

ENHANCEMENT OF *Pseudomonas fluorescense* WITH CERTAIN MICROELEMENTS FOR CONTROLLING MAIZE STALK ROT CAUSED BY *Fusarium verticillioides* (SACC.) UNDER GREENHOUSE CONDITIONS

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ABSTRACT

F. verticillioides (Sacc.) Nirenberg (G) is the major pathogen of maize worldwide causing seedling, stalk and ear rots. Five isolates of *F. verticillioides* have ability to infect maize Pioneer cultivar plants. The isolates varied in their infectivity, *F. verticillioides* isolate 3 gave the highest stalk rot severity in both 2008 and 2009 growing seasons, followed by isolate 4, while isolate 5 was the lowest pathogenic one. *In vitro*, the microelements [zinc (Zn), manganese (Mn) and copper (Cu)] reduced linear growth of the pathogen. Manganese exhibited the highest toxic effect followed by zinc while, copper exhibited the lowest toxic effect. The antagonistic effects of 3 *P. fluorescences* isolates on mycelial growth of *F. verticillioides* were in the range of 55.14 to 79.82%, *P. fluorescences* isolate (Pf2) considerably more antagonistic than other isolates. Under greenhouse conditions, treatment of maize plants with microelements decreased significantly disease severity. Manganese treatment was more effective than copper and zinc treatments. The biocontrol with *P. fluorescences* (Pf2) provided moderate level of protection when used alone, while combination of Pf2 with microelements treatment significantly improved the biocontrol activity and significantly reduced disease severity compared with microelements alone. Microelements content increased with *P. fluorescences* treatment comparing with control treatment.

INTRODUCTION

Maize (*Zea mays* L) is attacked by several diseases. Fusarium stalk rot of maize caused by *F. verticillioides*, represents the common stalk rot in many areas of the world such as Egypt. Management of Fusarium stalk rot has little success with classical methods as fungicides. There is a serious need for alternative control. Among different suggestions, there is an increasing interest in the introduction of bacterial and fungal biocontrol agent for managing different pathogens to avoid using chemical fungicides and their side effect on human health and environment. Rhizospheric *Pseudomonas fluorescens* bacterium has used in the biological control of plant diseases because of several advantages compared to other biocontrol agents.

Different mechanisms are involved to interrupt the importance of plant growth-promoting (PGP) rhizobacteria, one of them is induction of resistance (Geels and Schippers, 1983, Fravel 1988 and Weller, 1988). It has been demonstrated that the rhizospheric bacteria like pseudomonads stimulate the growth of shoot and roots of different plants (Arndt *et al.*, 1998). Plant growth promoting bacteria, *Pseudomonas* spp. can suppress pathogenic strains of *F. oxysporum* by siderophore-mediated competition (Van

Peer et., al.1990, Lemanceau et al., 1992, Duijff et. al., 1993 and Zhou and Paulitz, 1994). Pure culture of *P. fluorescens* used as seed treatment and as spray treatment enhanced the growth parameters and reduced the incidence of *F. verticillioides* compared with the other treatments (Nayaka et. al., 2009).

Also using of microelements, such as manganese and zinc, for inducing resistance is an alternative method, (Marschner, 1986, Abd El-Hai et. al., 2007 and El Baz, 2007). An adequate supply of manganese and several other microelements are important in most of active defense mechanisms mediated through the shikimate pathway.

This work was planned to study enhancement of fluorescent bacterium (*Pseudomonas fluorescences*) with certain microelements (Cu, Mn and Zn) for controlling Fusarium stalk rot of maize.

MATERIALS AND METHODS

Source of organisms:

F. verticillioides and *P. fluorescences* isolates were supplied from stock culture of Plant Pathology Dept., Faculty of Agriculture, Assiut University.

Maize cultivar (Pioneer) was, kindly, supplied from Agriculture research center, Giza, Egypt.

Pathogenicity test:

F. verticillioides isolates were examined for their pathogenicity of stalk rot on the corn cultivar Pioneer under greenhouse conditions in the corn growing seasons 2008 and 2009. For inoculation of stalks, the toothpick technique was used as described by Jardine and Leslie (1992). Round wooden toothpicks about 6 cm. long were boiled several times (about 1 hr. each time) in tap water to remove any possible toxic substances that might inhibit the growth of the tested fungi. Boiled toothpicks were washed in fresh tap water after each boiling, then dried and packed into glass jars. Potato dextrose broth, supplemented with 0.1% yeast extracts was added to moisten the toothpicks until slight excess of the medium accumulated at the bottom of each jar and autoclaved immediately at 1.5 cm² for 20 min. Autoclaved toothpicks were inoculated with 8 mm. in diameter discs of *F. verticillioides* cultures and incubated at 27 °C about 3 weeks until the fungal growth covered the toothpicks.

