

Evaluation of Different Control Measures against Damping off in Greenhouse *Solanaceous* Crops

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Abstract

Increased losses due to damping off disease have been reported recently on protected *Solanaceous* crops in Lebanon; however, no efficient treatment has been identified. In-pot and field experiments were done in greenhouses to assess the efficacy of several commercial formulations of pesticides in controlling damping off, and determine their effect on plant growth. The treatments were two commercial formulations of biological control agents, *Pythium oligandrum* and *Bacillus subtilis*, and a mixture of Mefenoxam+Azoxyastrobin. The results showed that *Pythium oligandrum* has reduced the incidence of damping off by the half comparing to the positive untreated control and was more efficient than *B. subtilis*, with a disease incidence of 22.2% and 42.2%, respectively, in the pot experiment. In field experiment, the average incidence of the disease was generally low independently from the treatment performed (5.2%) and the evaluation of the growth parameters indicated that both *B. subtilis* and *P. oligandrum* formulations were able to enhance the growth of plants compared to the untreated control. Therefore, *P. oligandrum* could be used in the management of damping-off; however, further research studies are needed in order to optimize its application in the field.

Keywords:

Biological control agents; *Pythium oligandrum*; *Bacillus subtilis*; Disease management; Growth promoters

Introduction

Damping-off is a destructive disease of plant seedlings, caused by several soil-borne fungi including *Rhizoctonia solani*, *Pythium* spp., *Phytophthora* spp., *Sclerotinia* spp. and *Fusarium* spp. [1]. In particular, pre- and post-emergence damping-off caused by *Pythium* spp. in vegetable crops is economically very important worldwide [2]. *Pythium* spp. are fungal-like organisms (Oomycetes), commonly referred to as water molds, which naturally exist in soil and water as saprophytes, feeding on organic matter. Some *Pythium* spp. can cause serious diseases on greenhouse vegetable crops resulting in significant crop

losses. Infections by *Pythium* lead to damping-off in seedlings and crown and root rot of mature plants. Several *Pythium* spp., including *P. aphanidermatum*, *P. irregulare* and *P. ultimum* are known to cause damping off and crown and root rot in greenhouse cucumber, pepper and tomato crops [3]. Several measures including chemical, cultural and biological control are available for the management of damping off. Chemical treatments commonly used in nurseries and fields are mainly based on metalaxyl, hymexazol, propamocarb or others. Removal and disposal of diseased plants, crop rotation, improved soil drainage and air circulation, reduced nitrogen fertilization and soil solarisation are also essential for the successful management of damping off disease [4]. Because of the potential negative impacts of chemical pesticides on the environment and consumers, several Biological Control Agents (BCAs) genera of fungi and bacteria have been recently integrated in programs of disease management and their effectiveness in controlling soil borne diseases is well-documented [5-7].

Pythium oligandrum Dreschler, in particular, is a soil inhabitant oomycete, considered as the most promising BCA for use in agriculture. It interacts directly with the fungal pathogens through distinct or combined mode of actions, such as mycoparasitism, antibiosis, nutrient and space competition, and/or indirectly by inducing resistance in the plants [8,9]. An impressive number of soil-borne diseases of various plants can be controlled by *P. oligandrum*, such as: black scurf of potato caused by *Rhizoctonia solani* [10]. Grapevine trunk wood disease caused by *Phaeoemoniella chlamydospora* [11]. Verticillium wilt of pepper caused by *Verticillium dahliae* damping off and root diseases of sugar beet caused by *Pythium ultimum* [12,13] among others. Today, the emergence of resistant pathogen strains to currently used pesticides, the increasing consumer demand for food products without toxic residues and the international objective to preserve as much as possible the environment and human health compromise the use of pesticides and favour the introduction of novel alternatives. Among these, activating plant defence responses by using BCAs is a promising strategy for replacing or at least reducing chemical applications [9].

Because plant diseases cause billions of dollars in harvest loss annually with huge consequences in developing countries, the improvement of management strategies that can be reliable and safe for the environment is urgently needed. Particularly in Lebanon, farmers of protected crops on coastal areas have long suffered from severe losses of their seedlings at the early phases of the growing season and yet, none of the treatment type adopted was satisfying. Therefore, the aim of this study was to assess the efficacy of a *Pythium oligandrum* commercial formulation in controlling damping off disease caused by *Pythium* spp. on *Solanaceous* hosts *in vivo* trials, and determine its effect on plant growth.

Materials and Methods

Two experiments (in-pot and in-soil experiments) were carried out in Amchit, Jbeil district, Mount Lebanon at the sea level in two separate greenhouses during the period August-October 2016.

