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# EFFICACY OF *GLOMUS AGGREGATUM* AND *BACILLUS SUBTILIS* AS BIOCONTROL AGENTS FOR REDUCING FUSARIUM ROOT-ROT IN SOYBEAN PLANTS

**MIKHAEEL F. T.[1], FATMA A. SHERIEF[2], R. Y. RIZK[2] AND F. M. ABDALLA[2]**

*1 Agric. Microbiol. Res. Dept.*

*2 Soil Microbiol. Lab., Sakha Agric. Res. Station.*

*Soils, Water & Environ. Res. Inst., ARC, Giza, Egypt*

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***Abstract***

A mycorrhizal fungus *Glomus aggregatum* (VAM), and a bacterial isolate *Bacillus subtilis* (Bs), were evaluated individually or in combination with *Bradyrhizobium japonicum* (Rh) as biocontrol agents for reducing root-rot disease caused by *fusarium oxysporum* (F.O.) in soybean plants, under greenhouse conditions. In non-inoculated (control) plants, infestation of soils with F.O. drastically reduced plant growth parameters compared to non-infested ones. However, the detrimental effects caused by F.O. infestation were less detected in VAM and/or Bs inoculated treatments, being the least pronounced in the VAM+Bs treatment.

In plants inoculated with *B. japonicum*, fusarium infestation resulted in variable decreases in number and dry weight of nodules, plant growth and seed yield. However, rhizobial inoculated-plants could withstand the stress of fusarium infestation when biologically controlled with *G. aggregatum* and/or *B. subtilis*. Results pointed out that the observed prophylactic effects of mycorrhizal inoculation was not only related with plant nutrition, but also related with reduction of disease severity.

## INTRODUCTION

Incited root-rot disease by *Fusarium* spp. is considered a menace to soybean cultivars at different areas in Egypt. Several fungicides are traditionally used as a fungal diseases control may cause environmental pollution. In addition, some of these chemicals disrupt symbiosis occuring between plants and $N_2$-fixing bacteria. Michael and David (1990) mentioned that, legume-Rhizobium relationship is not exempted from the effects of agricultural chemicals.

The real need for controlling plant diseases, together with eliminating the risk of fungicides application without hindering the biological $N_2$-fixation, have prompted the search for biological alternatives.

Mycorrhizal inoculation has been explored as an alternative agent used to reduce the incidence of root diseases. St-Arnaud *et al.*, (1997) concluded that, mycorrhizal inoculation with *Glomus intraradices* clearly reduced symptoms caused by *Fusarium oxysporum* in the non-VAM species *Dianthus caryophyllus* when co-cultured with VAM species *Tagetes patula.* Recently, Filion *et al.* (1999) used an in vitro system which permitted the isolation of soluble substances released by the extraradical mycelium of mycorrhizal fungus *Glomus intraradices.* They found that, conidial germination of *F. oxysporum* f. sp. *chrysanthemi* was reduced in the presence of these substances.

For using bacteria as a biocontrol agent, Sharga and Lyon (1998) summarized that, *Bacillus subtilis* BS 107 showed antagonism against a broad spectrum of bacterial and fungal species.

The main goal of the current study was to evaluate the validity of inoculation with *Glomus aggregatum* and *Bacillus subtilis* either individually or in combination as an integrated system for controlling root-rot disease caused by *F. oxysporum,* as well as their effects on biological $N_2$-fixation in soybean plants.

## MATERIALS AND METHODS

### Soil

A clay loam soil collected from Sakha Agric. Res. Station, Agric. Res. Center at Kafr El-Sheikh, Egypt, was used in this study. The soil has a pH of 7.8; total nitrogen, 0.12%; total phosphorus, 0.0093%; organic carbon, 0.81%, C/N ratio, 6.8. The soil was air dried, passed through a 2-mm sieve and distributed into earthenware pots (35 x 45 cm) at the rate of 6 kg $pot^{-1}$.

### Seeds

Soybean seeds (*Glycine max L. c.v. crowford*) were obtained from Legume Crops Section, ARC, Giza. Seeds were sown in the prepared pots, then thinned to three seedlings per pot after 7 days of germination.