Five of surface sterilized grains of the tested cultivar (pioneer) were sown in sterilized pots (30 cm. in diameter), containing autoclaved soil. Inoculation was performed at second internode from the ground surface of forty days old plants by using an ice pick to create a hole at the second internode. A toothpick was then inserted into the hole. Sterile toothpicks were inserted into the second internode of control plants. One month after inoculation, the stalks were cut longitudinally and stalk discoloration was determined using the following rating scale ranging from 1 to 5 (Hooker, 1956): 1= 0-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of the inoculated internode diseased and 5 = 100% with infection extending into the adjacent internode. For each replicate a disease index (DI) similar to that described by Liu et al. (1995) was calculated as follows: $DI\% = \frac{\sum(1A + 2B + 3C + 4D + 5E)}{5}$

5T X 100 where, A, B, C and D are the number of plants corresponding to the numerical grade, 1, 2,3,4 and 5, respectively, and 5T is the total number of plants (T) multiplied by the maximum discoloration grade 5.

In vitro experiments:

Effect of certain microelements on the fungal linear growth:

To study the effect of manganese, zinc and copper at 100, 200, 300, and 400 ppm it were separately mixed in PDA medium before solidification, and then poured in sterile Petri dishes (9 cm). Five plates for each treatment were used as replicates, while each plate was inoculated with 5 mm fungal disc and incubated at 25 °C. The linear growth of the fungus was measured when the full growth of tested isolate was observed in the check treatment and the average growth diameters were calculated. The experiment was carried out twice:

Antagonistic effect of different *P. fluorescences* isolates on the pathogenic fungus:

This study was in the Plant Pathology Dept., Faculty of Agriculture, Assiut University. Agar disks carrying mycelium of *F. verticillioides*, each was placed at the centre of PDA plate between two parallel streaks of the tested bacteria (3 isolates of *Pseudomonas* were tested), which were 5 cm apart. Plates inoculated with the fungus alone served as control, when the fungal growth of the control approached the edge of the plates. *In vitro* antagonistic effect was assessed by relating mycelial diameter on plates inoculated with bacteria to mycelial diameter on control plates and computing inhibition percentages. Five plates were used for each treatment. The experiment was carried out twice.

Preparation of *P. fluorescences* inoculum:

Pseudomonas isolate (Pf2) of the 48 h-old-culture in Kings medium B was centrifuged at 10.000 rpm for 10 min. Pellets (bacterial cells) were washed twice with sterilized distilled water by centrifugation. The optical density of the solution was adjusted to 0.45 (A610 nm) to obtain 10^8 CFU / ml with the help of a UV-Visible spectrophotometer.

Effect of certain microelements and *P. fluorescences* (Pf2) on disease severity:

Under greenhouse conditions in 2009 and 2010 maize growing season, surface sterilized grains of Pioneer cultivar were sown in sterilized pots (30 cm in diameter) containing sterilized soil. After forty days, toothpick technique was used for inoculating *F. verticillioides* isolate F3 (the highly virulent isolate) as mentioned above. Plants were sprayed with *Pseudomonas* at concentration of 10^8 CFU / ml after 45 days from planting then sprayed with Zn (as EDTA), Cu (as CuSO_4), and Mn (as EDTA) separately at concentration of 300 ppm. The same treatments were repeated after 60 days from planting. Plants not inoculated with *Pseudomonas* served as control. Normal NPK was applied as recommended and the plants were irrigated when necessary. Disease severity was assessed as disease index as mentioned in pathogenicity tests experiment.

Analysis of microelements in plants:

At the end of the seasons, leaves samples were collected and washed with tap water and then with deionized water three times. After that,

the leaves samples were dried at 70 °C for 48 hours in electric oven. The oven dried leaves samples were grinded properly in mortar with pestle to make it homogenous and 0.5 gram of sample was treated with a 2 : 1 HNO₃ : HClO₄ acid mixture until the solution became clear (Usman and Mohamed, 2009). Digested samples were filtered using filter papers and made up to 50 mL with HNO₃. Four replicates from each treatment were used in this experiment. Mn, Zn and Cu were determined with a GBC Model 906 atomic absorption spectrophotometer (AAS) from digestion solution mined volumetrically by EDTA (versene) method (Page *et al.*, 1982).

