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Impact of PGPM in Pepper Immune-Response to Fusarium Wilt Disease

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Original Research Article

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Abstract: Plant growth promoting microorganisms (PGPM) are the rhizosphere bacteria and Vesicular Arbuscular Mycorrhizal spores (VAM) that enhance plant growth and suppress plant diseases.The current study was carried out at Ahmed Orabi Agricultural Cooperative Association, Ismailia, Egypt, to investigate the efficient antagonistic bacterial isolates and VAM against Fusarium wilt disease in pepper plant under filed experiment. A total of 49 bacterial isolates were isolated, out of which 4 (P1, P11, P19 and P42) were selected based on their antagonism of phytopathogens. Phylogenetic analysis of 16SrRNA sequences identified these isolates as new strains of *Bacillus subtilis*, *Bacillus aerius, Achromobacter xylosoxidans* and *Lysinibacillus fusiformis*. The selected isolates produced high levels siderophore and hydrogen cyanide. Disease symptoms, disease index percent, phytochemicals and Metabolic indicators of resistance in plant as response to induction of systemic resistance (SR) in pepper plants were recorded. VAM and P42 were the best isolates and reduced percent disease indexes by 10 and 15 % respectively. P11, and PGPR (P1, P11, P19, P42) gave the same result 17.5%. and came next P19, (VAM+PGPR) and P1 which recorded 20,22.5and 30 % respectively. The beneficial effects of the used elicitors were extended to increase not only total phenol, total soluble protein content but also the activities of peroxidase and polyphenoloxidase enzymes in comparison with control. At the same time, the results appeared that pepper infected plants treated with applied elicitors showed variation in number, molecular weight of protein bands as well as variability in number of polypeptide peroxidase and polyphenol oxidase isozymes in the leaves.

Keywords: Pepper plant – *Fusarium oxysporum* – *Achromobacter xylosoxidans*- Vesicular Arbuscular Mycorrhizal - *Bacillus subtilis* – Immune response.

INTRODUCTION

The sweet pepper (*Capsicum annuum* L.) is one of the highly favorite vegetables cultivated in most parts of the world especially in temperate regions. In recent years, increasingly frequent occurrence of fusarium wilt of sweet pepper has been observed on open-field-grown peppers. Fusarium wilt is a disease caused by the soil pathogen *Fusarium oxysporum* which negatively affects crop yield, significantly decreasing the quantity and the quality of the crop [1-3].

Disease suppression by using biocontrol agents is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community around the plant and the physical environment [4]. Biological control has potential for the management of many diseases [5]. The induction of plant resistance using non-pathogenic or incompatible microorganisms is also a form of biological control [4]. Moretti *et al*., **[5]** isolated A. xylosoxidans MM1 and evaluated for its ability to suppress Fusarium wilt of tomato under glasshouse conditions. Result of different trials showed that this bacterial strain reduced wilt incidence by about 50%. No phytotoxicity was observed on healthy plants treated with the bacterial suspension and, on the contrary, these showed a growth stimulation if compared to untreated plants. Rhizobacterial strains of *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Alcaligenes* and *Bacillus* spp. Also, have been used to reduce wilt disease caused by *F. oxysporum* f. sp. *Lycopersici* [7-10]. The use of antagonist mixtures may also provide improved disease control over the use of single organisms [5]. Arbuscular mycorrhizae are ubiquitous symbiotic associations that are important to normal plant growth of most crops and as such tend to optimize crop production [11]. AM fungi are known to reduce soil borne diseases or the effects of wilt disease in pepper caused by fungal pathogens [12]. Suppression of the diseases by AM fungi is also attributed to increased nutrient uptake (particularly phosphorus) by mychorrhizal plants [13].

This investigation aimed to study the positive performance of biological agents against wilt of pepper plant caused by *Fusarium oxysporum* which considered among the most difficult crop diseases to control. Furthermore, to evaluate the effect of PGPM as alternative and safety method in Integrated

Management programs to management the fusarium wilt disease.

MATERIALS AND METHODS

Plant material and growing conditions

For the present investigation, four weeks age pepper (Capsicum *annuum* L.) seedlings were obtained from agricultural research center (ARC), ministry of agriculture, Giza, Egypt.

Isolation and maintenance of *F. Oxysporum*

Fusarium oxysporum f.sp. *capsici* was isolated from infected wilted pepper plants according to [14] and identified Morphological macroscopic and microscopic according to [15[. The isolated fungus was maintained on PDA at 24°C. To induce sporulation, cultures were transferred on 24°C for 6 days on PDA. Conidial suspensions were prepared as described in Boedo *et al*., [16]. Spore density was counted by a hemocytometer and adjusted to 10^7 spores per mL, then pathogen was confirmed by pathogenicity test according to [17].