## Rhizobial inoculum

Two active *Bradyrhizobium japonicum* strains namely 110 and Sb6 obtained from Biological Nitrogen Fixation Unit, ARC, Sakha, Kafr El-Sheikh, were used. Cultures were grown in yeast extract mannitol (YEM) broth (Vincent, 1970) on a rotary shaker at 25 °C for 7 days. Inoculation with rhizobia was done one week after seed germination by pippetting 2 ml of mixed culture containing 1.5 x $10^7$ cells $ml^{-1}$ around the base of each seedling. Uninoculated treatments were received rhizobia-free medium.

## VA-mycorrhizal inoculum

The mycorrhizal fungus was *Glomus aggregatum* (Schenck and Smith emend. Koske), was obtained from Dr. Habte. M., Dept. of Agron. and Soil Science, Univ. of Hawaii, Honolulu, USA. It was propagated on *Sorghum bicolor* in sterilized pot culture for 3 months prior to the start of the experiment. Soil from 12 week-old pot culture was used as a crude inoculum. The mycorrhizal propagule density was determined by MPN technique (Daniels and Skipper, 1982). For mycorrhizal inoculation, portions of 50 g crude inoculum containing 500-700 viable propagules were placed 2-4 cm below the soil surface in each pot before seed sowing. Uninoculated pots were received a filtrate of soil inoculum free from VAM propagules.

## *Bacillus subtilis*

*Bacillus subtilis* strain was obtained from Plant Pathology Research Institute, Agric. Res. Center, Giza. It was maintained on nutrient agar (Difco) at 6 °C. The culture was grown on fresh medium composed of peptone, 5.0; Beef extract, 3.0; Yeast extract, 1.0; Glucose, 5.0 g/L of distilled water and incubated on rotary shaker (120 r.p.m) at 28 °C for 3 days. Then, the growth was harvested by centrifugation (2000 r.p.m) and it was suspended under aseptic conditions in sterile distilled water, 2 ml aliquots of bacterial suspension (1.8 x $10^6$ cells $ml^{-1}$) was added over each seed at sowing.

## *Fusarium oxysporum*

The fungus was originally isolated from wilted soybean plants, kindly provide by Dept. of Legume Diseases, ARC., Giza. Spore inoculum was prepared by growing *F. oxy-*

*sporum* on potato dextrose agar (PDA) plates for 15 days at 25 °C, and the spores were harvested in sterile distilled water. The suspension was containing 0.5 x $10^6$ spore $ml^{-1}$. Soil infestation was done by adding 100 ml of a spore suspension in each pot 2 weeks after seed germination.

## Mineral fertilization

The mineral fertilization treatment (M f) was carried out by adding urea (46% N) at the rate of 60 kg N per feddan and superphosphate (15.5% $P_2O_5$) and potassium sulphate (48% $K_2O$) at the rates of 60 kg and 50 kg per feddan, respectively.

There were nine treatments as follows:

1. uninoculated soil [control C],
2. uninoculated and fertilized with NPK fertilizers (Mf),
3. inoculated with a mixture of *B. japonicum* strains 110 and Sb6 (Rh),
4. inoculated with mycorrhizal fungus *G. aggregatum* (VAM),
5. inoculated with *B. subtilis* (Bs),
6. inoculated with (Rh+VAM),
7. inoculated with (Rh+Bs),
8. inoculated with (VAM+Bs),
9. inoculated with (Rh+VAM+Bs).

Each of the nine treatments was either infested with the pathogenic fungus *F. oxysporum* or remained without infestation. Pots were arranged in a greenhouse of 25-35 °C and watered when needed. The experimental design was a completely randomized block with nine replicates for each treatment.

After 60 and 90 days of planting, 3 replicates from each treatment were taken. Number and dry weight of nodules as well as dry weight of shoots were recorded. Nitrogen and phosphorus contents of plant shoots and seeds were determined according to Chapman and Pratt (1961). Mycorrhizal colonization ratios of soybean roots were estimated after staining (Phillips and Hayman, 1970). At the end of the experiment the seed yield was recorded. Data were statistically analyzed by the least significant difference according to Snedecor and Cochran, (1980).

# RESULTS AND DISCUSSION

## Number and dry weight of nodules

Data of number and dry weight of nodules are shown in Table (1). Plants grown in soil infested with *F. oxysporum* and inoculated with *B. japonicum* gave the lowest number of nodules with average reduction of 62.3 and 56.9% after 60 and 90 days of sowing when compared with non-infested ones. Such reduction in nodule number could be explained by the detrimental effect of the pathogen (*F. oxysporum*) with the survival of rhizobia in the rhizosphere and nodule formation. These results confirmed the observations recorded by Ghobrial *et al.* (1996).

