing season 2009.

Characteristics	Tested isolates									
	***R*$_1$**	***R*$_2$**	***R*$_3$**	***R*$_4$**	***R*$_5$**	***R*$_6$**	***R*$_7$**	***R*$_8$**	***R*$_9$**	***R*$_{10}$**
Short Rod	**Short Rod**	**Short Rod**	**Short Rod**	**Short Rod**	**Short Rod**	**Short Rod**	**Short Rod**	**Short Rod**	**Short Rod**	**Short Rod**
Gram staining	-	-	-	-	-	-	-	-	-	-
Spores formation	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+
Production of fluorescent on KB	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-

Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Redaction of nitrate	+	+	+	+	+	+	+	+	+	+
Levan production	-	-	-	-	-	-	-	-	-	-
H_2S production from peptone	-	-	-	-	-	-	-	-	-	-
Potato soft rot	-	-	-	-	-	-	-	-	-	-
Growth at 41°C	-	-	-	-	-	-	-	-	-	-
Growth in 1% NaCl	+	+	+	+	+	+	+	+	+	+
Growth at 2 % NaCl	-	-	-	-	-	-	-	-	-	-

+ = positive reaction, - = negative reaction and *R*= *Ralstonia solanacearum* .

Table (2b): Biochemical characteristics of *R. solanacearum*, isolates during the growing seasons 2009.

Carbohydrates		Tested isolates									
		R_1	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**	**R_7**	**R_8**	**R9**	**R10**
Glucose		A	A	A	A	A	A	A	A	A	A
Mannose		W	A	A	A	A	W	A	A	A	A
Fructose		A	A	W	A	W	A	A	W	A	W
Lactose		A	-	-	-	W	A	-	-	-	W
Maltose		-	-	A	-	-	A	-	-	-	A
Mannitol		A	A	A	A	-	A	A	A	A	-
Sorbitol		W	A	W	-	A	W	A	W	-	A
Dulicitol		A	W	-	-	A	A	W	-	-	A

- = Negative reaction, A= Acid production and w= Weak acid production **&** *Rs= Ralstonia solanacearum*

GNA+TZC medium

SMSA **medium**

Plate (1): Colonial types of *R. solanacearum* grown on glycerol nutrient agar medium containing triphenyl tetrazolium chloride (TZC) and selective media of south Africa (SMSA) medium.

techniques

i. Immunofluorescent antibody stains (IFAS):

Identification of tested bacterial isolates using Immunofluorescent **antibody** stains (IFAS) gave positive results which confirmed that these tested isolates are ***R. solanacearum.*** In this respect, the morphology of bacterial cells appeared as short rod and green fluorescent with specific fluorescent-labaled anteiserum in Plate (2). **These results were agreement** with (Pastrik, *et al*., 2002 and Fikre, *et al*., 2010)

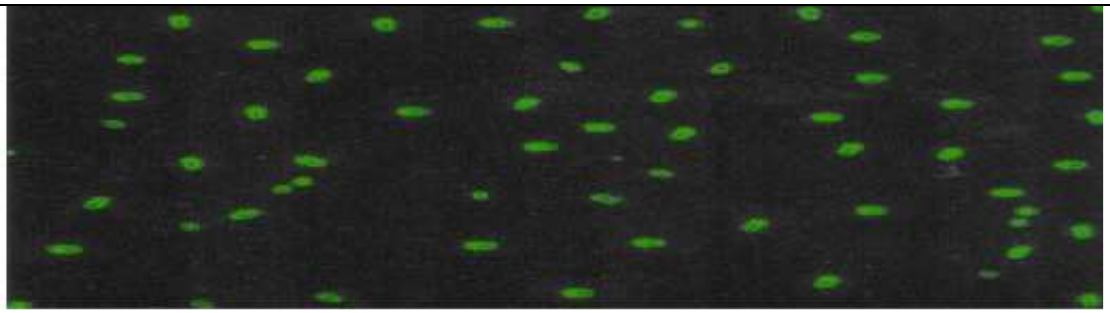
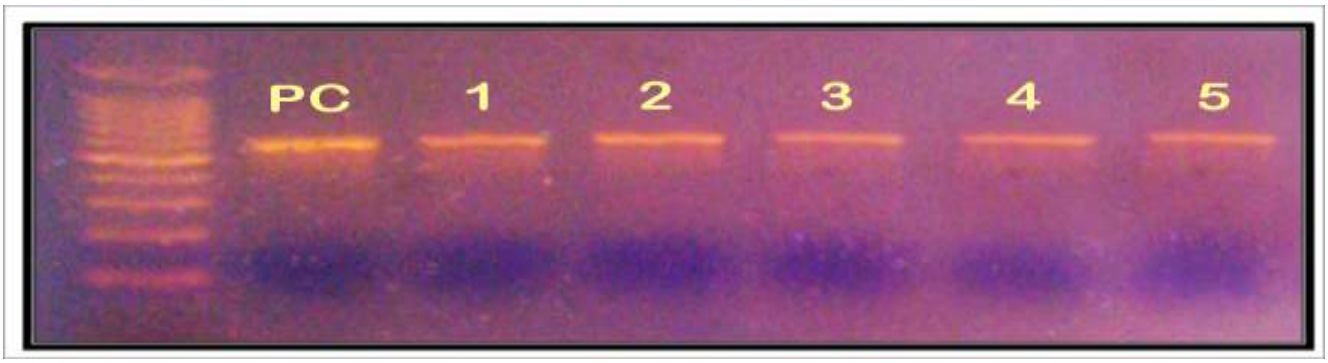


