Biocontrol of *Verticillium* wilt of Berseem by bacterization

Biocontrole de la verticilliose du Berseem par bacterisation

**EL AISSAMI A.**<sup>1</sup>, **BENTATA F.**<sup>2</sup> & **EL ALAOUI FARIS F. E.**<sup>1</sup>

<sup>1</sup>Laboratory of Botany, Mycology and Environment, Faculty of Sciences, Mohammed V-Agdal University, B.P. 1014. Avenue Ibn Battouta B, Rabat, Morocco.

<sup>2</sup>Breeding, Conservation and Valorisation of Phytopathogenic Resources Unit, Laboratory of Phytopathology, CRRAR,INRA, B.P 6356, Avenue Mohamed Belarbi Alaoui Rabat, Morocco.

**ABSTRACT**

*Verticillium* is a strong plant pathogenic fungus, causing severe wilt and death and average reductions in shoot and root growth in the survived plants. The control of this disease has become a priority in biological research. *Rhizobium* sp. strain showing *in vitro* the highest antagonistic activity against *Verticillium dahliae* and *V. Albo atrum* was tested in this study, for its efficiency in controlling *Verticillium* wilt of Berseem (*Trifolium alexandrinum*). Bacterial seed treatments increased significantly the percentage of healthy plants as well as the fresh weight of plants. The success of this protection depends on the time of pre-inoculation with the protective bacteria. In our conditions, Berseem seed treatments with *Rhizobium* before pathogenic infection conferred the best results with 83% of healthy plants against 7% in control (inoculated with *Verticillium* only). Bioprotection of Berseem against aggressive strains of *Verticillium* is closely related to the inoculum of the protector agent. The maximum reduction of the disease was achieved by the highest concentration 10<sup>9</sup> cells / ml, which was higher than the pathogenic inoculum (10<sup>6</sup> spores / ml). The existence of an optimal density for induction of a systemic resistance suggests that the host plant has unique requirements to be able to effectively oppose pathogen inoculation. Berseem seed inoculated with *Rhizobium* sp. before inoculation with *Verticillium* showed reduced severity of disease. This suggests that the colonization of seedling by bacteria before the attack of the pathogen reduces the potential of *Verticillium* to colonize root and to cause symptoms of disease. These requirements would reflect physiological processes leading to host resistance. This may suggest new alternatives for the biological control of *Verticillium* Disease.

**Key words:** Bacterization, Berseem, *Verticillium* wilt.

**RÉSUMÉ**

Le *Verticillium* est un redoutable champignon phytopathogène causant le flétrissement sévère, la mort et le nanisme des plants ayant survécu à l’attaque. La recherche d’un moyen de lutte biologique contre cette maladie est devenue une priorité.

*Rhizobium* sp. montrant *in vitro* une grande activité antagoniste contre *Verticillium dahliae* et *V. albo atrum* a été testé dans cette étude en vue de contrer la verticilliose du Bersim
Des traitements bactériens des graines ont augmenté significativement aussi bien le pourcentage des plantes saines que leur poids frais. Le succès de cette opération dépend du temps de préinoculation avec la bactérie protectrice. Dans nos conditions, le traitement des graines de Bersim avec le *Rhizobium* avant l’attaque pathogène a donné la meilleure protection avec 83% de plantes saines contre 7% rappelant le témoin malade (inoculé avec le *Verticillium* seul).

La bioprotection du Bersim contre l’attaque agressive de *Verticillium* est étroitement liée à l’inoculum de l’agent protecteur. La réduction maximale de la maladie a été obtenue avec la concentration de $10^9$ cellules/ml dépassant largement celle de l’inoculum pathogène ($10^6$ spores/ml). L’existence d’une densité optimale conférant une résistance systémique suggère que la plante hôte possède des exigences particulières pour pouvoir s’opposer efficacement à l’inoculation pathogène. Les graines de Bersim inoculées avec *Rhizobium* sp avant l’inoculation par *Verticillium* ont montré une réduction de la maladie. Ceci suggère que la colonisation des jeunes plants par la bactérie avant l’attaque de l’agent pathogène réduit le pouvoir de *Verticillium* à coloniser la racine et causer les symptômes de la maladie. Ceci refléterait des processus physiologiques conduisant à la résistance. Ces résultats peuvent mener à de nouvelles alternatives de contrôle contre la verticilliose.

