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Biological Controls of Cucumber Wilt Disease Caused by *Fusarium Oxysporum*.

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ABSTRACT

In this study, it is attempted to investigate the antagonistic mechanisms of Bacillus subtilis against Fusarium oxysporum, which is the cause of cucumber wilt disease. The fungal organism has been isolated from the infected rhizosphere of cultivated area with cucumber plants. Pot experiments were designed, firstly by seeds treatment, spraying and irrigated with crude extract, culture of Bacillus subtilis and biofertilizers. The results revealed that the best treatment was the combination of biofertilizer1 (sting) and the Bacillus subtilis crude extract, which is significantly promoted the growth of cucumber plants and decrease the incidence of disease (83.8%). The antifungal compounds were determined from the crude extract of Bacillus subtilis. The extract was purified by ammonium sulphate and identified using Gas Chromatography Mass spectroscopy (G-C Mass). This compound was identified as pseudojervine.

Keywords: Biocontrol, Fusarium oxysporum, Bacillus subtilis, cucumber wilt disease,pseudojervine.

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INTRODUCTION

Cucumbers are commercially cultivated as a seasonal vegetable crop worldwide[1,2].Fusarium wilt is a classic vascular wilt disease in which the fungus occludes the xylem vessels causing water blockage. It survives in soil for long periods and thus susceptible genotypes cannot be grown in an infested field for up to 30 years [3].Various control measures have been practiced to manage this disease, including destruction of diseased plants, sanitary measures, use of disease-free tissue culture planting material, use of tolerant variety and other integrated management methods. For the management of this disease chemicals are also widely utilized. As an alternative approach, biocontrol agents are being used for the management of various diseases [4, 5].

Bacillus subtilis is known as one of the most important antagonistic (biocontrol agent) and plant-growth promoting bacteria (PGPR) that is isolated from rhizosphere of different kinds of plants [6, 7, 8, 9].

Commercial products including enzymes, antibiotics, amino acids and insecticides are produced by *Bacillus* sp. The potential of *Bacillus* species to secrete various peptides which have shown distinct capacities to inhibit plant pathogens, such as fungi and bacteria with high concentrations, have been known for more than 50 years. Many researches indicated that the *Bacillus* sp. strains themselves and their antimicrobial substances had huge application potential in bio-control of plant diseases. Some antibiotics have been a certain degree of practical application [10].

Bacillus species are attractive due to their potential use in the biological control of fungal diseases [11]. *Bacillus subtilis*has been widely used as a biological control agent.This bacterium was reported to produce antibacterial and antifungal substances, such as surfactin, iturin, and fengycins[12].Antagonistic compounds are suppression factors and play a major role in biocontrol of soil-borne diseases. Several reports have described *Bacillus* strains worthy to be used as biocontrol agents for plant diseases [13]. The biocontrol efficiency of *B. subtilis, B. cereus*, *B. amyloliquifaciens, B. licheniformis* and *B. pumilis* has been proven by number of studies[14, 15, 16].

Plant proteinase inhibitors are polypeptides or proteins which occur in a wide variety of plants [17]. The most common and widely studied group of plant proteinase inhibitors are those that inhibit the animal serine proteases, which include trypsin and chymotrypsin. The function of proteinase inhibitors in plants has not been clearly established; they may serve a regulatory function, and/or a protective role [18, 19].

The aim of this research is to find out a biological control of cucumber wilt disease caused by *Fusarium oxysporum.*

MATERIALS AND METHODS

Isolation and characterization of fungal organism:

The fungal organism has been isolated from the infected rhizosphere of cultivated area with cucumber plants. Soil samples were collected, air-dried, then one gram of the rhizosphere is weighed. For isolation of the fungal isolate, serial soil dilution technique has been used[20]. The plates were incubated at 28℃ for 10 days. The fungus has been characterized morphologically and also biochemically and by scanning electron microscopy.

Bacterial organism:

Bacillus subtilis was purchased from Animal Health Research Institute (Agriculture research center), Giza, Egypt.