Plate (2):: Cell morphological of *R. solanacearum* using the serological immunofluorescent antibody staining (IFAS) technique where cells appeared as short rods and stained evenly as bright green fluorescent under IF microscope (600X).

ii. Polymerase chain reaction (PCR):

Data in **Plate. (3)** confirm the identification of five bacterial isolates among the ten isolates of those isolated from Beheira and Qalubiya governorates during season 2009 which was previously identified as *R. solanacearum* using the traditional and IFAS techniques. In this respect, PCR technique using two oligonucleotide primers namely forward primer *Rs*-l-F (5\ ACT AAC GAA GCA GAG ATG CAT TA-3\) and reverse primer RS- 1-R (5\ CCC AGT CAC GGC AGA GAG T- 3\) visualized the specific DNA band with molecular weight 718 bp in the five tested bacterial isolates and the positive control one under UV light. The results reveal also that there were very close similarity without any variation among the five tested isolates and the positive control one under investigation to confirm that these five tested bacterial isolates are ***R. solanacearum*. These results were in agreement with (Pastrik, *et al*., 2002 and Fikre, et al., 2010)**



Pc =positive control. 1 & 3 isolates = from Behara governorate while 2,4 & 6 isolates = from Qalubiya governorate

Plate (3): Single DNA band in the five tested *R. solanacearum* isolates and the positive control one with very close similarity among them at MW 718 bp..

IV. Pathogenicity test of different *R. solanacearum* isolates:

Data in Table (3) and Plate (4.a & b) reveal the pathogenicity of ten *R. solanacearum* isolates on potato plants (Cara cv.) in sterilized soil. All tested *R. solanacearum* isolates caused bacterial wilt disease symptoms on potato plants compared with the un-inoculated control. In this respect, *R. solanacearum* (No.6) record the highest infection % where it gave 100% with 5% disease severity at 35 day post inoculation the sterilized soil with brown rot pathogens. *R. solanacearum* (No.3) came in second rank where it caused 62.67% infection with 4.67%

disease severity at 35 day post inoculation followed by *R. solanacearum* (No.1). The least infection% was recorded by *R. solanacearum* (No.8) where it recorded 37.33% followed by *R. solanacearum* (No.5). The determined disease severities % were increased with increasing the incubation time from 7-35 days compared with the un-inoculated control under greenhouse conditions, these results were agreement with **(Hayward, 1991 and Shinohara *et al.*, 2004**).

As cleared in **Plate (4.a & b)**, symptoms of brown rot disease caused by *Ralstonia solanacearum* on infected potato plants (Cara cv.) under greenhouse conditions could be seen in form of yellowing or sudden leaf wilting with down bending of these leaves before appearance the whole death onto potato plants. Also, whitish exudates might be seen onto the cut surface of tubers, a wet breakdown initiated at the point of attachment of the stolon and the eyes of tubers. A light-brown breakdown of water-conducting tissues have been seen in tuber crosses. Light milky fluid is squeezed from this discolored area in infected potato tubers**, these results were in agreement with (Haywrd, 1991 and Anonymous, 1995)**.