**Mots clés:** Bactérisation, Bersim, Verticilliose.

**INTRODUCTION**

The application of soil microbial antagonists seems to be a good alternative to chemical control for bioprotection of plants. Before dispersing such microorganisms in the environment, we must ensure not only that they are not pathogenic to humans or animals, but also that they are atoxinogenic on food (Dupuy et al., 1992; El Aissami, 1999; El Aissami et al., 2002 and El androusse & El Aissami, 2006).

Some authors have used bacterization to prevent natural hazards by surrounding the seed with a beneficial bacterial population. Bioprotective Bacteria are of the genus *Pseudomonas* (Digat et al., 1990 and Digat, 1992), *Bacillus subtilis* (Hall et al., 1986), which were used for bioprotection against *Verticillium* of maple. The *Rhizobium* was also reported as a bioprotector against plant root diseases (Chou & Schmithner, 1974; TU, 1980; Beagle-Ristaino & Risler, 1983; Hwang et al., 1992; 1993).

Many works on biological control have shown that the success of the phenomenon of bioprotection can be significant, if the period of preinoculation is carefully determined. This delay is 0 minute for bioprotection against *Verticillium* of alfalfa by *Gliocladium roseum* (Millar et al., 1984) and cotton by *Talaromyces flavus* (Murray et al., 1997), 3 days to protect maple with *Bacillus subtilis*, against the same disease (Hall et al., 1986), seven days in the case of protecting the eggplant by *Trichoderma harzianum* (Henni, 1987). The density of the preinoculum must be taken into account too. Several studies have reported that a threshold density of the protective agent must be respected for a better expression of biological control (Murray et al., 1997 and El Aissami 1999). Wymore & Baker (1982) reported that this concentration should be the same as the inoculum of the aggressive agent to fight *Fusarium* of the tomato. However, Elad & Hadar (1981) and Henni (1987) used increasing...
ncentrations of non-pathogenic preinoculum and found that there was a positive correlation between the density of preinoculum and the degree of plant protection against plant pathogenic agents.

The present study was undertaken, to use *Rhizobium* sp. strain, which was highly antagonistic against three *Verticillium* strains *in-vitro* to evaluate its effectiveness in triggering off the Berseem plant defence mechanisms against *Verticillium* wilt.

**MATERIALS AND METHODS**

1. **Bacterization of seeds**

Disinfected Berseem seeds were soaked during 30 min in the Bacterial inoculums suspension (BIS) containing $10^9$ cells / ml. The BIS-inoculated Berseem seeds were then sown in pots containing sterile soil. Five seeds were planted in each pot. There were 6 replicates pots per treatment.

2. **Inoculation with the pathogenic strain of *Verticillium***

After 5 days, Berseem seedlings were watered by a suspension of *Verticillium* spores ($10^6$ spores / ml) freshly prepared from 3 pathogenic strains of the fungus, naturally in the host, this heaving of the roots causes injuries, which increase the rate of penetration.

Preinoculated controls are watered by sterile distilled water. Diseased checks are watered with the aggressive inoculum only. All plants were watered every two days with a nutrient solution (El Aissami, 2006). None inoculated Plants with *Rhizobium* were watered with the same nutrient solution containing fifteen times more KN03.

3- **Evaluation of plant protection against disease**

The evaluation of plant protection against disease was made by:
- an assessment of foliar damage in an attempt to evaluate the reduction of leaf symptoms in treated plants compared to the BIS untreated plants.
- Percentage of healthy plants
- Fresh weight of plant

**RESULTS**

**Effect of bacterial treatment with *Rhizobium* sp. on wilt disease expression**

1. **Effect on the foliar damage**

The bacterial treatment before pathogenic inoculation protects Berseem. In fact, these plants have minimal leaf damage compared to all treated plants (Figure 1).