Table 1. Number and dry weight of nodules on soybean roots grown in soil infested or non-infested with *Fusarium oxysporum.* (Values per pot)

| Treatments* | No. of nodules | | Dry weight of nodules (mg) | | No. of nodules | | Dry weight of nodules (mg) | |
|---|---|---|---|---|---|---|---|---|
| | *Fusarium* infestation | | | | | | | |
| | - | + | - | + | - | + | - | + |
| | 60 days ** | | | | 90 days ** | | | |
| C | - | - | - | - | - | - | - | - |
| Mf | - | - | - | - | - | - | - | - |
| Rh | 40.67 | 15.33 | 163.44 | 55.19 | 58.00 | 25.00 | 540.33 | 120.35 |
| VAM | - | - | - | - | - | - | - | - |
| Bs | - | - | - | - | - | - | - | - |
| Rh+VAM | 47.33 | 43.00 | 190.00 | 157.00 | 71.67 | 62.33 | 660.00 | 556.60 |
| Rh+Bs | 44.33 | 39.00 | 176.60 | 144.70 | 62.00 | 54.33 | 562.00 | 500.00 |
| VAM+Bs | - | - | - | - | - | - | - | - |
| Rh+VAM+Bs | 48.67 | 44.32 | 210.00 | 175.00 | 81.70 | 75.00 | 690.00 | 623.34 |
| LSD at 0.05 | NS | | NS | | 4.68 | | 33.15 | |
| 0.01 | NS | | NS | | 6.50 | | 46.01 | |

* C, control; Mf, mineral NPK fertilization; Rh, *Bradyrhizobium japonicum;* VAM, mycorrhizal fungus *Glomus aggregatum;* Bs, *Bacillus subtilis.*

** Days after sowing NS, non-significant.

When *F. oxysporum* was biologically controlled by inoculating the soil with mycorrhizal fungus *G. aggregatum* and/or bacterial isolate *B. subtilis*, the negative effect caused by *F. oxysporum* infestation on nodule formation was less detected particularly in Rh+VAM+Bs treatment, the average reduction was 8.9 and 8.2% after 60 and 90 days of sowing, respectively, compared with non-infested ones. The aforementioned trend holds true for nodule dry weights (Table 1). It was found that higher dry weights of nodules were recorded by plants grown under Rh+VAM+Bs treatment either Fusarium-infested or non-infested, the average values of this treatment were 210.0 and 175.0 mg/pot after 60 days of sowing and 690.0 and 623.34 mg/pot after 90 days of sowing for Fusarium non-infested and infested pots, respectively. In this respect, Dar *et al.* (1997) indicated that, inoculation with VAM fungi in the presence of the pathogens reduced the detrimental effect of the pathogens on nodule formation.

## *VA-mycorrhizal* colonization ratios

Data in Table (2) showed that, soybean roots either inoculated or non-inoculated with *G. aggregatum* gave variable ratios of VAM colonization being more pronounced with VAM-inoculated treatments. These results confirmed the observation estimated by Tinker (1980) who reported that, the local mycorrhizae may be relatively ineffective and/or insufficient to give a useful level of infection. Infestation of soil with *F. oxysporum* resulted in a variable effect on mycorrhizal colonization ratios. For treatments inoculated with mycorrhizal fungus (*G aggregatum*) the VAM colonization levels were not affected by Fusarium infestation (St-Arnaud *et al.*, 1997). However, mycorrhizal colonization ratios in plants colonized with native VAM fungi were significantly reduced due to Fusarium infestation except *B. subtilis* inoculated treatment in which VAM colonization ratios were non-significantly affected by Fusarium infestation. This might be due to the antagonistic effect of *B. subtilis* against *F. oxysporum.* Sharga and Lyon (1998) found that *B. subtilis* BS 107 showed antagonism against many organisms including *Fusarium* spp.

## Plant growth

Data of shoot dry weights, nitrogen and phosphorus contents of soybean plants are shown in Tables (3) and (4). Infestation of soil with *F. oxysporum* resulted in variable decreases in plant growth parameters. However, the detrimental effect due to fusarium infestation was less detected in *G. aggregatum* and/or *B. subtilis* inoculated treatments after 60 and 90 days of planting.

