

BIOCONTROL OF MACHROPHOMINA PHASEOLINA BY *Bacillus* AND *Pseudomanas cepacia* ON SOYBEAN SEEDLINGS.

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ABSTRACT

Two isolates of *B. subtilis* and one of *P. cepacia* were tested for their antagonistic effect to control *M. phaseolina*. For measuring the bioagent actively the fungicide benlate was used as a standard. The *in vitro* tests showed that *B. subtilis* weakly inhibited the growth of *M. phaseolina*. while *P. cepacia* strongly inhibited its growth. However *in vivo* studies proved that *B. subtilis* isolate No.1 and that isolate of *P. cepacia* effectively controlled soybean root rot caused by *M. Phaseolina*. While *P. cepacia* strongly inhibited its growth. The tested fungicide gave the similar results as the pre-emergence of seedlings was increased. The tested bioagents were more effective than the fungicide in decreasing root rot disease incidence and increasing the number of survival seedling. *B. subtilis* isolate No. 1 gave the best results when it was used as seed treatment while *P. ecepacia* was very effective as a soil drench and seed treatment.

INTRODUCTION

Nowadays soybean grow in many parts of the world and are important source of legume oil and protein and for its low cost as well as nutritional balance (Abdelal *et al*, 1984 & Mahrous and Ibrahim, 1984). As a result of increasing cultivated area ; soybeen diseases have increased in number and severity. In Egypt Bekhit (1957) described charcoal rot disease caused by *Macrophomina phaseolina* which also attacks lupine, sesame and sunflower.

The causal organisms of root rot and damping off disease of soybean in Egypt include *Fusarium solani*, *Rhizoctonia solani*, *M. phaseolina* and *Pythium ultimum* (Abdel Aziz, 1970 and Fadl and Hessien, 1978).

Biological control differs fundamentally from conventional chemical control of plant pathogens. Bioprotectant can grow and colonize plant parts such as root systems and protect the entire root system from soil born land pathogens (Harman *et al*, 1989).

Several plant pathogenic fungi were controlled using *Bacillus subtilis* as a seed treatment. The antagonistic mechanism of *B. subtilis* can be attributed to antibiotic production (Loeffler *et al* 1986). Cubeta *et al*, (1985) studied the interaction between *B. subtilis* and 26 fungi associated with soybean seeds. They found that autoclaved culter filtrates of *B. subtilis* inhibited growth and stroma formation of *phomopsis sp*. They used suspensions of *B. subtilis* as seed treatment to reduce soybean stem infection caused by *phomopsis sp*. Siddiqui and Mahmood (1995) studied the biocontrol effect of *B.subtilis* alone or in combination with the fungal filtrates of *Aspergillus niger*, *Curvularia tuberculata* and *Penicillium*

coriophilium as seed treatments for the management of chickpea root rot disease caused by *M. phaseolina* and *Meloidigyne incognita* race 3. In general treatments of tested agents alone or in combination reduced nematode multiplication, galling formation root rot index.

Campo et al, (1994) found that *B. subtilis* and *Pseudomonas. cepacia* applied as seed treatment were able to inhibit the growth of *M. phaseolina* and reduced the disease incidence on bean seedlings. Sanchez et al, (1994a) reported that seed treatment of *Phaseolus vulgaris* with *P. cepacia* strongly antagonist the growth of *M. phaseolina*.

This work was carried out to study the antagonistic effect of two isolates of *B. subtilis* and one of *P. cepacia* to control *M. phaseolina* the causal of damping off and root rot of soybean seedlings , The fungicide benlate was used as standard.

MATERIALS AND METHODS

1- Source of isolates:

M. phaseolina was isolated from soybean seeds (Clark variety) obtained from Field Crop Inst. Res. (A.R.C). The suppressive bacteria *B. subtilis* (Eherenberg) Cohn isolate No.1 was previously isolated (Hassanein and EL Goorani , 1991). *B. subtilis* isolate No.2 was isolated from soybean seeds during the present work.

An isolate of *P. cepacia* (*Burkholderia cepacia*) (ex. Burkholer) NCSU 5.5 (ATCC 55344) was obtained from plant Pathology Department, North Carolina State University. Culture Colletion (Hassanein 1997). Pure cultures were maintained on glycerol agar medium (GAM 5g. peptone, 5g beaf extract, 20 ml glycerol, 15 g a gar and 1000 ml water) at 27° C.