![Figure 1: Effect of preinoculation by *Rhizobium* (RH) on foliar damage induced by *Verticillium* strains, P3S, L9 and E27 in Berseem plants](image-url)
Pretreated plants with symbiotic bacteria before pathogenic infection show very few symptoms at the cotyledons and the leaflets of the lowest shoots. The foliage of *Rhizobium* treated Berseem plants look like the foliage of plants in healthy controls. The plants react to the isolates of *Verticillium* P3S, L9 and E27 in the same way. Leaf alterations were also appreciated and the results are presented in Figure 1. *Verticillium* strains induce the most spectacular leaf damage. Pretreatment with *Rhizobium* sp. before the pathogen, confers protection for Berseem plants.

2. Effect on the percentage of healthy plants

Berseem seeds treated with *Rhizobium* showed a high percentage of healthy plants. The seed inoculation with the *Verticillium* alone has caused an important reduction of the number of healthy plants, 7% compared to 83 % in the Controls (T) (Figure 2).

3. Effect on fresh weight of plants

Two months after inoculations, fresh weight of the surviving plants showed that seed bacterization before inoculation with *Verticillium* improved the fresh weight of plants in comparison with the diseased plants inoculated with the *Verticillium* only (Figure 3). Nevertheless, this improvement of fresh weight did not attain the level of Controls (T) nor does it reach the level of fresh weight of plants treated with the *Rhizobium*. In fact, *Rhizobium* showed a stimulating effect on the growth of Berseem plants. The effect of preinoculation by the symbiotic bacteria before aggressive inoculation has shown well on the fresh weight of Berseem plants. The lowest fresh weights are those of plants inoculated by *Verticillium* strains alone (Figure 3). All the treated plants by the symbiotic bacteria have a fresh weight significantly higher than that of diseased plants.

**DISCUSSION AND CONCLUSION**

Bacterization of Berseem seeds with *Rhizobium* sp. protected them against aggressive action of *Verticillium*. The success of this protection is closely related to the inoculum of the *Rhizobium* sp. The reduction of the disease was achieved by the concentration $10^9$ cells / ml, which was higher than the pathogenic inoculum ($10^6$ spores / ml). This is in agreement with the results of Hall & Schreiber (1984), and Hall et al., (1986), which used a higher concentration of *Bacillus subtilis* to protect maple against a pathogenic strain of *Verticillium dahliae*. Similar observations were made by Henni (1987), who also used a higher density of *Trichoderma viride* to fight

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**Figure 2:** Effect of bacterial seed treatments with *Rhizobium* (RH) before inoculation with *Verticillium* strains (P3S, L9 and E27) on the percentage of healthy Berseem plants. 8 weeks after inoculation.
Verticillium of eggplant and Millar et al., (1984); who protected the alfalfa against Verticillium albo-atrum by inoculation with a spore suspension of Gliocladium roseum larger than the pathogenic agent. The protection depends also on the time interval between Berseem seed treatment with the biocontrol agent and exposure to Verticillium. Thus, without bacterial pretreatment delay; seedlings were weakly protected. On the other hand, the protection was largely improved when the bacteria was inoculated to the seeds before Verticillium. This condition allowed obtaining a maximum and durable protection as expressed by a higher percentage of healthy plants and a higher fresh weight of plants.

The time interval seems necessary for the triggering of the plant defence mechanisms in response to primary infection by protective bacteria. This phenomenon consists in the activation of a number of genes that allow the induction and the keeping of plant resistance against pathogen (Compant et al., 2005). Manifestation of systemic acquired resistance, depends consequently on the time interval, and on the combination of host plant, protective agent and aggressive pathogen. This interval is of 0 minute in the biocontrol of alfalfa against Gliocladium roseum (Millar et al., 1984) and of 48h when biocontrol is conducted by Sinorhizobium meliloti (El Aissami et al., 2002) and Pseudomonas aeruginosa (El Androusse & El Aissami, 2006).

Berseem seed inoculated with Rhizobium sp. before inoculation with Verticillium showed reduced severity of disease. This suggests that Berseem seeds bacterization reduces the potential of the Verticillium attack. When the symbiotic bacteria were introduced to Berseem after inoculation with Verticillium, no significant reduction in disease was recorded (El Aissami, 1999). This indicates that the symbiotic species did not compete with the pathogen once it had infected the seedling and become established in the root.

From these results, we conclude that Rhizobium sp. used in our experiments has a protective effect on Berseem against Verticillium and stimulates the growth of plants. This may suggest new alternatives for the biological control of Verticillium diseases.
LITERATURE CITED


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