In the control treatment, *F. oxysporum* drastically reduced plant growth compared to non-infested ones. Decreases in plant growth parameters caused by *F. oxysporum* infestation were 33.8, 28.9 and 52.4% after 60 days of planting and were 40.2, 45.6 and 42.9% after 90 days of planting for shoot dry weight, N and P contents, respectively.

Table 2. VA-mycorrhizal colonization ratios in soybean roots grown in soil infested or non-infested with *Fusarium oxysporum.*

| Treatments | *Fusarium* infestation | | | |
|---|---|---|---|---|
| | - | + | - | + |
| | 60 days | | 90 days | |
| C | 16.4 | 10.8 | 18.6 | 12.0 |
| Mf | 9.8 | 10.7 | 12.6 | 6.5 |
| Rh | 20.5 | 12.8 | 26.3 | 21.4 |
| VAM | 41.8 | 42.0 | 56.3 | 52.2 |
| Bs | 19.9 | 16.8 | 24.8 | 21.2 |
| Rh+VAM | 52.0 | 50.9 | 70.1 | 67.5 |
| Rh+Bs | 22.6 | 19.4 | 33.6 | 30.1 |
| VAM+Bs | 45.7 | 44.9 | 68.3 | 68.7 |
| Rh+VAM+Bs | 66.2 | 64.6 | 80.4 | 76.8 |
| LSD 0.05 | 5.3 | | 4.6 | |
| 0.01 | 7.1 | | 6.1 | |

Generally, non significant differences in plant growth parameters due to pathogen infestation were detected in soybean plants inoculated with *G. aggregatum* and/or *B. subtilis*. Decreases in shoot dry weights due to Fusarium infestation were 8.4, 4.5 and 2.3% after 60 days of planting and 9.6, 9.2 and 4.6% after 90 days of planting for VAM, Bs and VAM+Bs treatments, respectively. The same trend was observed with N and P contents of soybean shoots (Table 4). The obtained results indicated that, efficiency of the combined mixture of both *G. aggregatum* and *B. subtilis* to reduce detrimental effect was more effective than the individual ones. In this respect, Duponnois and Garbaye (1992) found that *B. subtilis* increased the percentage of mycorrhizal infection of Douglas fir seedlings colonized with ectomycorrhizal fungus *Laccaria laccata.*

Table 3. Shoot dry weight (g $pot^{-1}$) of soybean plants grown in soil infested or non-infested with *Fusarium oxysporum*.

| Treatments | *Fusarium* infestation | | | |
|---|---|---|---|---|
| | - | + | - | + |
| | 60 days | | 90 days | |
| C | 14.01 | 9.27 | 17.75 | 10.62 |
| Mf | 21.74 | 12.33 | 28.67 | 14.95 |
| Rh | 23.34 | 11.50 | 29.67 | 12.71 |
| VAM | 19.65 | 18.00 | 24.70 | 22.32 |
| Bs | 17.21 | 16.44 | 24.88 | 22.60 |
| Rh+VAM | 25.22 | 23.45 | 31.94 | 29.14 |
| Rh+Bs | 23.11 | 22.00 | 28.93 | 25.25 |
| VAM+Bs | 22.00 | 21.50 | 26.61 | 25.37 |
| Rh+VAM+Bs | 27.11 | 26.23 | 35.46 | 33.42 |
| LSD 0.05 | 1.82 | | 2.80 | |
| 0.01 | 2.44 | | 3.75 | |

Table 4. Nitrogen (N) and Phosphorus (P) contents (g $pot^{-1}$) in soybean plants grown in soil infested or non-infested with *Fusarium oxysporum*.