Antagonistic activity in vitro:

After the activation of *Bacillus subtilis*in (LB) broth media [21];*Bacillus subtilis* suspension was tested for antagonism against *Fusarium oxysporum* on PDA. Equal amounts of the sterilized media were poured in sterilized Petri dishes. *Fusarium oxysporum* was inoculated by spreading 1 µl of the fungus suspension (1×

10^8 CFU ml^{-1}) to the whole agar surface. Four wells were made by sterile cork borer in the PDA plate, then, 0.01 µl of *Bacillus subtilis* suspension (1× 10^4 CFU ml^{-1}) was added to each well; the test performed in duplicate. Thenthe plates were incubated at 28℃ for seven days; and the antagonistic effect was assessed by measuring of the inhibition zone formed around the wells (Figure2).

The pot experiment:

According to Yu *et al.*(2011)the pot experiment has been designed as follows;

1) Sterilized clay soil and sterilized seeds which represented by control (1).
2) Pots containing fumigated clay soil by *Fusarium oxysporum* and sterilized seeds (25 seeds/pot) which is represented by control (2).
3) The treated pots were kept as:

i) Seeds soaked in bacterial suspension (1× 10^4 CFUml^{-1}) represented by treatment (A).
ii) Soil irrigated with bacterial suspension (1× 10^4 CFUml^{-1}) astreatment (B).
iii) The soil sprayed with bacterial suspension (1× 10^4 CFUml^{-1}) astreatment (C).
iv) Seeds sown in bacterial suspension as treatment (D).
v) Seeds sown in bacterial crude extract this treatment has two symbols treatment (E) in the first experiment, and treatment (C") at the second experiment.
vi) Treated seeds with biofertilizers; a) symbion-p this treatment represented by treatment (A"),and b)sting that represented by treatment (B")
vii) Treated seeds with combination of biofertilizer and bacterial crude extract, which is represented astreatment (D") for the combination of biofertlizer a) sympion-p with bacterial crude extract and treatment (E") which is a representative for the combination of biofertilizer b)sting with bacterial crude extract.

All experiments were carried out in triplicates. Growth parameters (root and shoot growth), fresh and dry weightand(enzymatic protease activity and protease inhibitor activity test),weredetermined according[23].

Purification of the antifungal compounds

The inoculum for production of antifungal metabolites was prepared by growing the bacterial strain in 5ml of Potato Dextrose Broth (PDB, Difco Laboratories) at 30°C for 24hours. Cultures were incubated at 30°C and 100rev min−1 for 48hours on a shaker incubator. At the end of the incubation period, a cell-free supernatant fluid was obtained by centrifuging the culture at 15000 g and 5°C for 15minutes. Antifungal metabolites were isolated from the cell-free supernatant fluid by precipitation using solid ammonium sulphate which was gradually added to the supernatant fluid to achieve a 40% concentration (w/v). The mixture was slowly stirred at 5°C for 1hour and left to stand overnight at 5°C. This resulted in the formation of a precipitate which was removed after centrifugation at 15000 g for 15minutes at 5°C. The precipitate was dissolved in distilled water. Traces of ammonium sulphate were removed from the solution by exhaustive dialysis in a tubular cellulose membrane against distilled water. Insoluble residues were removed by centrifugation at 20000 g and 5°C for 15minutes. Finally, the solution was freeze-dried at −50°C. The resultant solid residue was stored at −20°C pending characterization[24].

Identification of the active components by GC-MS

The analysis for both the crude extract and the precipitate were carried out using a GC interfaced with a mass-selective detector equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i. d. and 0.25 µm film thicknesses). The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature.

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Statistics:

All the experiments conducted in this work were performed in 3 replicates and the results obtained were analyzed using ANOVA test.

RESULTS AND DISCUSSION

In this study, fungal organism was appeared on the surface of agar substratum medium, was isolated from infected rhizosphere of cultivated area with cucumber plants. This isolate was purified and maintained on potato dextrose agar for further investigation. The morphological and cultural (macroscopic, microscopic), physiological characterization of the isolate was tested for identification and taxonomic studies. The cultural characteristics were observed after 7 days and other tests were carried out as mentioned in material and methods. All the tests determined that fungal fungal organism is "*Fusarium oxysporum*". This is in agreement [25].