Statistical analysis:

All data were subjected to statistical analysis and means were compared using L.S.D. range test as described by Gomez and Gomez, 1984.

RESULTS

Pathogenicity tests:

The pathogenicity test showed that five isolates of *F. verticilliodes* have ability to infect maize pioneer cultivar (Table 1). *F. verticilliodes* isolates varied in their infectivity on maize plants. Data show that *F. verticilliodes* F. 3 gave the highest stalk rot severity in both 2009 and 2010 growing seasons, followed by F. 4 isolate, then F. 1 isolate, while F. 5 isolate was the least pathogenic one.

Table (1): Index of stalk rot disease on maize plants caused by the tested *F. verticilliodes* isolates

<i>F. verticilliodes</i> isolate	Disease index %	
	2008 growing season	2009 growing season
F. 1	28.33	30.66
F.2	22.66	23.33
F.3	52.75	49.50
F.4	37.20	36.33
F.5	19.75	20.33
Control	0.00	0.00
L.S.D. at P<0.05	16.50	14.20

Effect of certain microelements on fungal linear growth

Data in Table 2 show inhibitory effect of zinc, manganese and copper on mycelial linear growth of the pathogen. In general, all tested microelements reduced linear growth of *F. verticilliodes* *in vitro*. Mn exhibited the highest toxic effect followed by zinc. Cu exhibited the lowest toxic effect on *F. verticilliodes*. Microelements concentrations varied in their toxic effect, in which, 400 and 300 ppm exhibited the highest reduction of mycelial growth followed by 200 ppm, at 100 ppm, the lowest toxic concentration of the tested microelements was obtained. Data also indicated that there is no significant difference between 300 and 400 ppm.

Table (2): Reduction percent in mycelial growth of *F. verticillioides* influenced by different concentrations of some microelements.

Microelement	Microelement concentration (ppm)				Mean
	100	200	300	400	
Zn	45.50	55.20	70.30	71.50	60.63
Mn	55.90	58.70	70.10	72.50	64.30
Cu	20.80	28.60	33.70	35.60	29.68
Control	0.00	0.00	0.00	0.00	0.00
Mean	30.55	35.63	43.53	44.90	

L.S.D. ($P \leq 0.05$) ; Microelements (A): 4.12 Concentration (B) 5.66 Interaction (AxB) 7.12

Antagonistic effect of *P. fluorescense* isolates on fungal mycelial growth *In vitro*

The inhibitory effect of *Pseudomonas fluorescense* isolates on mycelial growth of *Fusarium verticillioides* was shown in Figure (1). Results indicate that the 3 isolates gave positive reaction of antagonism to the pathogen *in vitro*. The antagonistic effects of *P. fluorescense* against *F. verticillioides* were in the range of 55.14 – 79.82 %. *P. fluorescense* (Pf2) considered more antagonistic to *F. verticillioides* than the other isolates. *P. fluorescense* Pf2 isolate gave the highest effect (79.82) followed by Pf3 isolate (59.2) and then Pf1 isolate (55.14).

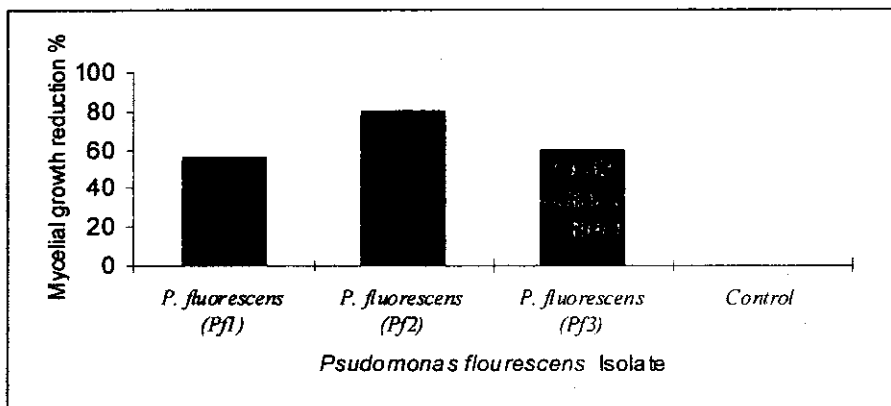


Figure (1): Antagonistic effect of *P. fluorescense* isolates on linear growth of *F. verticillioides*