Source, isolation and identification of PGPR from rhizosphere:

Rhizosphere was collected from pepper field and suspended by added 10 g in 90 mL of sterile distilled water. Serial dilution technique was performed up to 10^{-2} to 10^{-6}. Aliquots of 0.1 ml (10^{-2} to 10^{-6}) were spread on sterile petri plates containing Nutrient Agar Medium. The petri plates were incubated in bacteriological incubator for 48 hours at 30°C [18, 19]**.** The Gram reaction was performed as described by Vincent and Humphrey, [20]. According to Sujatha and Ammani, [21] Siderophore production was detected. The isolates were screened for the productions of hydrogen cyanide by adopting the slight modification of method of Geetha *et al*., [22]. The 16S rRNA gene of the selected bacteria was PCR amplified by using universal primer forward and reverse primers, F (5' AGA GTT TGA TCC TGG CTC AG -3') and R (5'-GGT TAC CTT GTT ACG ACT T -3'), respectively. The nucleotide sequence of purified PCR products was analyzed at Sigma Scientific Services Company, Lebanon Square, El Giza, Egypt.
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Extraction and identification of Vesicular arbuscular mycorrhizal spores (VAM) inoculum

Vesicular arbuscular mycorrhizal spores (VAM) were extracted from pepper fields of different provinces (El-Kalubia, El-Sharkia and El-Fayoum). VAM spores (mixture contained different genera and species of VA-mycorrhizas being; *Glomus mosseae*, *Glomus fasiculatum*, *Glomus cladonicum*, *Glomus clarum*, *Gigaspora margarita* and *Acaulospora laevis*. were collected from rhizosphere soil wet sieving and decanting technique, a method adopted by Gerdemann and Nicolson, [23]. The spores were extracted used to prepare the standard inoculum for field experiment.

Field experiment

Applied elicitors were added at one week before infection with *F. oxysporum*. The field trials were conducted at the Experimental garden of Ahmed Orabi Agricultural Cooperative Association, Ismailia, Egypt in 2017 growing season. seedlings were planted in 9 groups as following; :(1) plants without any treatments were referred as healthy control, (2) plants infected with *F. Oxysporum* as infected control, (3) plants treated with P1 then infected with *F. Oxysporum*, (4) plants treated with P11 then infected with *F. Oxysporum*,(5) plants treated with P19 then infected with *F. Oxysporum* ,(6) plants treated with P42 then infected with *F. Oxysporum* (7) plants treated with PGPR of (P1,P11,P19 and P42) then infected with *F. Oxysporum*,(8) plants treated with VAM then infected with *F. Oxysporum* (9) plants treated with PGPR of (P1,P11,P19 ,P42 and VAM) then infected with *F. Oxysporum* . Disease development was recorded 15 days after inoculation. Disease severity was recorded. The plant samples were collected for morphological and biochemical indicators for resistance analysis when the plants were 32 days old (Stage I) and 47 days old (Stage II).

Disease symptoms and Disease index

Disease symptoms were assessed 60 days after inoculation and the disease index was evaluated according to [10] with slight modifications using score consisting of five classes: 0(no symptoms), 1(slight yellow of lower leaves), 2(moderate yellow plant), 3(wilted plant with browning of vascular bands), 4(plants severely stunted and destroyed). Percent Disease index (PDI) was calculated using the five-grade scale according to the formula: PDI= $(1n_1+ 2n_2 + 3n_3 + 4n_4)100/4n_t$. Where n_1-n_4 the number of plants in the indicated classes, and N_t total number of plants tested. Percent protection by PGPR strains and VAM were calculated using following formula: Protection % = A–B/A × 100% Where, A = PDI in infected control plants B = PDI in infected- treated plants.

Metabolic and biochemical resistance indicators in plant

Determination of total soluble proteins (mg/100g of dry wt) according to the method of Lowery *et al*., [24] using casein as a standard protein. Determination of phenolic compounds (mg/100g of dry wt) was carried out according to that method described by Daniel and George, [25]. Peroxidase activity enzyme was determined according to the method adopted by Srivastava [26]. The activity of polyphenol oxidase enzyme was determined according to the method adopted by Matta and Dimond, [27]. Protein finger print was analyzed using Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS - PAGE) according to Studier [28]. Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among the studied plants using two isozyme systems according to Stegmann *et*

al., [29]. Peroxidase (Px) isozyme was determined according to the method [30]. Polyphenoloxidase (PPO) isozyme was determined according to the method Baaziz *et al.*, [31].

D- Statistical analyses

Experimental data were subjected to one-way analysis of variance (ANOVA) and the differences between means were separated using Duncans multiple rang test and the (L.S.D) at 5% level of probability using Co-state software [32].