Table (3): Pathogenicity test of ten ***R. solanacearum*** isolates on tested potato plants (**Cara cv.**) in sterilized soil under greenhouse conditions.

Tested *Ralestonia* isolates	**Disease severity % after days of inoculation**						**Infection %**
	7days	**14dys**	**21das**	**28dys**	**35days**	**Mean**	
R_1	1.33	2.33	3.33	3.67	5.00	3.13	60.00
R_2	1.33	2.33	3.33	3.67	5.00	3.13	58.67
R_3	1.33	2.67	3.00	3.33	4.67	3.00	62.67
R_4	2.33	2.33	2.67	3.67	5.00	3.20	58.66
R_5	1.33	1.33	2.00	2.67	4.33	2.33	41.33
R_6	2.00	3.67	4.00	5.00	5.00	3.93	100.0
R_7	1.00	1.67	2.67	3.33	4.33	2.60	46.67
R_8	1.00	1.00	2.00	3.00	4.33	2.27	37.33
R_9	1.00	1.33	2.67	3.33	4.33	2.53	44.00
R_{10}	1.00	1.00	2.67	3.33	4.33	2.47	41.33
Control	1.00	1.00	1.00	1.00	1.00	1.00	0.00
Mean	1.333	1.879	2.67	3.273	4.303		
R= *R. solanacearum* &Control=Non-inoculated.							
L.S.D at 5%: **isolates=0.368 , weeks = 0.248 and interaction=0.822**							

Control (non-infected) | Infected with *Rs* after 14 days from injection | Infected with *Rs* after 21 days from injection

Plate (4a):Symptoms of brown rot disease (*R. solanacearum*) on plants (above) and tubers (below) of potato (cv. Cara) under greenhouse conditions.

Healthy tuber Infected tuber by *R. solanacearum*

Plate (4b): Potato tuber internal typical symptoms of infected tubers with brown rot disease symptoms caused by *R. solanacearum*.

IV. The effect of combination of tested bio- and abiotic agents with 10 % compost on:

1. The disease incidence of potato plants:

Data in **Table (4)** indicate that effect of combination between tested bio- and abiotic agents with 10% compost was significantly efficient to reduce brown rot disease caused by ***R. solanacearum***. All treatments reduced the disease compared with the control treatment. The highest reduce in infection (disease) was induced by treatment of (***B. subtilis*****+** Salicylic acid + K_2HPO_4) (18%) followed by treatments of ***B. subtilis*****, (*B. subtilis*** **+** Salicylic acid), (***B. subtilis*** **+** K_2HPO_4), ***P. fluorescens***, (***P. fluorescens*****,** **+** Salicylic acid), (***Ps. fluorescens*** **+** K_2HPO_4), (***P. fluorescens*** **+** Salicylic acid+ K_2HPO_4), (***B. subtilis*** **+** ***P.fluorescens*** **+** Salicylic acid), (***B. subtilis*****+** ***P.fluorescens*** **+** K_2HPO_4), and (***B. subtilis*****+** ***P.fluorescens*** **+** Salicylic acid + K_2HPO_4), respectively compared with the control treatment. While, the least reduce in infection (disease) was induced by ***B. subtilis*** **+** ***P.fluorescens*** (46.67%). These results were in agreement with **Abdalla *et al.* (1999); Budi *et al* (2003); Zayed (2004); Venglovsky *et al.* (2005) and Yadessa *et al* (2010) who** found that bioagents and abiotic were efficient in reduce of disease.

Table (4): Effect of combination of tested bio- and abiotic agents on ***R. solanacearum*** infection of potato plant (**Cara cv.**) in sterilized soil amended with 10% mixed compost under greenhouse conditions**.**

Bioagents	Abiotic factors (4.5 mM)	Disease severity	Infection %	Efficacy %
B. subtilis	Without	1.22	33.33	-58.34
	Salicylic acid (SA)	1.18	20	-75.00
	K_2HPO4	1.16	20	-75.00
	SA+K2H	1.07	18	-77.50
Pseudomonas fluorescens	Without	1.27	33.33	58.34
	Salicylic acid	1.07	20	-75.00
	K_2HPO4	1.07	20	-75.00
	Sal. +K2H	1.07	20	-75.00
B. subtilis **+** ***P.fluorescens***	Without	1.40	46.67	-41.66
	Salicylic acid	1.16	20	-75.00
	K_2HPO4	1.22	20	-75.00
	Sal. + K_2H	1.07	20	-75.00
R. solanacearum		2.67	80.00	
Control		1.00	0.00	
LSD at 5 % : Treatments*=0.22 interaction =0.49 and percentage =16.0				

2.