| Treatments | N | | P | | N | | P | |
|---|---|---|---|---|---|---|---|---|
| | *Fusarium* infestation | | | | | | | |
| | - | + | - | + | - | + | - | + |
| | 60 days | | | | 90 days | | | |
| C | 0.214 | 0.152 | 0.021 | 0.010 | 0.364 | 0.198 | 0.049 | 0.028 |
| Mf | 0.398 | 0.218 | 0.026 | 0.019 | 0.640 | 0.299 | 0.071 | 0.035 |
| Rh | 0.457 | 0.170 | 0.028 | 0.013 | 0.682 | 0.297 | 0.086 | 0.037 |
| VAM | 0.327 | 0.291 | 0.032 | 0.030 | 0.527 | 0.480 | 0.098 | 0.084 |
| Bs | 0.282 | 0.245 | 0.027 | 0.025 | 0.428 | 0.386 | 0.076 | 0.063 |
| Rh+VAM | 0.557 | 0.520 | 0.041 | 0.038 | 0.707 | 0.658 | 0.102 | 0.087 |
| Rh+Bs | 0.447 | 0.397 | 0.034 | 0.033 | 0.667 | 0.627 | 0.093 | 0.080 |
| VAM+Bs | 0.388 | 0.353 | 0.039 | 0.037 | 0.546 | 0.509 | 0.103 | 0.094 |
| Rh+VAM+Bs | 0.667 | 0.632 | 0.051 | 0.049 | 0.858 | 0.811 | 0.122 | 0.107 |
| LSD 0.05 | 0.038 | | 0.003 | | 0.049 | | NS | |
| 0.01 | 0.051 | | 0.004 | | 0.065 | | NS | |

With regard to rhizobial inoculated treatments, plants received *B. japonicum* and grown in Fusarium infested soil showed decreases in dry weight, N and P contents compared with rhizobial treatments of non-infested soil. Thus the loss in dry matter due to pathogen infestation reached to 50.7 and 57.2% after 60 and 90 days of planting, respectively. Similarly, N and P contents of shoots were decreased (Table 4). Therefore, the obtained results revealed that weak growth of soybeans was not only a direct effect of soil-borne pathogen, but could be attributed to poor nodulation. These results were found to be in agreement with those reported by El-Bahrawy (1983) and Ghobrial *et al.* (1996). On the other hand, rhizobial plants which inoculated with *G. aggregatum* and/or *B. subtilis* could withstand the stress of Fusarium infestation better than those inoculated with *B. japonicum* only. However, protection of soybean plants against *F. oxysporum* was observed in Rh+VAM+B treatment. Decreases in plant growth parameters due to fusarium infestation in this treatment were 3.2, 5.2 and 3.9% after 60 days of planting, and 5.7, 5.5 and 12.3% after 90 days of planting for dry matter yield, N and P contents, respectively.

## Seed yield

Highly significant decreases of yield, N and P contents of seeds (42.2, 50.7 and 54.2%, respectively) were recorded with *Fusarium* control plants compared to non-infested plants (Table 5). Increases of seed yield, N and P contents were obtained when *Fusarium* infested plants were inoculated with *G. aggregatum* and/or *B. subtilis*. These treatments enhanced rhizobial inoculated plants whereas more pronounced increases were recorded with Rh+VAM+Bs treatment. Increases in seed yield, N and P contents of soybean seeds in this treatment were 52.2, 53.1 and 53.8%, respectively, compared with rhizobial inoculated plants growing in *Fusarium* infested soil. The obtained results could confirm that inoculation with VAM fungi and/or *B. subtilis* had a pronounced effect on reducing the severity of the disease caused by *F. oxysporum.*

As for the mineral fertilization (Mf) treatment, in spite of plant growth improvement due to NPK additives the protecting effects against *Fusarium* infestation seemed minor. Therefore, a significant decrease in growth parameter of infested plants compared with non-infested ones was observed (Table 3). On the other hand, VAM inoculation with *G. aggregatum* could confer good protection against this pathogen. For instance, decreases in shoot dry weights due to *Fusarium* infestation in Mf treatment in comparison with VAM treatment were 43.3 vs 8.4% and 47.9 vs 9.6% after 60 and 90 days of sowing, respectively. According to these results, it could be suggested that sufficient NPK nutrition was not enough to reduce the damage caused by this pathogen. Therefore, the prophylactic effects observed for the mycorrhizal inoculated as compared with non-inoculated plants could not be solely attributed to phosphorus availability of the VAM plants. These results strongly support the observation of St-Arnaud *et al.* (1997).