RESULTS

Bacterial Strains Isolation and Molecular Characterization

We obtained total of 49 rhizobacterial strains from soil pepper field. Four isolates–P1, P11, P19and P42 were selected based on antagonistic against causal pathogen and their ability to produce siderophore and hydrogen cyanide. With one exception, isolates selected were reacted positively to the Gram staining. The exceptional case was p19 which was negative gram stain (Table-1). Phylogenetic trees constructed from 16SrRNA sequences showed that the selected isolates were mainly members of genus *Bacillus, Achromobacter,* and *Lysinibacillus*. The sequences of the isolate P1 showed 99% similarity with *Bacillus subtilis* and was submitted to Gen Bank under accession numbers NR_118383 (Table-1). Isolate P11 had 99% homology with *Bacillus aerius* and was submitted to Gen Bank under accession number NR_118439. Isolate P19 had 99% homology with *Achromobacter xylosoxidans* and was submitted to Gen Bank under accession number NR_113733. Isolate P42 showed 99% sequence homology with *Lysinibacillus fusiformis* and were submitted to Gen Bank under accession number NR_112569, (Table-1). The data recorded in Table (1) showed that all bacterial isolates produced HCN ranging from dark brown (++++) to faint brown (+++). Also, data recorded in Table (1) revealed that *Bacillus subtilis* the best isolates to produce siderphore (2.1 µM DFOM) followed by *Bacillus aerius, Lysinibacillus fusiformis* and Achromobacter *xylosoxidans (*1.4,1.3 and 1.1 µM DFOM) respectively.

Table-1: Biochemical and molecular analysis of PGPR isolates

PGPR Isolates	Biochemical analysis			Molecular analysis
	Gram	HCN	Sidophore µM DFOM	**Identification based on 16S rRNA gene sequencing**
P1	+	++++	2.1	*Bacillus subtilis* strain SBMP4
P11	+	+++	1.4	*Bacillus aerius* strain 24K
P19	-	+++	1.1	*Achromobacter xylosoxidans* strain NBRC15126
P42	+	+++	1.3	*Lysinibacillus fusiformis* strain NBRC15717

Note: µM DFOM: standard solution of deferoxaminmesylate

Control of wilt disease caused by *F. oxysporum* using tested applied elicitors

All applied elicitors were effective in reducing disease index compared to the infected control (table 2). VAM and P42 were the best isolates and reduced percent disease indexes by 10 and 15 % respectively. P11, and PGPR (P1, P11, P19, P42) gave the same result 17.5% and came next P19, (VAM+PGPR) and P1 which recorded 20,22.5and 30 % respectively.

Table-2: Control of wilt disease caused by *F. oxysporum* using tested elicitors

Treatment	Classes					Disease index (DI) %	Protection %
	0	1	2	3	4		
F. oxysporum	0	1	1	2	6	80	0
P1 + *F. oxysporum*	6	1	0	1	2	30	62.5
P11 + *F. oxysporum*	6	1	1	0	1	17.5	78.1
P19 + *F. oxysporum*	7	2	1	0	1	20	75
P42 + *F. oxysporum*	7	1	1	1	0	15	81
PGPR + *F. oxysporum*	6	2	1	1	0	17.5	78.1
VAM + *F. oxysporum*	8	1	0	1	0	10	87.5
(PGPR + VAM) + F. oxysporum	7	0	1	1	1	22.5	71.8

P1: *Bacillus subtilis* strain SBMP4, P11: *Bacillus aerius* strain 24K,
P19: *Achromobacter xylosoxidans* strain NBRC15126
P42: *Lysinibacillus fusiformis* strain NBRC15717
PGPR: P1 + P11 + P19 + P42
VAM: Vesicular Arbuscules Mycorrhizal spores

Fig-1: wilt disease symptoms on pepper plant showed (a, b) slight yellow of lower leaves,(c, d) wilted plant with browning of vascular bands and plants wilted plant (e) moderate yellow plant , (f) severely stunted and destroyed

Total soluble protein contents

The results in Table (3) showed that, total soluble protein contents in shoots & roots of pepper plants highly significantly decreased due to *F. oxysporum* infection through two stages of growth. All tested bio inducers showed considerable increase in total soluble protein contents of pepper shoots compared to the infected control. P42, P1 and VAM were the best isolates which gave highly significant increase in total soluble protein contents of pepper shoot infected plants. On the other hand, it was found that (VAM, PGPR & P42) showed considerable increase in total soluble protein contents in roots of pepper plant followed by P11and (PGPR + VAM), respectively. and came next p1 and P19 at two stages of growth in comparison with *F. oxysporum* - infected plants. These observed increased were found to be statically significant.