II- Pathogenicity :

M. Phaseolina was grown for 2 weeks at 25°C in 500 ml/bottles containing autoclaved sand barley medium (25g clean sand, 75g barley grains and enough water to cover the mixture). For soil infestation the culture contents of the bottles (2 weeks old) were thoroughly mixed with sterilized soil at the rate of 4% of soil weight then filled in previously sterilized plastic pots (20 cm) at the rate of one kg/pot . Four replicates were used. The infested soil was left for seven days to secure establishment of the inoculated isolate, then sown with soybean seeds (Cv clark) strelized with 25% clorax Ten seeds were sown in each pot. The plants were weekly inspected for 30 days.

III- In vitro assays:

The bacterial isolates of *B. subtilis* and *P. cepacia* were tested in the present work for their possible antagonistic effect against *M. phaseolina*. Inocula of the antagonistic bacteria were streaked in a single line on the surface edge of glycerol agar medium and some of the plates were then incubated at 27°c for 2 days. Incula of *M. phaseolina* (5 mm in diameter) which previously grow on PDA medium were placed at the centre of the same

plates containing antagonistic bacterial growth. Another set of GAM were streaked with the tested bacterial isolates and inoculated with the pathogenic fungus at the same time. Plates containing GAM inoculated with the tested fungus were served as control. All treatments were incubated at 27°C for 7 days. While the growth of *M. phaseolina* covered the surface of control plates, the degree of the growth inhibition of the fungus was estimated according to their growth extension in the vicinity of *B. subtilis* and or *P. cepacia* growth. Four replicates were prepared for each treatment.

IV- In vivo test:

Cell suspensions of *B. subtilis* and *P. cepacia* isolates were prepared by growing the organism in Petri dishes on GAM and incubated at 27°C for 3 days. The resulting growth was washed from the plates with sterile water. Concentration of viable cells in the preparation was adjusted to be 10^8 – 10^9 colony – forming units (CFU) per milliliter (Baker 1962).

The tested fungus was grown for 2 weeks at 27°C in bottles (500 ml), containing autoclaved sand barley medium. Soil was inoculated with *M. phaseolina* as previously mentioned. Another set of pots was left without inoculation to serve as a control. At the same time suspensions of the antagonistic bacteria were added as soil drench at the rate of 100 ml/pot. The treated pots were moistened and mixed thoroughly every other day for one week to ensure even growth and distribution of the inoculated fungus and bacteria. Control consisted of two treatments in the first the soil was mixed with sterilized sand barley medium, and in the second treatment the soil was mixed with inoculated barley medium with the tested fungus.

In all treatments the soybean seeds were surface sterilized with 25% chlorax solution for 2 minutes and washed several times with sterile water. After that soybean seeds were left with bacterial suspensions for 30 minutes to be fully covered with such bacteria and dried at laminar flow. Arabic gum was used as adhesive agent. In a control treatment the fungicide benlate at the rate of 3g/kg seeds was used as comparative control treatment. Ten seeds were sown in each pot and a set of 4 pots was used for each treatment. Disease incidence was recorded 10 days after sowing for pre-emergence damping off and after 30 days for post-emergence and survivors. The obtained results were statistically analyzed according to Snedecor and Cochran (1967).

RESULTS

Pathogenicity test:

Pathogenicity of the tested fungus against soybean Clark variety showed that the pre-emergence damping off was increased than rot root disease.

In vitro test :

Results obtained during the *in vitro* studies revealed that the two tested isolates of *B. subtilis* weakly inhibited the growth of *M. phaseolina* (inhibition zone was 4 mm at the middle of the plate after 7 days from the

inoculation of fungus and bacteria. Isolate of *P. cepacia* strongly inhibited the growth with *M. phaseolina*. The inhibition zone was 22mm at the middle of the plate and 30mm at the edge of the fungal growth, when the inoculation of the fungus was after 2 days from the inoculation of bacteria . When the inoculation of fungus and bacteria was at the same time, the inhibition zone was 12mm (fig 1).

The in vivo test:

Results obtained from the *in vivo* test were presented in table 1. It is clear that any of the tested bacteria gave a good control to *M. phaseolina* as they reduced the pre-emergence damping off of soybean seedlings. *B subtilis* isolate No.1 and *P.cepacia* gave the same result in controlling soybean damping off.