On induction the Peroxidaze, catalase and polyphenoloxidase activity:

Data in **Table** (**5**) show that all combination of tested bio- and abiotic agents with 10% compost increased enzymes activity (peroxidaze, catalase and polyphenoloxidase) compared with the control treatment. The highest activity of peroxidase was induced by ***(B. subtilis* + *P.fluorescens*** + Salicylic acid + K_2HPO_4) (172.46%) followed by (***B. subtilis*** + Salicylic acid + K_2HPO_4), ***(P. fluorescens*** + Salicylic acid + K_2HPO_4), (***P. fluorescens*** + Salicylic acid), (***B. subtilis* + *P.fluorescens*** + Salicylic acid), (***P. fluorescens*** + K_2HPO_4), (***B. subtilis*** + Salicylic acid), (***B. subtilis*** + ***P.fluorescens*** + K_2HPO_4), (***B. subtilis*** + K_2HPO_4) and ***R. solanacearum*** increased it (168.99, 153.91, 143.48, 115.94, 111.59, 106.09, 101.74, 88.99 & 66.96 %), respectively over control treatment. The highest activity of catalase and polyphenoloxidase was induced by ***(B. subtilis* + *P.fluorescens*** + Salicylic acid + K_2HPO_4) followed by (***B. subtilis*** + Salicylic acid + K_2HPO_4) and ***R. solanacearum*** , respectively. These results were similar to those found by **Mosa (2002); El-Habbak (2003); Mazen (2004); Zhao *et al.* (2005) and Hajhamed (2011)**.

Table (5): Effect of combination of bio- and abiotic agents on enzymes activity of tested potato leaves (**Cara cv.**) infested by ***R. solanacearum*** in sterilized soil amended with 10 % mixed compost after 45 days from planting under greenhouse conditions**.**

Biotic agents	Abiotic factors (4.5 mM)	Enzymes activity (mg/gfw)					
		Peroxidase	**Eff. %**	Catalase	**Eff. %**	PP	**Eff. %**
Bacillus subtilis	***Sal. acid***	7.11	106.09	8.40	172.73	8.10	159.62
	K_2HPO_4	6.52	88.99	7.72	132.14	7.15	129.17
	***Sal.*+K_2HPO_4**	9.28	168.99	8.56	183.44	8.73	180.45
Pseudomonas fluorescens	***Sal. acid***	8.40	143.48	7.72	150.56	8.32	166.7
	K_2HPO_4	7.30	111.59	7.18	133.12	8.19	162.50
	***Sal.*+K_2H.**	8.76	153.91	8.37	171.75	8.49	172.12
B. subtilis*+ *P.fluorescens	***Sal. acid***	7.45	115.94	8.28	168.83	8.15	161.22
	K_2HPO_4	6.96	101.74	8.28	168.83	8.70	178.85
	***Sal.* +K_2H**	9.40	172.46	9.52	207.79	9.48	203.85
R. solanacearum		5.76	66.96	5.80	88.31	5.16	65.06
Control (untreated)		3.45		3.08		3.12	

PP= Polyphenoloxidase and Eff.%=Efficacy %.

3

. On phenols compounds:

Data in **Table** (6) indicate that phenols content (free, conjugated and total phenols) were affected by combination between of bio- and abiotic agents with 10% compost. All treatments increased the free and total phenols compared with the control. The highest increase in free and conjugated phenols were induced by *B.* ***subtilis* + *P. fluorescens*** + Salicylic acid + K_2HPO_4 followed by ***B. subtilis*** + Salicylic acid + K_2HPO_4.