Total phenols

Data generated in Table (3) showed that, *F. oxysporum*, cause a marked significant increase in total phenols of shoots & roots of the infected plants. Concerning the effect of tested bio inducers on total phenols of the challenged plants with *F. oxysporum*, it was found that all the applied elicitors, with one exception, gave markedly increased than that of corresponding infected control. The exceptional case was represented by significant decrease in total phenol contents of shoot at stage II of growth due to the treatment with P11.

Peroxidase (POD) activity

The results in Table (4) show that, all applied elicitors significantly increased peroxidase activity compared with infected control throughout the various stages of growth. It was found that VAM, P19 and P42 increased peroxidase activity by 67, 53 and 26% respectively, followed by, P11 and P1 were the least effective and increased peroxidase activity by 8 %. This was throughout the first stage of growth. While at stage II of growth it was found that, VAM, P1, PGPR and P42 induced the highest activity of peroxidase by (49 ,48 ,47 and 40 %) respectively, followed by P11 and VAM + PGPR that increased peroxidase activity by (19 and 11%) respectively. While, P19 was the least effective and increased peroxidase activity by 5 %.

With one exception, *F. oxysporum* -infected plants treated with applied elicitors showed significant increases in peroxidase activity of roots throughout the two growth stages. The exceptional case was represented by significant decrease in peroxidase activity at the first stage due to the treatment with P42 by (0 %). P1, PGPR and P19 increased peroxidase activity by 178, 70 and 52 % respectively and came next PGPR + VAM and VAM increased peroxidase activity by 35 and 17 %. On the other hand, P11 was the least effective and increased peroxidase activity by 4 %. This was throughout the first stage of growth. While at stage II of growth it was found that, P1, PGPR + VAM, VAM, and PGPR induced the highest activity of peroxidase (102 ,92 ,76 and 57 %) respectively, followed by P42 and P19 that increased peroxidase activity by (45 and 33%) respectively. While, P11 was the least effective and increased peroxidase activity by 20 %.

Polyphenoloxidase (PPO) activity

Results of the present work (table 4) revealed that, pepper plants infected with *F. oxysporum* gave highly significant increases in PPO activity in shoots and roots related to healthy pepper plants (un-infected) at the first and second stages of growth. All applied elicitors significantly increased PPO activity in shoots and roots compared with infected control throughout the various stages of growth. The results in Table show that, all applied elicitors significantly increased peroxidase activity compared with infected control throughout the various stages of growth. It was found that VAM, VAM+PGPR, P42 and P19 increased PPO activity during the two stages of growth by (125 – 93), (57 – 54), (56 – 46) and (38 – 43) % respectively. While, PGPR was the least effective and increased PPO

activity by (14 -3) %. These observed increased were found to be statically significant during two stages of growth.

Also, results in table (4) showed that application of VAM, P19, PGPR and P42 increased PPO activity of roots by 300, 244,172 and 105% respectively, followed by, PGPR +VAM, P11 and P1 were the least effective and increased PPO activity by 83,61 and 39 %. This was throughout the first stage of growth. While at stage II of growth it was found that, VAM, PGPR and P42 induced the highest activity of PPO by (165 ,71 and 58 %) respectively, followed by VAM + PGPR and P19 that increased PPO activity by (44 and 24%) respectively. P1and P11 were the least effective and increased PPO activity by 11 %.

Table-3: Effect of Bio-elicitors on the total soluble protein contents and Phenolic compounds of pepper plant infected with *F. oxysporum*

Treatments	Protein content Total soluble mg/g d.wt				Phenolic compounds mg/100g d.wt (g)			
	Shoot		Root		Shoot		Root	
	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II
Control (H.)	14.74^{e}	35.38^{c}	16.22^{f}	32.9^{c}	1.93^{g}	0.26^{g}	0.53^{g}	0.99^{h}
Control (Inf.)	10.43^{f}	28.99^{f}	14.83^{g}	29.51^{e}	1.96^{g}	0.39^{f}	1.99^{e}	1.05^{g}
P1	38.28^{b}	38.69^{b}	15.12^{g}	23.69^{f}	3.95^{e}	0.85^{d}	1.01^{f}	1.18^{d}
P11	14.98^{e}	29.57^{ef}	20.35^{d}	32.31^{cd}	4.78^{c}	0.31^{g}	3.27^{d}	1.08^{f}
P19	19.11^{d}	34.92^{c}	13.21^{h}	31.88^{d}	4.28^{d}	0.95^{c}	1.09^{f}	1.21^{c}
P42	41.33^{a}	45.67^{a}	22.13^{c}	32.79^{c}	3.75^{f}	0.54^{e}	4.46^{a}	1.17^{e}
PGPR	14.79^{e}	30.67^{e}	25.49^{b}	35.24^{b}	5.18^{a}	1.48^{a}	4.46^{a}	1.27^{a}
VAM.	22.35^{c}	38.28^{b}	31.42^{a}	38.23^{a}	4.98^{b}	1.91^{b}	3.69^{c}	1.23^{b}
PGPR+VAM.	15.54^{e}	32.01^{d}	17.38^{e}	32.53^{cd}	2.06^{g}	0.84^{d}	1.09^{f}	1.18^{d}
LSD at %5	1.21	1.32	0.47	0.88	0.19	0.08	0.17	0.01