Table 5. Seed yield, nitrogen and phosphorus contents (g pot$^{-1}$) in seeds of soybean plants grown in soil infested or non-infested with *Fusarium oxysporum*.

| Treatments | Seed yield | | N-content | | P-content | |
|---|---|---|---|---|---|---|
| | *Fusarium* infestation | | | | | |
| | - | + | - | + | - | + |
| C | 12.63 | 7.30 | 0.597 | 0.294 | 0.048 | 0.022 |
| Mf | 16.02 | 9.36 | 1.154 | 0.692 | 0.076 | 0.046 |
| Rh | 16.57 | 8.76 | 1.009 | 0.603 | 0.074 | 0.042 |
| VAM | 15.51 | 14.21 | 0.913 | 0.810 | 0.082 | 0.071 |
| Bs | 13.77 | 12.75 | 0.783 | 0.687 | 0.059 | 0.052 |
| Rh+VAM | 18.95 | 17.08 | 1.232 | 1.098 | 0.097 | 0.082 |
| Rh+Bs | 16.32 | 14.42 | 1.172 | 1.080 | 0.074 | 0.062 |
| VAM+Bs | 15.94 | 14.77 | 0.851 | 0.776 | 0.087 | 0.077 |
| Rh+VAM+Bs | 19.95 | 18.32 | 1.372 | 1.285 | 0.099 | 0.091 |
| LSD 0.05 | 3.82 | | 0.089 | | 0.013 | |
| 0.01 | 5.12 | | 0.120 | | 0.018 | |

Different hypotheses have been proposed to explain the possible effect of mycorrhizal fungi on plant disease such as stimulation of host plant disease–resistance mechanisms (Caron *et al.* 1986) and direct interaction between VAM fungi and soil microorganisms, which might lead to changes in microbial equilibrium detrimental to pathogens (Filion *et al.* 1999). Although it is not possible to support one hypothesis more than the other, our results pointed out that the reduction of disease severity was clearly not related to plant nutrition only.

More studies are needed in this subject for understanding complex interactions between VA-mycorrhizal fungi and plant pathogens.

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# كفاءة كل من *Bacillus subtilis*، *Glomus aggregatum* كعوامل مقاومة حيوية لمقاومة مرض تعفن الجذور الفيوزاريومى فى نباتات فول الصويا

فتحى توفيق ميخائيل[1]، فاطمة أحمد شريف[2]،
رزق يوسف رزق[2] وفكرى مرقص عبدالله[2]

١ قسم بحوث الميكروبيولوجيا الزراعية
٢ معمل ميكربيولوجيا الأراضى – محطة البحوث الزراعية بسخا
معهد بحوث الأراضى والمياه والبيئة – مركز البحوث الزراعية – الجيزة

فى تجربة أصص تم تقييم كل من فطر الميكورهيزا *G. aggregatum* والعزلة البكتيرية *B. subtilis* فى وجود أو عدم وجود بكتريا الريزوبيوم *B. japonicum* وذلك كعوامل مقاومة حيوية لمرض تعفن الجذور الفيوزاريومى فى نباتات فول الصويا، وقد دلت نتائج التجربة على الآتى:

١- بالنسبة لمعاملة الكنترول، أدت إصابة التربة بفطر الفيوزاريوم إلى نقص حاد فى كل مقاييس النمو المختلفة وذلك مقارنة بمثيلاتها والتى لم تلقح بالفطر.

٢- لوحظ ان الآثار الضارة لفطر الفيوزاريوم كانت اقل وضوحاً فى المعاملات الملقحة بكل من الميكورهيزا وبكتريا *B. subtilis* كما أظهرت المعاملة VAM+ Bs أقل نسبة اختزال فى نمو النبات الناتج عن العدوى بالفيوزاريوم.

٣- بالنسبة للنباتات الملقحة بالريزوبيا، أدت العدوى بالفيوزاريوم الى مستويات نقص مختلفة فى كل من العدد والوزن الجاف للعقد الجذرية وأيضا فى نمو النبات وامتصاص العناصر ومحصول البذور.

٤- لوحظ أن النباتات الملقحة بالريزوبيا فقط كانت أكثر تأثراً بالعدوى بالفيوزاريوم، وعلى العكس من ذلك فقد وجد أن النباتات الملقحة بالريزوبيا والتى لقحت ايضا بالميكورهيزا *G. aggregatum* أو بكتريا *B. subtilis* أو بكليهما، استطاعت ان تقاوم التأثيرات الضارة والناتجة عن العدوى بالفيوزاريوم.

٥- بينت نتائج هذه الدراسة أن التأثيرات الوقائية والتى لوحظت فى المعاملات الملقحة بالميكورهيزا، لم تكن فقط بسبب التحسن فى تغذية النبات.