Table (1):The antagonistic effects of two isolates of *B.subtilis* and one of *P.cepacia* against *M. phaseolina* on soybean seeding (*)

Treatment	Pre – emergence %	Post – emergence %	Survival - seedlings %
Benlate (benomyl)	16.66 (26.6) ** C	10 (18.4) ab	73.33 (59) bC
<i>Basillus subtilis</i> isolate No.1	20 (26.6) bC	6.66 (13.1) bC	73.33 (59) bC
<i>B .subtilis</i> isolate No.2	23.33 (28.8) bC	10 (18.4) ba	66.66 (54.8) bC
<i>Pseudomonas cepacia</i>	16.66 (23.86) C	6.66 (13.1) bC	76.66 (61.73) b
<i>Macrophomina phaseolina</i>	36.66 (37.2) a	20 (26.6) a	43.33 (41.13) d
Soil drensh			
<i>M.phaseolina</i> + <i>B.subtilis</i> 1	20 (26.6) bC	3.33 (7.8) C	76.66 (61.2) b
<i>M.phaseolina</i> + <i>B.subtilis</i> 2	26.6 (31) b	10 (18.4) ab	63.33 (52.8) c
<i>M.phaseolina</i> + <i>P.cepacia</i>	20 (26.6) bC	3.33 (7.8) C	76.66 (61.2) b
Sead treatment			
<i>M.phaseolina</i> + <i>B.subtilis</i> 1	10 (18.4) d	3.33 (7.8) c	86.66 (68.86) a
<i>M.phaseolina</i> + <i>B.subtilis</i> 2	16.6 (23.86) C	13.33 (21.13) ab	70 (57) bC
<i>M.phaseolina</i> + <i>P.cepacia</i>	10 (18.4) d	3.33 (7.8) C	86.66 (68.86) a
Uninfsted control	26.66 (31) b	10 (18.4) ab	63.33 (52.8) c
L . S . D . 5 %	5.31	10.6	7.56

- Mean of four replicates (*)
- Values between brackets are the angular transformation (**)
- Values with similar letters indicate none significant values . (***)

Fig. (1) : Antagonistic effect of *P.cepacia* on *M. phaseolina* grown on glycerol agar medium .
Left plate , the inoculation with the fungus was after 2 days from the inoculation of bacteria .
Right plate , the inoculation with fungus and bacteria was at the same time .

The percentage of pre-emerge damping off was reduced from 36.66% in infested pots untreated with bacterial suspension to 20%, when the bacteria were used as soil drench.

Seed treatment with *B. subtilis* isolate No1, *P. cepacia* and the fungicide benlate gave better results to control *M. phaseolina*. They reduced the percentage of pre-emergence damping off from 36.66% in infested pots sowed with untreated soybean seeds to 10% and 16.66% in pots sowed with seeds treated with *B. subtilis* isolate No. 1 *P. cepacia* and benlate respectively.

The post emergence root rot results revealed that the use of bacteria as soil drench gave a good control to *M. phaseolina*. *B. subtilis* isolate No.1 and *P. cepacia* reduced the percentage of the post emergence root rot from 20 in pots sowed with untreated seeds to 3.33 in pots treated with either *B. subtilis* isolate No.1 or *P. cepacia*.

All the tested treatment increased the survival seedlings compared with the infested control with *M. phaseolina*. The bioagents *P. cepacia* and *B.*

subtilis isolate No.1 as soil drench or seed treatment increased the survival seedlings from 43.33 to 76.66 and 86.66% respectively.

Benlate gave similar results to those obtained in case of *B. subtilis* isolate No.2 when it is used as seed treatment (the percentage of survival seedlings were 73.33 and 70%, respectively.)

On the other hand no undesired effect on soybean seedlings was observed with duce the tested isolate of *B. subtilis* and *P. cepacia*. Meanwhile significant enhancement of soybean survival seedlings was detected.

DISCUSSION

Several plant pathogenic fungi are controlled by using *B. subtilis* and *P. cepacia* as a seed treatment. The antagonistic mechanism of bacteria has taken place through antibiotic production and nutrient competition (Utkhede and Rahe 1980, Cubeta et al, 1985, Tsuchiya et al, 1995).

In vitro test showed that the antagonistic behavior of the two tested isolates of *B. subtilis* is in agreement with the result obtained by Cubeta et al (1985) who reported that several plant pathogenic fungi isolated from soybean seedlings were controlled by using *B. subtilis*. They also reported that the ability of micro organism to produce antibiotic depends on the selection of nutrient media.