P1: *Bacillus subtilis* strain SBMP4, P11: *Bacillus aerius* strain 24K, P19: *Achromobacter xylosoxidans* strain NBRC15126, P42 : *Lysinibacillus fusiformis* strain NBRC15717
PGPR: P1 + P11 + P19 + P42, VAM: Vesicular Arbuscules Mycorrhizal spores
LSD: Least Significant Difference
Control (H.): untreated pepper plant
Control (Inf.): pepper plant infected with *Fusarium oxysporum* only

Table-4: Effect of Bio-elicitors on the activity of Peroxidase enzyme (ug/g fresh weight) shoots and roots of pepper infected with *F. oxysporum*.

Treatments	Peroxidase (POD) ug/g. f.wt							
	Shoot		Efficacy %		Root		Efficacy %	
	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II
Control (H.)	0.76^{d}	1.01^{d}	00	00	0.2^{f}	0.36^{f}	00	00
Control (Inf.)	1.01^{cd}	1.31^{c}	00	00	0.23^{e}	0.49^{ef}	00	00
P1	1.09^{cd}	1.95^{a}	8	48	0.64^{a}	0.99^{a}	178	102
P11	1.09^{cd}	1.56^{bc}	8	19	0.24^{ef}	0.59^{de}	4	20
P19	1.55^{ab}	1.38^{c}	53	5	0.35^{c}	0.65^{cde}	52	33
P42	1.27^{bc}	1.83^{ab}	26	40	0.23^{ef}	0.71^{bcd}	00	45
PGPR	1.11^{cd}	1.92^{a}	10	47	0.39^{b}	0.77^{bc}	70	57
VAM	1.68^{a}	1.95^{a}	67	49	0.27^{d}	0.86^{ab}	17	76
PGPR+VAM	1.35^{abc}	1.45^{c}	34	11	0.31^{d}	0.94^{a}	35	92
LSD at %5	0.39	0.27	-----	-----	0.03	0.16	-----	----

P1: *Bacillus subtilis* strain SBMP4, P11: *Bacillus aerius* strain 24K, P19: *Achromobacter xylosoxidans* strain NBRC15126, P42: *Lysinibacillus fusiformis* strain NBRC15717
PGPR: P1 + P11 + P19 + P42, VAM: Vesicular Arbuscules Mycorrhizal spores
LSD: Least Significant Difference
Control (H.): untreated pepper plant
Control (Inf.): pepper plant infected with *Fusarium oxysporum* only

Table-5: Effect of Bio-elicitors on the activity of poly phenol oxidase enzyme (ug/g fresh weight) shoots and roots of pepper infected with *F. oxysporum*

Treatments	Polyphenoloxidase (PPO) ug/g f.wt							
	Shoot		Efficacy %		Root		Efficacy %	
	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II	Stage I	Stage II
Control (H.)	2.92^{d}	1.24^{g}	00	00	0.14^{g}	0.36^{g}	00	00
Control (Inf.)	3.45^{cd}	1.34^{fg}	00	00	0.18^{fg}	0.66^{g}	00	00
P1	4.44^{bc}	1.48^{e}	29	24	0.25^{ef}	0.73^{f}	39	11
P11	4.63^{bc}	1.38^{ef}	34	10	0.33^{d}	0.73^{f}	83	11
P19	4.75^{bc}	1.91^{c}	38	43	0.62^{b}	0.82^{e}	244	24
P42	5.39^{b}	1.95^{bc}	56	46	0.37^{d}	1.04^{c}	105	58
PGPR	3.94^{bcd}	1.66^{d}	14	3	0.49^{c}	1.13^{b}	172	71
VAM	7.78^{a}	2.58^{a}	125	93	0.72^{a}	1.75^{a}	300	165
PGPR+VAM	5.42^{b}	2.07^{b}	57	54	0.29^{de}	0.95^{d}	61	44
LSD at %5	1.52	0.13	-----	-----	0.07	0.03	-----	----