Figure 1: The microscopic appearance of *Fusarium oxysporum* mycelium

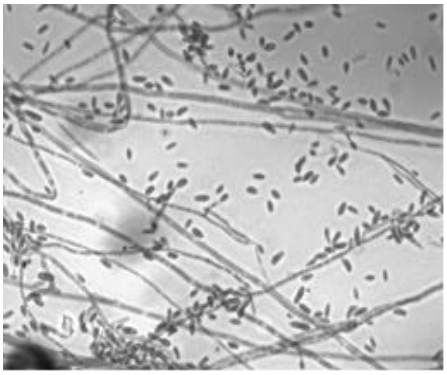
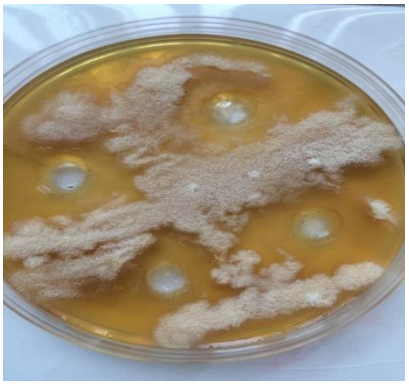


Figure 2: The antagonistic effect between *Bacillus subtilis* and *Fusarium oxysporum*as inhibition zone



The object of this experiment was to study the potentiality of the locally obtained *Bacillus subtilis* strain to produce antifungal substances in its culture medium capable of inhibiting growth of *Fusarium oxysporum*.

As shown in Fig (2) the inhibitory effect of *Bacillus subtilis* suspension is clear and *Bacillus subtilis* could inhibit 70%-75% of the growth of *Fusarium oxysporum(in vitro).*

The screening of the biological control of *Bacillus subtilis* strain *in vivo* figured out the best way to reduce *Fusarium* wilt disease incidence in cucumber plants were illustrated in tables (1), (2), (3), (4), (5) and (6).

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Table 1: Measurements of different parameters of seedlings at first week:

Measurements (1") / Treatments	Disease incidence (%)	Seedling length (in cm)	Seedling fresh weight (in mg)	Seedling dry weight (in mg)	Protease activity	Protease inhibitor activity
Control (1)	9.23±2.3	22.9±0.75	1.076±0.01	0.112±0.039	0.184±0.005	0.561±0.024
Control (2)	81.01±23.2	18.57±0.91	0.688±0.05	0.0657±0.009	0.162±0.012	0.613±0.047
Treatment(A)	8.56±2.3	20.87±1.14	0.99±0.10	0.083±0.0102	0.188±0.01	0.588±0.015
Treatment(B)	7.43±2.31	20.83±0.70	1.011±0.04	0.095±0.014	0.213±0.024	0.419±0.020
Treatment(C)	33.3±2.31	18.667±1.07	0.886±0.09	0.085±0.0059	0.188±0.01	0.533±0.011
Treatment(D)	4.28±2.31	23.533±1.05	1.109±0.13	0.129±0.024	0.361±0.006	0.398±0.019
Treatment(E)	1.37±2.3	23.95±0.577	1.15±0.094	0.144±0.005	0.37±0.013	0.34±0.02

Table (1) showed the measurements of different parameters after one week of sowing with different treatments, the disease incidence records a highest percentage in control (2)that presents the soil fumigated with *Fusarium oxysporum* only (which is a positive control). The lowest percentage is recorded at treatment(E);(which represents the seeds treated with *Bacillus subtilis*crude extract and the soil irrigated with the same solution) (as a best result). Other growth parameters such as; seedling length, fresh weight and dry weight of the seedling and protease activity test recorded low value in control (2) and high value in treatment (E) as a best result. The protease inhibitor activity test record a high value in control (2) and the lowest was in treatment (E), which proved that treatment (E) is the best result. These results in agreement [26, 27, 28, 29] whom used the bacterial strains as biocontrol applications. Many studies have reported that *Bacillus* spp. can produce a broad range of metabolites with antifungal and/or antibacterial activities [30, 31, 32].