Effect of tested microelements and *P. fluorescense* (Pf2) biocontrol on disease incidence

Results in Table (3) indicate that treatment of maize plants with 300 ppm Cu, Mn and Zn decreased significantly disease severity. Manganese treatment was more effective than copper and zinc treatments. The biocontrol agent (Pf2) isolate provided a moderate level of protection, reducing disease index from 58.50 to 29.67 % and from 65.00 to 42.31 % in 2010 and 2011, maize growing seasons, respectively. Combination of Pf2 with minerals significantly reduced disease severity compared with the minerals alone. The combination between Pf2 and zinc was more effective in

this respect than zinc only in two experimental seasons followed by combination between Pf2 and manganese treatment. .

Table (3): Effect of certain microelements and *P. flourescens* (Pf2) on maize stalk rot disease under greenhouse conditions in 2010 and 2011 maize growing seasons

Treatment	2010 growing season		2011 growing season	
	Disease index %	Reduction %	Disease index %	Reduction %
Cu	28.83	50.71	39.42	39.35
Mn	16.25	72.22	36.09	44.48
Zn	35.20	39.83	40.67	37.43
<i>P. flourescens</i> (Pf2)	29.67	49.28	37.50	42.31
Pf2 + Cu	22.42	61.67	33.50	48.46
Pf2 + Mn	13.50	76.92	30.00	53.85
Pf2 + Zn	16.92	71.08	18.09	72.17
Control	58.50	-	65.00	-
L.S.D. at $P \leq 0.05$	5.60		6.72	

Microelements contents in maize leaves

Cu, Mn and Zn contents in leaves of maize plants were determined as mg /Kg fresh weight. Results in Table (4) indicate that the copper and zinc contents in the leaves of the treated plants with *P. flourescens* (Pf2) were significantly higher than those of manganese compared with untreated plants (control) in the both experimental seasons.

Table (4): Content of microelements (mg/Kg fresh weight) in maize leaves as affected by *P. flourescens* (Pf2)

Treatment	Cu		Mn		Zn	
	2010	2011	2010	2011	2010	2011
<i>P. flourescens</i>	25.28	22.98	59.95	57.86	35.88	47.80
Control	19.03	11.64	55.05	53.95	25.31	39.88
L.S.D. 0.05	2.17	5.25	5.21	4.88	7.12	6.13

DISCUSSION

F. verticillioides may cause seedling blight and stalk rot as well as ear rot on maize (Thomas and Buddenhagen, 1980; Asran and Aboul-Nasr, 2011). The results of this investigation revealed that all tested isolates able to infect maize plants and cause stalk rot disease. The isolates, however, varied considerably in disease severity. Bacon *et al.* (1996), Asran and Buchenauer (2002) and Asran and Aboul-Nasr (2011) also reported considerable variability in pathogenicity of *F. verticillioides* isolated to maize plants. Microelement amendments have been used commercially on a limited scale to manage certain soil borne diseases including Fusarium diseases (Engelhard, 1989). Data in our investigation show that all tested microelements reduced growth of *F. verticillioides* *in vitro*. Manganese exhibited the highest toxic effect followed by zinc, while, copper exhibited the lowest toxic effect. Pathogen suppression, by using microelement, is most often attributed to direct inhibition of fungal growth and activity (Huber, 1989 and Asran 2012).

Testing antagonistic capabilities of certain *P. fluorescens* isolates was investigated *in vitro*. Data revealed that all the tested isolates were able to inhibit the mycelial growth of the pathogen with different degrees of antagonistic capability. Several researchers have successfully employed antagonistic bacteria to control plant diseases (Hassan and Abd El-Rehim, 2002 and Hussein *et al.*, 2007).