P1: *Bacillus subtilis* strain SBMP4, P11: *Bacillus aerius* strain 24K, P19 : *Achromobacter xylosoxidans* strain NBRC15126, P42 : *Lysinibacillus fusiformis* strain NBRC15717
PGPR: P1 + P11 + P19 + P42, VAM: Vesicular Arbuscules Mycorrhizal spores
LSD: Least Significant Difference
Control (H.): untreated pepper plant
Control (Inf.): pepper plant infected with *Fusarium oxysporum* only

Expressed protein as response to induction of Systemic Resistance

The pepper plants treated with biotic inducers inoculated with *F. oxysporum* showed variation in number, molecular weight of protein bands. The variability analysis among tested inducers appeared 126 protein bands. The molecular weight of polypeptides ware determined related to protein markers ranged from 250 to 5.829 KDa. The most prominent specific polypeptide alteration (polymorphic bands) ranged molecular weight from 250 to 8.110 KDa with percentage 4 %. These bands may be related to biotic inducers. The prominent polypeptide bands in all inducers (monomorphic or common polypeptide) were ranged molecular weight from 91.710, to 5.829 kDa with percentage 12 %. These bands may be related to pepper plant.

Table-6: Protein fractions in leaf of fusarium infected pepper plants treated with biotic inducers using SDS-PAGE

MW	Lane1	Lane2	Lane3	Lane4	Lane5	Lane6	Lane7	Lane8	Lane9	Frequency	Polymorphism
250.000	-	+	+	+	+	+	+	+	+	0.889	Polymorphic
91.710	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
80.823	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
66.459	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
33.099	+	+	+	+	+	+	+	+	-	1.000	Monomorphic
27.664	+	+	+	+	+	+	+	+	-	1.000	Monomorphic
23.122	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
18.629	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
14.527	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
12.803	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
10.921	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
9.863	+	+	+	-	-	+	-	-	-	0.444	Polymorphic
8.517	-	-	-	+	+	+	+	-	-	0.444	Polymorphic
8.110	+	+	-	-	-	-	-	-	-	0.222	Polymorphic
7.475	+	+	+	+	+	+	+	+	+	1.000	Monomorphic
5.829	+	+	+	+	+	+	+	+	+	1.000	Monomorphic

Table-7: Polymorphism and genetic markers in leaf of fusarium infected pepper plants treated with biotic inducers using SDS-PAGE.

Polymorphism	Lane1	Lane2	Lane3	Lane4	Lane5	Lane6	Lane7	Lane8	Lane9	Polymorphism%
Mono	12	12	12	12	12	12	12	12	12	85.7
Poly	2	3	2	2	2	3	2	1	1	14.3
Unique	0	0	0	0	0	0	0	0	0	0
Total bands	14	15	14	14	14	15	14	13	13	126

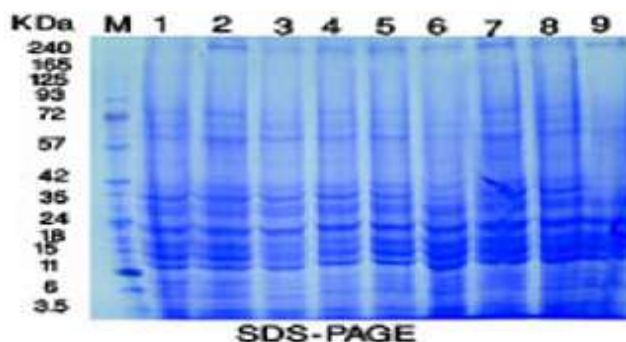

Fig -2: Protein fractions of pepper plants treated with biotic inducers using SDS-PAGE. M: Marker. 1: P1+ fusarium. 2: P11+ fusarium. 3: *P19*+ fusarium. 4: *P42* + fusarium. 5: PGPR (P1, P11, P19, P42) + fusarium. 6: VAM. + fusarium. 7: (PGPR +VAM.) + fusarium 8: Control (H.) 9: Control (Inf.). Monomorphic (Common polypeptide). Polymorphic (Specific polypeptide). Unique (Polypeptide marker) or (genetic marker). - =Absence of band and + = presence of band

Oxidative enzymes

Peroxidase isozyme

The bio inducers and *F. oxysporum* -infection treatments showed variation in number, relative mobility and density polypeptide bands compared with healthy ones. VAM, PGPR gave 5 Isozyme with strong density bands. while P1, PGPR+ VAM gave the same number of bands (5 Isozyme*)* with moderate density. P11, P19, P42 gave the lowest number of bands (4 Isozyme) with moderate density. In addition, infected control plants gave (5 Isozymes) bands with strong density of isozymes Table-8 & Fig-3.