Table 2: Measurements of different parameters of seedlings at second week:

Measurements / Treatments	Disease incidence (%)	Seedling length (in cm)	Seedling fresh weight (in mg)	Seedling dry weight (in mg)	Protease activity	Protease inhibitor activity
Control (1)	11.43±2.3	26.5±1.595	1.293±0.104	0.131±0.008	0.401±0.017	0.242±0.026
Control (2)	87.95±9.45	22.07±1.60	0.673±0.187	0.059±0.01	0.276±0.012	0.332±0.02
Treatment(A)	10±2.3	23.87±1.05	1.123±0.108	0.077±0.006	0.297±0.011	0.344±0.034
Treatment(B)	10.44±6.1	24.93±0.86	1.069±0.063	0.097±0.0169	0.411±0.004	0.244±0.010
Treatment(C)	37.88±4	21.87±1.20	0.909±0.909	0.081±0.0117	0.312±0.014	0.303±0.022
Treatment(D)	7.1±4.6	28.43±1.65	1.307±1.267	0.158±0.044	0.415±0.007	0.196±0.073
Treatment(E)	1.37±2.31	28.62±1	1.389±0.032	0.163±0.032	0.423±0.006	0.082±0.043

Table (2) showed the measurements of different parameters of seedlings after two weeks of sowing, as it shown that the highest disease incidence presented in control (2). The highest morphological data presented in treatment (E).

Table 3: Measurements of different parameters of seedlings at third week

Measurements / Treatments	Disease incidence (%)	Seedling length (in cm)	Seedling fresh weight (in mg)	Seedling dry weight (in mg)	Protease activity	Protease inhibitor activity
Control (1)	14.29±6	32.3±2.272	1.783±0.14	0.194±0.053	0.349±0.011	0.402±0.038
Control (2)	91.39±24.33	24.67±1.159	0.89±0.019	0.087±0.006	0.228±0.015	0.376±0.007

Treatment(A)	11.43±4	28.167±1.301	1.2±0.104	0.094±0.009	0.267±0.015	0.459±0.037
Treatment(B)	13.4±6.1	29.267±1.779	1.114±0.153	0.116±0.011	0.391±0.02	0.303±0.017
Treatment(C)	40.9±2.3	25.67±0.611	1.047±0.053	0.101±0.004	0.271±0.01	0.431±0.022
Treatment(D)	10±4.62	31.47±1.234	1.495±0.156	0.157±0.011	0.384±0.003	0.352±0.036
Treatment(E)	2.7±2.3	33.67±1.527	1.662±0.0907	0.178±0.091	0.392±0.004	0.280±0.01

Table (3) showed the measurements of different parameters of seedlings after three weeks of sowing, as it shown that the highest disease incidence presented in control (2). The highest morphological data presented in treatment (E).

From these results, treatment (E) was the best result (crude extract irrigation and sowing). This is in agreement [26, 27, 28, 29]whom used the bacterial strains as biocontrol applications.

Table 4: Measurements of different parameters of seedlings at first week:

Measurements / Treatments	Disease incidence (%)	Seedling length (in cm)	Seedling fresh weight (in mg)	Seedling dry weight (in mg)	Protease activity	Protease inhibitor activity
Control (1)	8.69±4.62	20.87±1.12	1.039±0.015	0.177±0.0057	0.297±0.056	0.472±0.02
Control (2)	81.38±4.62	16.9±1.99	0.890±0.028	0.133±0.0377	0.154±0.02	0.643±0.05
Treatment(A")	8.57±4.62	20.2±0.82	1.015±0.021	0.171±0.0025	0.352±0.016	0.240±0.05
Treatment(B")	10±4.62	19.5±0.87	1.031±0.059	0.178±0.0099	0.340±0.03	0.264±0.01
Treatment(C")	2.74±4	19.5±0.56	0.979±0.026	0.163±0.0078	0.382±0.026	0.225±0.06
Treatment(D")	2.8±6.1	19.77±0.75	1.020±0.027	0.174±0.0041	0.385±0.03	0.210±0.02
Treatment(E")	1.35±2.31	23.23±0.87	1.106±0.060	0.187±0.0091	0.400±0.004	0.202±0.01

Table (4) showed the measurements of different parameters of seedlings after one week of sowing, as it showed that the highest disease incidence presented in control (2). The highest morphological data presented in treatment (E").