Under greenhouse conditions, treatment of maize plants with 300 ppm copper, manganese and zinc decreased significantly disease index. Manganese treatment was more effective than copper and zinc treatments. Mandal and Sinha (1992) suggested that zinc and other minerals reduce Fusarium wilt of tomato by inducing host resistance. Disease reduction, by using microelement, is most often attributed to improve nutrition that boosts host defenses or to direct inhibition of fungal growth and activity. Pathogen suppression may result indirectly from modification of chemical and physical properties of soil and rhizosphere (Simon and Sivasithamparam, 1989) or from modification of host root exudates to disfavor pathogen activity (Huber, 1989).

Data herein show that the biocontrol agent; *P. fluorescense* (Pf2) provided a moderate level of protection when used alone. *P. fluorescense* is a biocontrol agent with activity against diseases caused by soil borne fungal pathogens (Voisard *et al.*, 1994). We found that microelement significantly improved the biocontrol activity of *P. fluorescense* (Pf2) against *F. verticillioides*. Zinc in combination with *P. fluorescense* improve biocontrol of Fusarium crown and root rot of tomato and repress the production of pathogen metabolites (Duffy and Défago, 1997). There are a few cases, though, in which minerals appear to reduce disease e.g., exerting an indirect beneficial effect on indigenous and introduced antagonistic microorganisms (Elmer, 1995). Zinc soil content has been found to be positively correlated with the biocontrol activity of introduced *P. fluorescense* 2-79 (Weller and Thomashow, 1994).

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تحسين المقاومة البيولوجية لبكتريا *Pseudomonas fluorescense* باستخدام العناصر الصغرى لمقاومة عفن الساق في الذرة الشامية المتسبب عن الفطر *Fusarium verticilliodes*

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- يعتبر الفطر *F. verticilliodes* من الفطريات التي تسبب لفة البادرات وأغصان الساق والكيزان لنباتات الذرة الشامية وقد أجرى هذا البحث بغرض تحسين المقاومة البيولوجية للبكتريا *P. fluorescense* لمقاومة مرض عفن الساق الفيوزاريومي باستخدام بعض العناصر الصغرى مثل المنجنيز والنحاس والزنك وقد تم الحصول على النتائج الآتية:-
- 1- تم اختبار القدرة المرغوبة لـ ٥ عزلات من الفطر *F. verticilliodes* على صنف الذرة الشامية Pioneer وقد اختلفت العزلات في قدرتها المرضية وكانت العزلة رقم ٣ اقوى العزلات بينما أظهرت العزلة رقم ٥ أقل درجة إصابة لنباتات الذرة الشامية خلال موسمي الإختبار.
 - 2- في المعمل تم اختبار تأثير ٣ عناصر صغرى (زنك - منجنيز - نحاس) على نمو العزلة رقم ٣ من الفطر الممرض بتركيزات مختلفة (١٠٠ ، ٢٠٠ ، ٣٠٠ ، ٤٠٠ جزء في المليون) وكان المنجنيز أكثرها تأثيراً على إعاقه نمو الفطر في الاطباق يليه الزنك ثم النحاس.
 - 3- كذلك معملياً تم اختبار تضاد ٣ عزلات من البكتريا *P. fluorescense* على الفطر وكانت العزلة Pf2 أكثر العزلات البكتيرية المختبرة تأثيراً على النمو الفطري. وأكثرها تثبيطاً لنمو الفطر .
 - 4- في إختبارات الصوبة أدت لمعاملة بالعناصر الصغرى منفردة إلى خفض معنوي لشدة الإصابة وكان المنجنيز أكثر العناصر فاعلية مقارنة بالنحاس والزنك، وكذلك أدت المعاملة بالبكتريا إلى مقاومة متوسطة للإصابة بالمرض بينما أدت المعاملة بالبكتريا مع المعاملة بالعناصر المعدنية إلى تحسين معنوي في تحسين كفاءة البكتريا *P. fluorescense* في مقاومة مرض عفن الساق مقارنة باستخدام البكتريا منفردة أو استخدام العناصر الغذائية كل على حده.
 - 5- تم تقدير العناصر الصغرى في النباتات المعاملة بالبكتريا وكذلك بالنباتات غير المعاملة وتبين أن محتوى أوراق النباتات من عنصرى النحاس والزنك قد ازداد في النباتات المعاملة بالبكتريا زيادة معنوية في موسمي الدراسة مقارنة بعنصر المنجنيز .

قام بتحكيم البحث

كلية الزراعة - جامعة المنصورة
مركز البحوث الزراعية

أ.د / محمد الششتاوى عبد ربه
أ.د / وسام الدين اسماعيل على صابر