Table-8: Disc-PAGE banding patterns of peroxidase isozymes in infected pepper plants treated with biotic inducers

Peroxidase Groups	Relative Mobility (R.M)	1	2	3	4	5	6	7	8	9
PX1	0.296	1^{+}	1^{+}	1^{+}	1^{+}	1^{+}	1^{+}	1^{+}	1^{+}	1^{+}
PX2	0.617	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}
PX3	0.790	1^{++}	1^{-}	1^{-}	1^{-}	1^{++}	1^{++}	1^{+}	1^{++}	1^{++}
PX4	0.874	1^{++}	1^{-}	1^{-}	1^{-}	1^{++}	1^{++}	1^{+}	1^{++}	1^{++}
PX5	0.957	1^{+}	0	0	0	1^{++}	1^{++}	1^{-}	1^{-}	1^{++}

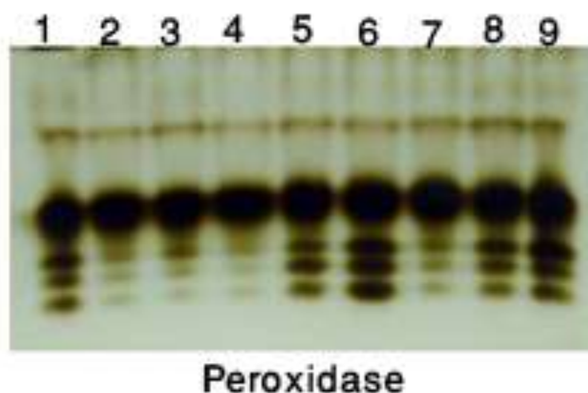

Fig-3: 1: P1+ fusarium. 2: P11+ fusarium. 3: *P19*+ fusarium. 4: *P42* + fusarium. 5: PGPR (P1, P11, P19, P42) + fusarium. 6: VAM. + fusarium. 7: (PGPR +VAM.) + fusarium 8: Control (H.) 9: Control (Inf.). 1^{+++} = strong density. 1^{++} = high density. 1^{+} = moderate density. 1^{-} = low density. 0= absence of band.

Polyphenol oxidase isozyme:

The bio inducers and *F. oxysporum* -infection treatments showed variation in number, relative mobility and density polypeptide bands compared with healthy ones. VAM and P1 gave 2 Isozyme with strong density bands. While P11, P19, P42, PGPR and PGPR + VAM gave the same number of bands (1 Isozyme*)* Table-9 & Fig-4.

Table-9: Disc-PAGE banding patterns of peroxidase isozymes in infected pepper plants treated with biotic inducers

Poly Phenyl Oxidase Groups	Relative Mobility (R.M)	1	2	3	4	5	6	7	8	9
PPO 1	0.338	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}	1^{+++}
PPO 2	0.659	1^{+}	0	0	0	0	1^{++}	0	0	1^{+}

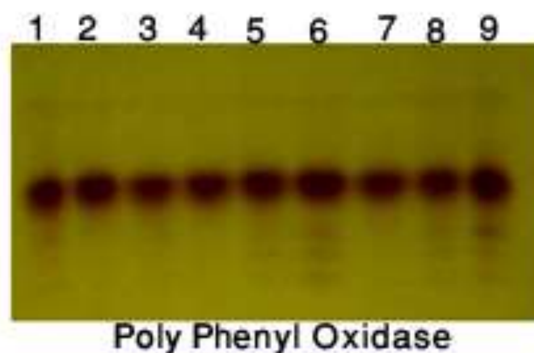

Fig-4: 1: P1+ fusarium. 2: P11+ fusarium. 3: *P19*+ fusarium. 4: *P42* + fusarium. 5: PGPR (P1, P11, P19, P42) + fusarium. 6: VAM. + fusarium. 7: (PGPR +VAM.) + fusarium 8: Control (H.) 9: Control (Inf.). 1^{+++} = strong density. 1^{+} = moderate density. 0= absence of band.