Table 5: Measurements of different parameters of seedlings at second week:

Measurements / Treatments	Disease incidence (%)	Seedling length (in cm)	Seedling fresh weight (in mg)	Seedling dry weight (in mg)	Protease activity	Protease inhibitor activity
Control (1)	10±2.31	26.30±1.701	1.858±0.109	0.256±0.025	0.390±0.064	0.373±. 0.02
Control (2)	83.07±4.62	21.30±1.20	1.108±0.113	0.158±0.016	0.149±0.039	0.686±0.036
Treatment(A")	8.57±6.1	31.17±1.06	2.017±0.040	0.271±0.026	0.365±0.06	0.233±0.031
Treatment(B")	12.86±2.31	26.77±1.387	1.729±0.027	0.229±0.007	0.370±0.01	0.240±0.04
Treatment(C")	4.1±4.62	25.60±1.136	1.803±0.067	0.255±0.007	0.389±0.02	0.204±0.024
Treatment(D")	4.17±2.31	30.83±1.082	1.873±0.096	0.281±0.006	0.395±0.05	0.110±0.01
Treatment(E")	1.35±4	31.93±1.320	1.950±0.077	0.279±0.008	0.440±0.010	0.075±0.04

Table (5) showed the measurements of different parameters of seedlings after two weeks of sowing, as it shown that the highest disease incidence presented in control (2). The highest morphological data presented in treatment (E").

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Table 6: Measurements of different parameters of seedlings at third week:

Measurements / Treatments	Disease incidence (%)	Seedling length (in cm)	Seedling fresh weight (in mg)	Seedling dry weight (in mg)	Protease activity	Protease inhibitor activity
Control (1)	11.59±2.31	32.63±1.46	2.956±0.276	0.7403±0.0606	0.36±0.009	0.285±0.04
Control (2)	86.5±2.31	24.57±0.71	2.394±0.442	0.5780±0.0945	0.14±0.084	0.62±0.02
Treatment(A")	10±4.62	35.10±2.55	4.001±0.211	0.9330±0.0614	0.359±0.02	0.240±0.05
Treatment(B")	14.3±2.31	30.30±0.92	3.290±0.443	0.7877±0.095	0.363±0.033	0.254±0.01
Treatment(C")	5.48±2.31	30.80±1.49	3.584±0.455	0.8127±0.1	0.38±0.013	0.225±0.008
Treatment(D")	5.56±4.62	34.53±1.45	3.930±0.065	0.9257±0.023	0.385±0.05	0.203±0.02
Treatment(E")	2.7±4.62	37.57±1.75	3.989±0.358	0.942±0.055	0.4±0.007	0.19±0.024

Table (6) showed the measurements of different parameters of seedlings after three weeks of sowing, as it shown that the highest disease incidence presented in control (2). The highest morphological data presented in treatment (E").

From the previous experiment it was found that the best results can be achieved by combining the biofertilizer 1 (sting) and *Bacillus subtilis* crude extract as a biological control for cucumber disease wilt and this is supported by combinations of biocontrol agents can result in more effective and robust control of plant diseases. From these results, we found that treatment (E") is the best results (combination between biofertilizer sting and crude extract in both sowing the seeds and irrigation of the plant). These results are in agreement [33, 34] that reported combination of biofertilizer and antagonistic microorganism is the best way to reduce incidence of wilt disease.

Figure 3: Chromatogram of bacterial crude extract using (G-C Mass):