Also , the highest increase in total phenols was induced by (***B. subtilis* + *P. fluorescens*** + Salicylic acid+ K_2HPO_4) (282.35 %) followed by ***(B. subtilis*** + Salicylic acid+ K_2HPO_4), (***P. fluorescens*** + Salicylic acid + K_2HPO_4), (***B. subtilis* + *P. fluorescens*** + Salicylic acid), (***B. subtilis*** + Salicylic acid), (***P. fluorescens*** + Salicylic acid),

(***B. subtilis*** + K_2HPO_4), (***B. subtilis*** + ***P. fluorescens*** + K_2HPO_4), (***P. fluorescens*** + K_2HPO_4) and ***R. solanacearum*** increased it by (197.69, 174.58, 153.57, 133.82, 114.08, 106.51, 97.27, 89.50 & 38.24 %), respectively over the control. These results were in agreement with **(Meena *et al* 2001; El-Habbak 2003 and Ahmad, 2004**).

Table (6): Effect of combination of bio- and abiotic agents on phenols content of potato leaves (Cara cv.) infested by ***R. solanacearum*** in sterilized soil amended with 10 % mixed compost after 45 days from planting under greenhouse conditions**.**

Biotic agents	Abiotic factors (4.5 mM)	Phenols content (mg/g fresh weight)					
		Free	Efficacy%	Conjugated	Efficacy%	Total	Efficacy%
B. subtilis	*Sal. acid*	7.16	438.35	3.97	15.74	11.13	133.82
	K_2HPO_4	6.65	400.00	3.18	-7.29	9.83	106.51
	SA + K	10.22	668.42	3.95	15.16	14.17	197.69
P.fluorescens	*Sal. acid*	6.39	380.45	3.80	10.79	10.19	114.08
	K_2HPO_4	5.22	292.48	3.80	10.79	9.02	89.50
	SA + K	9.89	643.61	3.18	-7.29	13.07	174.58
B. subtilis*+ *P..fluorescens	*Sal. acid*	7.59	470.68	4.48	30.70	12.07	153.57
	K_2HPO_4	5.89	342.86	3.50	2.04	9.39	97.27
	SA + K	11.50	764.66	6.70	95.34	18.20	282.35
R. solanacearum		5.41	306.77	1.17	-65.89	6.58	38.24
Control		1.33		3.43		4.76	

4.

The effect of combination of tested bio- and abiotic agents on protein electrophoretic pattern of tested potato leaves (cv. Cara) infested by *R. solanacearum* in sterilized soil amended with 10 % mixed compost:

Results in **Table** (7) and **Plate (5)** show the protein was extracted from (17) treated and untreated samples. The electrophoresis of extracted protein on polyacrylamide gel 12% was obtained for the different patterns of 16 different treated samples and one untreated sample (control). Some bands disappeared and others appeared **Fig (5)**. Some new bands appeared when compared with control. The new bands were 412,86, 87.72, 85.08, 79.63, 63.09, 44,24, 38.29 and 36.6KDa. The appearance of new bands may be due to effect of the used treatments and the active defense of potato plants under the effects of the aforementioned treatments. The protein analysis for four samples treated with combination between *B. subtilis* , salicylic acid and K_2HPO_4 indicated that**, t**he protein bands 183.38, 86.75 and 16.35 KDa disappeared in all treatments. On the other hand, new protein bands appeared as a response to these treatments such as 85.08 and 87.72 KDa in all treatments except for the first one in which we used *B. subilus* only without using abiotic factors in which the band 86.64KDa appeared in it. While the bands 72.04 and 35.69KDa appeared only in the case of using *B.subtilis* with salicylic acid and K_2HPO_4. The bands 50.83, 40.9, 35.69, 33.88, 27.46 and 13.12KDa appeared in the fourth treatment (*B. subtilis* +salicylic acid+ K_2HPO_4).This means that the appearance of new bands may be due to the effect of adding salicylic acid. Concerning of protein bands in potato treated by the combination of *Pseudomonas fluorescens* with salicylic acid and K_2HPO_4. There were new protein bands that appeared in different treatments as a response from the host against the pathogen such as 87.72, 85.08, 79.63, 63.09, 44.24, 38.29 and 36.6KDa when compared with control. While,