DISCUSSIONS

The objectives of this study were induction of systemic resistance in pepper plants against Fusarium wilt disease**.** Four PGPR classifieds *Bacillus subtilis* (P1), *Bacillus aerius* (P11), *Achromobacter xylosoxidans* (P19) and Lysinibacillus *fusiformis* (P42) were isolated from the rhizosphere of pepper plants. With one exception, all isolates selected were reacted positively to the Gram staining. The exceptional case was *Achromobacter xylosoxidans* (p19). Also, our results indicated that all bacterial isolates able to produce HCN and siderphore. *F. oxysporum* is a soil-borne in nature and invades vascular system of a plant internally. It is better to using biological agents as a safety method for human and environment to protect the entrance point of this fungus in plant instead of changing the entire soil mycoflora. For this purpose, some plant growth promoting rhizobacteria (PGPR) and Vesicular arbuscular mycorrhizal spores (VAM) can be used to induce resistance in plants for combating with this devastating pathogen. It has been found that all applied elicitors were effective in reducing disease index compared to the infected control (table 2). The first standard to govern the occurrence of systemic resistance in pepper plants, treatment with plant growth promoting microorganism were reduced percentage of disease index; our results similar to [11, 13] they reported that Plants inoculated with the VAM had improved growth which supports the findings of earlier workers. None of the VAM inoculated plants died. This might be due to increase in nutrient uptakes which conferred vigor to the pathogen infected plants. The inhibition of the pathogenic infection was as a result of lignifications of the VAM colonized roots cell wall and wound barrier formation as found in the highly thickened cells of those plants colonized by the mycorrhiza. These caused reductions in disease index and Fusarium wilt symptoms. Colonization of plant roots by nonpathogenic bacteria, [33, 5] can induce a distinct broad-spectrum resistance response in both below- and aboveground parts of the plant. Application of some *Bacillus* strains to the seedlings has been found effective for suppressing soil borne diseases and has successfully induced systemic resistance in the treated plants [33, 34]. Many beneficial functions of Achromobacter sp have been reported, including stimulation of ionic transport to promote plant growth and production of glutaryl-3-deacetoxy-7-aminocephalosporanic acid acylase, which is an important enzyme in the production of the antibiotic cephalosporin [35]. The inhibitory effect of our selected strains against *F. oxysporum* could be related to production of HCN and siderophores that limit the availability of iron. This hydroxamate siderophores have important roles in biocontrol because these compounds are comparatively stable, have high iron chelating ability and can impart suppression to soil. It could be speculated that *Achromobacter sp* strain also acts by induction of Systemic Resistance (ISR) in tomato [5]. It is already demonstrated that all selected strains can induce ISR in plant by improvement of NO3- uptake rate [36].

Total protein was determined as response to induction treatments. In the present work, biotic inducers (PGPR & VAM) showed significant increase in total soluble protein contents of shoots and roots in comparison with fusarium- infected plants. The indirect effects of PGPR in disease suppression are the activation of plant defense mechanisms when challenged with pathogens through production of proteins [37]. At the same time, Quantitative proteins of induced pepper plants infected with fusarium were determined using SDSPAGE, our results indicated that, a new pattern of proteins were produced according to the type of used elicitors. It has been suggested that, the induced proteins may help to limit spread or multiplication of pathogen [38-41]**.** Several investigators have indicated that induced resistance in plants was associated with a great increase in chitinase activities. Our results showed that presence of molecular weight**,** 33.099, 27.664, 7, and 32 KD (PR3 – Chitinase) was found in all plants treated with biotic inducers these induced proteins have been defined as pathogenesis related proteins, they implicated in plant defense because of their anti-pathogenic activities [42-44]**.** Phenolic compounds are known to play a major role in the defense mechanism of plants against various external infectious agents. Total phenols play a significant role in the regulation of plant metabolic process and over all plant growth as well as lignin synthesis [45]. In addition, phenols act as free radical scavengers as well as substrates for many antioxidant enzymes [46]. Our results indicated that, PGPR, VAM and P42 were showed highly significant increase in total phenols of shoots &roots of *F. oxysporum* -infected plants. It is quite evidence that, the greatest value of total phenols was achieved by using PGPR or VAM inoculation on the fusarium infected plants more than on the healthy plants, indicating induction of systemic acquire resistant (SAR). These are in accordance with Sudhakar *et al.*, [47].

Our results showed that antioxidant enzymes activity increased significantly in plants infected with fusarium. To obtain clearer indication on some defense-responsible enzymes, mean activities of peroxidase and polyphenoloxidase of the tested pepper plant were determined in this study. PO and PPO activities were greater in the plants treated with VAM or PGPR (either individual or combination) and challenged with fusarium, compared to infected plants (ones). In this respect, enhanced PPO activities against disease and insect pests have been reported in several beneficial plants–microbe interactions [48, 49]**.** Variation in isozyme reveals the information in biochemistry entity of resistant genes to physiological changes, genetic characteristics and development of different organisms [50, 39, 40, 51]**.** Moreover, their relative contents and activities could be used as biochemical indicator to identify whether a strain had resistant ability to an external stress [52]**.** In this study, clear differences existed not only in enzymatic activity but also in enzymatic composition between challenged plants without tested inducers and challenged plants treated with tested inducers. In general, activities of tested isozymes in challenged plants treated with all applied elicitors were higher than that in challenged plants without elicitors, which might be the potential factor for induction of SAR against fusarium according to previous findings.

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