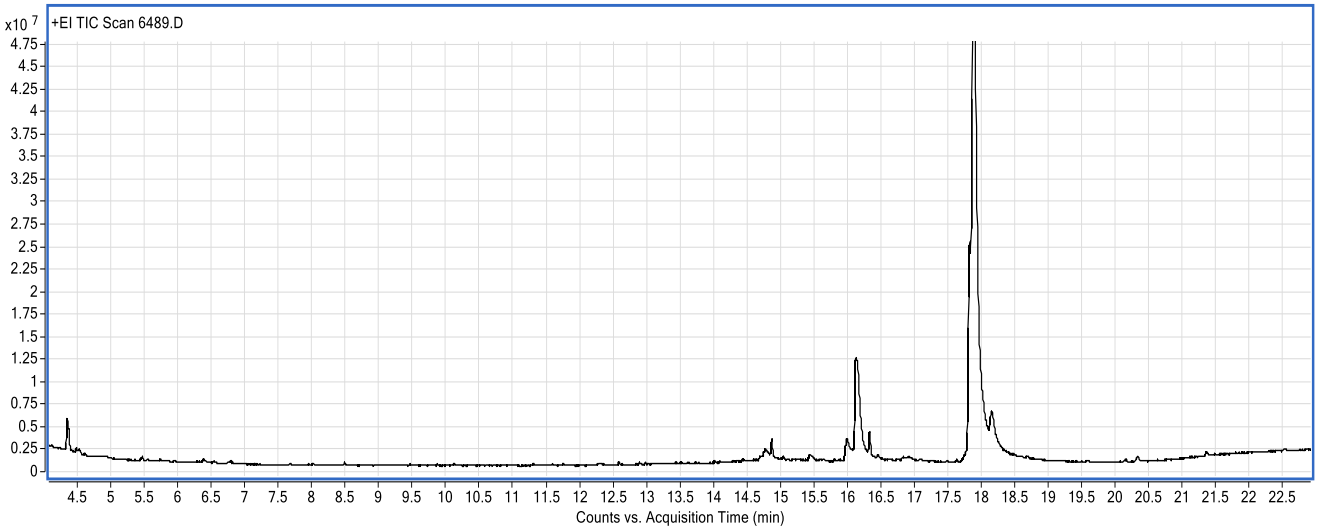

Table 7: Identified compounds in the bacterial crude extract

No.	RT	Compound name	Area Sum %
1	4.34	Paclitaxel	1.45
2	4.5	Muramic acid	0.46
3	5.5	Sinapic aldehyde	0.33
4	5.6	Streptovitacin	0.26
5	5.91	4,6-Benzylidene-α-methyl-D-glucoside	0.84
6	6.47	Pyrazole[4,5-b]imidazole, 1-formyl-3-ethyl-6-β-d-ribofuranosyl-	0.64
7	4.34	Paclitaxel	1.45

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8	4.5	Muramic acid	0.46
9	13.57	O,N-PERMETHYLATED N-ACETYLLYSINE	0.96
10	12.9	Pseudojervine	3.47
11	14.07	Rescinnamine	1.25
12	11.5	Scopoletin	2.93
13	9.817	Ferulic acid	1.17
14	10.155	Colchicine	0.98
15	10.34	Melibiose	0.47
16	11.62	(+) -α- Tocopherol	2.99
17	12.18	3`,4`,7- Trimethylquercetin	1.95

Figure 4: chromatogram of the precipitate

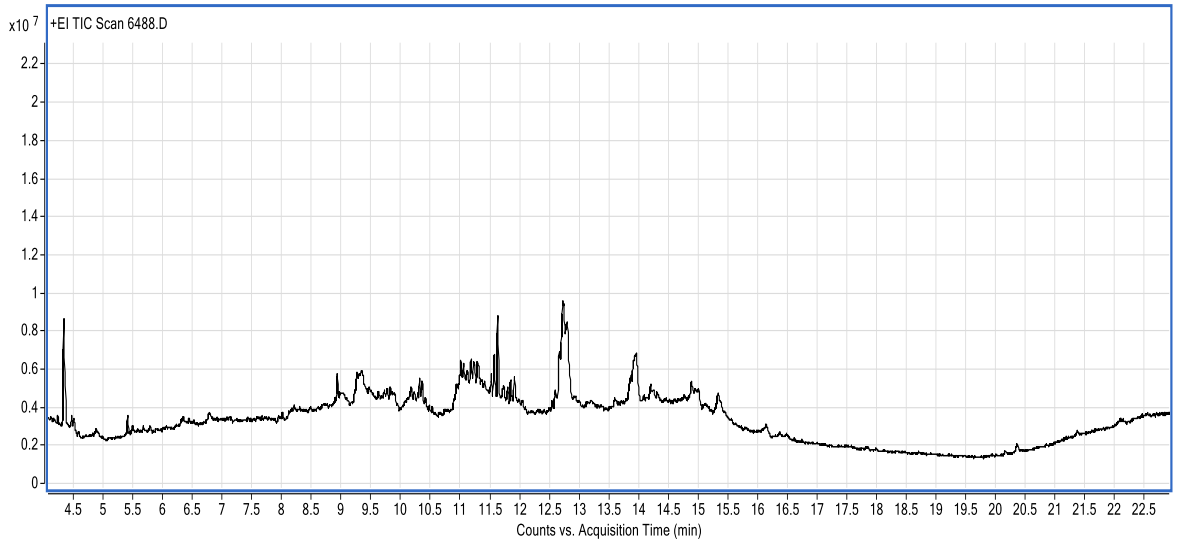
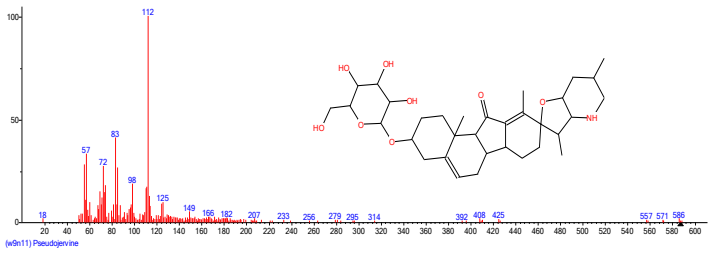