The strong inhibition of *M. phaseolina* by *P. cepacia* showed during the present work is similar to that reported by Sanchez et al (1984 b). They found that *P. cepacia* strain UPR 5C strongly antagonized the growth of *M. phaseolina* isolated from rotted roots of bean seedlings. Hassanien (1996) mentioned that *P. cepacia* had an antagonistic effect against *P. solnacearum* in *in vitro* studies.

The *in vivo* studies showed that *B. subtilis* significantly decreased the pathogenic effect of *M. phaseolina* on soybean seedlings. *B. subtilis* isolate No.1 was more antagonistic against *M. phaseolina* than *B. subtilis* isolate No.2. Similar results were reported by Osman et al (1986). They studied the biological control of lupin wilt caused by *Fusarium oxysporum* F.sp. *lupini* and found that the lowest percentage of wilt incidence was that when *B. subtilis* isolates VII, and IX and *Trichoderma harzianum*, were added to soils infested with *F.oxysporum* F.sp.*lupini*. Loeffler et al (1986) reported that *B. subtilis* produces different antibiotics namely: subtilin, bacillin, bacillomycin, subtenolin, mycosutenolin, toxymycin, bacitracin, xantobacidin iturin, subtilosin A and baclycin. According to Loeffler et al. (1986) the autheres suggest that the antagonistic effect of *B. subtilis* may be due to induction of one or more of the above mentioned antibiotics or others.

In vivo tests showed that *P. cepacia* had high antagonistic effect against *M. phaseolina* when it is used as seed treatment or soil drench. Similar results were obtained by other researchers. Campo et al(1994) found that the growth of *M. phaseolina* was inhibited by *B. subtilis* and *P. cepacia*. The disease incidence on bean seedlings was also decreased when these bacteria were applied as seed treatment. Homma et al, (1989) proved that *P.*

cepacia produced different antibiotics which showed high antifungal activity and less antibacterial activity.

The fungicide benlate gave a good result to control *M. phaseolina* but its effect is less than these of the tested bacterial biogents. These results are in agreement with those found by Whahid *et al* (1995). They reported that benlate improved germination of soybean seeds and gave a good control to the three *Fusarium* sp.

These results may prove the useful effect for using certain bacteria as biocontrol agents to reduce the soil pollution with fungicides. Field studies are required to confirm the present results.

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المقاومة الحيوية للفطر *Macrophomina phaseolina* باستخدام البكتريا *Bacillus subtilis* و *Pseudomonas* وذلك فى بادرات فول الصويا. نادية الصفوانى ، فريال حسنين و محمد رفعت رسمى معهد بحوث أمراض النبات ، مركز البحوث الزراعية ، محطة بحوث الصباحية بالأسكندرية.

استخدم فى هذه الدراسة عزلتين من البكتريا *B. subtilis* وعزلة واحدة من البكتريا *P. cepcia* وذلك لأختبار تأثيرهما الحيوى فى مقاومة الفطر *M. phaseolina* وقد أستخدم الفطر بنليت للمقارنة . وأظهرت الدراسات المعملية أن العزلتين المستخدمتين من *B. subtilis* وكان تأثيرهما ضعيفاً فى تثبيط نمو الفطر المختبر *M. phaseolina* بينما كان لعزلة البكتريا *P. cepcia* تحت ظروف المعمل تأثير قوى فى تثبيط نمو الفطر. الدراسة فى الأصص تحت ظروف الصوبة أثبتت أن العزلة الأولى رقم (1) من البكتريا *B. subtilis* وعزلة *P. cepcia* إستطاعا مقاومة تأثير الفطر *M. phaseolina* فى بادرات فول الصويا . المبيد الفطرى بنليت ساعد على خفض نسبة الإصابة بسقوط البادرات وزيادة نسبة إنبات فول الصويا ولكن البكتريا المستخدمة للمقاومة الحيوية كان تأثيرها أكبر فى تقليل الإصابة بأعفان الجذور وزيادة عدد البادرات السليمة . العزلة رقم (1) من *B. subtilis* أعطت أحسن النتائج عند إستخدامها فى معاملة بذور فول الصويا ولكن عزلة البكتريا *P. cepcia* أعطت أحسن النتائج فى الحالين عند إستخدامها كمعلق لمعاملة التربة وأيضاً عند إستخدامها فى معاملة البذور.