the bands 412.86 and 86.64KDa disappeared from all treatments. The bands 72.04, 63.09, 50.83 and 34.71KDa appeared in all treatments. The bands 13.12, 33.88, 34.71, 50.83, 63.09, 72.04 and 86.75KDa appeared in two treatments (*P. fluorescens and P. fluorescens* with salicylic acid). Meanwhile, the band 183.38KDa appeared only in the first treatment (*P. fluorescens*). On the other hand, the combination between *P. fluorescens* + *B. subtilis* with salicylic acid and K_2HPO_4 showed**,** new bands appeared when compared with control such as 87.72, 85.08,79.63, 63.09, 44.24 and 36.6KDa. On the other hand there were many bands that disappeared such as 412.86, 72.04, 50.83, 40.9, 38.29 and 35.69KDa, these bands disappeared in all treatments. Meanwhile, the combination between using salicylic acid and K_2HPO_4 reveal that, new bands appeared as a result of this treatments such as 412.86, 87.72, 79.63, 63.09 and 36.6KDa. The bands 412.86, 183.38, 87.72, 63.09, 36.6 and 27.46KDa appeared when we used salicylic acid with K_2HPO_4. On the other side, the protein analysis for infected leaves with *R. solanacearum* without treatments revealed that, The bands 412.86, 85.08, 72.04, 63.09, 44.24, 38.29, 37.84, 35.69, 33.88 and 16.35KDa disappeared. The bands 87.72, 79.63 and 36.6KDa appeared as a response and they considered new bands as they are not found in the control protein sample. Also, the bands 183.38, 86.75, 50.83, 40.9, 34.71, 31.22, 27.46 and 13.12KDa appeared in this infected sample as a response from the plant against the pathogenic bacterium (*R. solanacearum*).

These results agreed with **Gregory *et al.* (2003)** who stated that, the mechanism of plant defense against the pathogen might back for these protein bands. Recently, **Hajhamed (2011)** revealed that the number of protein bands detected in infected shoots of all cultivars was less than those in healthy shoots and the number of absent band due to infection in resistance cultivar is less than the number of absent band in susceptible or moderately susceptible cultivars. Meanwhile, he found that infected plants of susceptible cultivar (Lady rosetta) comparing with healthy plant gave only 14 bands with absence of bands number 11, 13, 15 and 16 with molecular weights 39.506, 32.675, 26.331 and 24.355 KDa, respectively. While, resistance cultivar (Lady balfor) comparing with healthy plant gave 15 bands with absence of band number 13, 15 and 16 with molecular weights 32.675, 26.331 and 24.355 KDa, respectively.

Table (7): Effect of combination of bioagents and abiotic factors with 10 % mixed compost on protein electrophoretic pattern of potato (*S. tuberosum*, L.) leaves infected with *R. solanacearum* at 45 days after planting during 2009 season.

Band number	M.W/KDa	*Bacillus subtilis*				*P. fluorescens*				*B. subtilis* + *P. fluorescens*				without bioagent			*R. solanacearum*	Control
		W. ab	SA	K_2HPO_4	SA. +K_2H	W. ab	SA	K_2HPO_4	SA+K_2H	W. ab	SA	K_2HPO_4	SA+K_2H	SA	K_2HPO_4	SA+K_2H		
1	412.86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
2	183.38	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	1
3	87.72	0	1	1	1	1	0	0	1	0	0	1	1	0	0	1	1	0
4	86.75	0	0	0	0	1	1	0	1	1	1	0	0	1	1	1	1	1
5	86.64	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
6	85.08	0	1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	0
7	79.63	0	1	1	0	0	1	0	1	1	1	1	0	1	1	1	1	0
8	72.04	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1

9	**63.09**	**1**	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**
10	**50.83**	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**
11	**44.24**	**0**	**0**	**1**	**1**	**1**	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**
12	**40.9**	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**
13	**38.29**	**0**	**1**	**0**	**1**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
14	**37.84**	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**0**	**1**
15	**36.6**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**0**
16	**35.69**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**1**
17	**34.71**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**
18	**33.88**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**1**
19	**31.22**	**0**	**1**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**1**
20	**27.46**	**1**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**1**
21	**16.35**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**0**	**0**	**0**	**1**
22	**13.12**	**0**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**1**