Figure 5: Spectrum of the identified compound -Pseudojervine



This spectrum shows the Pseudojervin compound. Our findings suggest that combination of sting (biofertilizer) and pseudojervine compound could be a possible alternative choice of any chemical, toxic compounds to control Fusarium wilt disease of cucumber.

REFERENCES

[1] Chen JC. Chiu NH. Nie RL. Cordell GA. Qiu AX. Cucurbitacins and cucurbitane glycosides: structures and biological activities, Nat. Prod. Rep. 2005; 22: 386-399.

[2] Mukherjee PK. Nema NK. Maity N. Sarkar BK. Phytochemical and therapeutic potential of cucumber.Fitoterapia 2013; 84: 227-236.

[3] Ploetz RC. Panama disease: a classic and destructive disease of banana. Online. Plant Health Progress 2000.

[4] Harish S. Kavino M. Kumar N. Balasubramanian P. Samiyappan R. Induction of defense-related proteins by mixtures of plant growth promoting endophytic bacteria against Banana bunchy top virus. Biological Control 2009; 51: 16–25.

[5] Kavino M. Harish S. Kumar N. Saravanakumar D. Samiyappan R. Induction of systemic resistance in banana (Musa spp.) against Banana bunchy top virus (BBTV) by combining chitin with root-colonizing *Pseudomonas fluorescens* strain CHA0. European J Plant Pathol 2008; 120: 353–362.

[6] Bais H.P. Fall R. Vivanco JM. Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 2004; 134: 307–319.

[7] Chen Y. Yan F. Chai Y. Liu H. Kolter R. Losick R. Guo JH. Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. Environ Microbiol2013; 15: 848–864.

[8] Mohammadipour, M.; Mousivand, M.; SalehiJouzani, G. and Abbasalizadeh, S. "Molecular and biochemicalcharacterization of Iranian surfactin-producing *Bacillus subtilis* isolates and evaluation of their biocontrol potential against *Aspergillus flavus* and *Colletotrichumgloeosporioides*".Can J Microbiol2009; 55: 395–404.

[9] Waewthongrak W. Pisuchpen S. Leelasuphakul W. Effect of *Bacillus subtilis* and chitosan applications on green mold (*PeniciliumdigitatumSacc.*) decay in citrus fruit. Postharvest Biol Technol.2015; 99: 44–49.

[10] Zhao X. Zhou Z. Han Y. Wang Z. Fan J. Xiao H. Isolation and identification of antifungal peptides from *Bacillus* BH072, a novel bacterium isolated from honey. Microbiological Research 2013; 168: 598-606.

[11] Zalila-Kolsi I. Mahmoud A. Ali H. Sellami S. Nasfi Z.Tounsi S. Jamoussi K. Antagonist effects of *Bacillus* spp. strains against *Fusarium graminearum* for protection of durum wheat (*Triticumturgidum*L. *subsp. durum*). Laboratory of Biopesticides, Centre of Biotechnology of Sfax, University of Sfax, P.O.Tunisia2016.

[12] Nagorska K. Bikowski M. Obuchowski M. Multi cellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. ActaBiochimicaPolomica 2007; 54: 495−508.

[13] Shoda M. Bacterial control of plant diseases. J BiosciBioeng 2000; 89:515–521.

[14] Li D. Nie F. Wei L. Wei B. Chen Z. Screening of high-yielding biocontrol bacterium Bs-916 mutant by ion implantation. ApplMicrobiolBiotechnol 2007; 75(6):1401–1408.

[15] Marten P. Smella K. and Berge G. Genotypic and phenotypic differentiation of an antifungal biocontrol strains belonging to Bacillus subtilis. J ApplMicrobiol2000; 89(3):463–471.