W.ab=without abiotic factors, SA=Salicylic acid and K_2H= K_2HPO_4

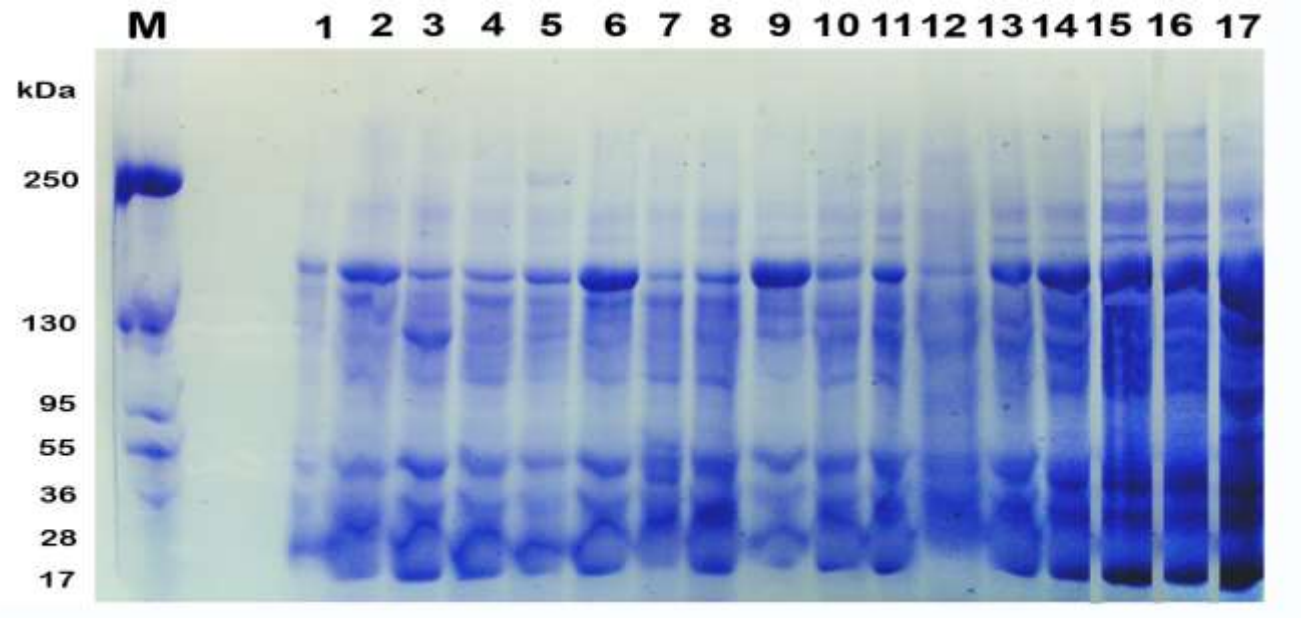


Plate (5): Effect of combination of bioagents and abiotic factors with 10 % mixed compost on protein electrophoretic pattern of potato (*Solanum tuberosum*, L.) leaves at 45 days after transplanting during 2009 season.

Where:

1. **With of *Bacillus subtilis* + Rs only. 2. *B. subtilis*+ salicylic acid at 4.5 mM .**
3. *Bacillus subtilis*+ K_2HPO_4 at 4.5 mM. 4. *B. subtilis*+ salicylic acid + K_2HPO_4at 4.5 mM
5. With of *Pseudomonas fluorescens* +*Rs* only.
6. *P. fluorescens* + salicylic acid at 4.5 mM .
7. *P. fluorescens* + K_2HPO_4 at 4.5 mM . 8. *P. fluorescens* + salicylic acid + K_2HPO_4 at 4.5 mM
9. *B. subtilis* + *P. fluorescens* at 4.5 mM. 10. *B. subtilis*+ *P. fluorescens* + Sal. acid at 4.5mM
11. *B. subtilis* + *P. fluorescens* + K_2HPO_4 at 4.5 mM .
12. *B. subtilis* + *P. fluorescens*+ salicylic acid + K_2HPO_4 at 4.5 mM .
13. Sal. acid at 4.5 mM . 14. K_2HPO_4 at 4.5 mM.

15. salicylic acid + K_2HPO_4 at 4.5 mM . 16. With *Rolstonia solanacearum* only

C: Control with compost 10 % only. M: Protein marker.

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