[16] Siddiqui S. Siddiqui ZA. Ahmad I. Evaluation of fluorescent *Pseudomonads* and *Bacillus* isolates for the biocontrol of a wiltdisease complex of pigeonpea. World J MicrobiolBiotechnol2005;21(5):729–732.

[17] Whitaker JR. Naturally occurring peptide and protein inhibitors of enzymes. In Impact of Toxicology on Food Processing (Ed. by Ayres J. C. and Kirschman J.), 1981, pp. 57-104.

[18] Richardson M. The proteinase inhibitors of plants and microorganisms.Phytochem 1977; 16: 159-169.

[19] Ryan CA. Proteolytic enzymes and their inhibitors in plants. A. Rev. PI. Physiol. 1973; 24: 173-196.

[20] Johnson LF. Curl EA. Bond JH. Fribourg HA.Methods for studying soil micoflora. Plant disease relationships, Burgess Pub.Co. 1959.

[21] Liu B. Huang L. Buchenauer H. Kang Z. Isolation and partial characterization of an antifungal protein from the endophytic *Bacillus subtilis* strain EDR4. Pesticide Biochemistry and Physiology 2010; 98: 305–311.

[22] Yu X. Ai C. Xin L. Zhou G. The siderophore-producing bacterium, *Bacillus subtilis* CAS15, has a biocontrol effect on Fusarium wilt and promotes the growth of pepper. European Journal of Soil Biol. 2011; 47: 138-145.

[23] Widnyana IK. Suprapta DN. Sudana IM. Rai IG. Temaja M. Pseudomonas alcaligenes, Potential Antagonist Against Fusarium oxysporumf.sp.lycopersicum the Cause of Fusarium Wilt Disease on Tomato. 2013; 7: 2224-3208

[24] Munimbazi C. Bullerman LB. Isolation and partial characterization of antifungal metabolites of Bacillus pumilus. Appl. Microbiol. 1998; 84: 959–968

[25] De Hoog GS.Guarro J. Gene J. Figueras MJ. Atlas of Clinical Fungi(2000).

[26] Abeysinghe S. Biological control of *Fusarium solani f. sp. phaseoli* the causal agent of root rot of bean using *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01, Ruhuna J. Sci. 2007; 2 : 82-88.

[27] Abeysinghe S. Effect of combined use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on biological control of *Rhizoctoniasolani* on *Solanum melongena* and *Capsicum annuum*, Plant Pathol. J. 2009; 8: 9-16.

[28] Dutta S. Kundu A. Chakraborty MR. Ojha S. ChakrabartiJ. Chatterjee NC. Production and optimization of Fe(III) specific ligand, the siderophore of soil inhabiting and wood rotting fungi as deterrent to plant pathogens, ActaPhytopathol. Entomol.Hungarica 2006; 41: 237-248.

[29] Janisiewica WJ. Korsten L. Biological control of postharvest diseases of fruits, Annu. Rev. Phytopathol. 2002; 40: 411-441.

[30] Chung S. Kong H. Buyer JS.Lakshman DK. Lydon J. KimS. Roberts DP. Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper.ApplMicrobiolBiotechnol 2008; 80 (1):115–123.

[31] HuangJF. Wei Z. Tan SY. Mei XL. Shen QR. Xu YC. Suppression of bacterial wilt of tomato by bioorganic fertilizer made from the antibacterial compound producing strain *Bacillus amyloliquefaciens* HR62. J Agricul. Food Chemist2014; 62: 10708−10716.

[32] Li XY. Yang JJ. Mao ZC. Ho HH. Wu YX. He YQ.Enhancement of biocontrol activities and cyclic lipopeptides production by chemical mutagenesis of *Bacillus subtilis* XF-1, a biocontrol agent of *Plasmodiophorabrassicae* and *Fusarium solani*. Ind. J. Microbiol2014; 54: 476−479.

[33] De Boer M. Borm P. Kindt F. Keurentjes JJB.Van der Sluis I. Van Loon LC. Bakker PAHM. Control of Fusarium wilt of radish by combining *Pseudomonas putida* strains that have different disease-suppressive mechanisms.Phytopathol2003; 93: 626-632.

[34] Leeman M. Ouden FM. Pelt JA. Cornelissen C. Matamala-Garros A. Bakker PAHM.Schippers B. Suppression of Fusarium wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. And root-colonizing fungi.EuroJ Plant Pathol 1996; 102: 21-31.