In-pot experiment

Artificial inoculation/Preparation of the broth: The culture liquid medium or broth was prepared based on the protocol [14] slightly modified by mixing 500 ml tomato sauce and 5 g CaCO₃ (Sigma-Aldrich, St Louis, Germany). After filtration through a cheesecloth, 250 ml of the mix were diluted with water to 1 L final volume and then autoclaved.

Inoculum preparation: Inoculum was produced as described by [15]. An isolate of *Pythium aphanidermatum* (PytA), obtained from the same greenhouse where plant previously showed symptoms of damping-off, was identified prior to this experiment based on morphological characteristics and molecular analysis and used in the experiment. The isolate was grown on Potato Dextrose Agar (PDA, Himedia Laboratories, India) medium at 31°C in the dark for 24 hours. Five agar plugs (5 mm) collected from the leading edges of mycelial growth were then transferred into 10 Petri dishes containing the broth. After incubating the plates at 31°C in the dark overnight, the mycelial mats obtained were washed with sterile deionized water three times, flooded in sterile deionized water, and incubated at room temperature of 24°C for 48 hours under white fluorescent lights. The mats were then blended in a clean blender on high speed for 2 minutes and then filtered through a four-ply layer of sterile cheesecloth to collect oospores. The residues on the cheesecloth were collected with sterile deionized water in a sterile 50 ml falcon tube. The concentration of *P. aphanidermatum* oospores in the resultant suspension was determined using a hemacytometer (Bright-Line, Sigma-Aldrich). The final concentration of the oospore's suspension (2 L) used in artificial inoculations used was 1×10^7 oospores/ml.

Inoculation: The pots (12 cm) were previously filled with the greenhouse soil in which the experiment was done. Each pot received 5 ml of sterile water then 10 ml of oospore suspension using a pipette. Inoculation was performed late in the afternoon to avoid the highest daily temperatures. Pots were placed on plastic film to prevent contamination of the greenhouse soil and kept in the greenhouse for 5 days for acclimatization. In each

pot, one tomato seedling at 2-3 leaf growth stage was planted in the inoculated soils. The hybrid DSW-8125 tomato by Seminis (Bayer) was used. During the whole period of the experiment, the greenhouse was tightly closed and pots were watered each two days, in order to create the favourable climatic conditions for *P. aphanidermatum* growth.

Experimental design: The experiment was done in a randomized complete block design (RCBD) with three blocks, five treatments-

- (Polyversum commercial formulation by Biopreparaty, Czech Republic),
- (Fulzyme Plus-SP commercial formulation by Biotech, California, USA) and
- Mefenoxam+Azoxystrobin (Uniform commercial formulation by Syngenta, North Carolina, USA),
- Negative control (non-inoculated soil and no damping off treatment),
- Positive control (Inoculated soil and no damping off treatment) and 15 replicates of potted tomato plants. The treatments, their composition, their conditions of application and their dose are shown in **Table 1**.

Treatment	Formulation	Conditions of application	
		In-pot experiment	In-soil experiment
Polyversum	<i>Pythium oligandrum</i> (1%)	Drenching directly after transplanting	Dipping the roots of seedlings in suspension for 10 min before planting
		Applied dose: 20 g for 2500 plants	Applied dose: 15 g for 2500 plants
Fulzyme plus-SP	<i>Bacillus subtilis</i> (2 × 10 ¹⁰ /gram)	Drenching directly after transplanting	Applied two days before planting by drenching
		Applied dose: 100 g for 2500 plants	Applied dose: 100 g for 2500 plants
Uniform	Mefenoxam (10.9%)	Drenching directly after transplanting	Applied directly after planting by drenching
	Azoxystrobin (28.2%)	Applied dose: 125 ml for 2500 plants	Applied dose: 125 ml for 2500 plants

Table 1: Treatments against damping off disease used in pot and soil experiments, their formulation, their application mode and dose.

Data collection: The incidence of damping off was determined each two days for a total period of two weeks, by calculating the percentage of dead plants in each group.

In-soil experiment

Experimental design: The second experiment was done in a greenhouse, in which the soil is naturally infected by *Pythium* spp., that measured 25 m in length and 7 m in width, and divided into 12 plots. Three treatments together with a negative

untreated control were set in RCBD design with three blocks and in average 40 eggplant plants of the hybrid Negra by Seminis (Bayer), at the 4-5 leaf stage, per treatment in each block. Application of the treatments was done respecting the recommendations on the labels in **Table 1**. Irrigation was done each 2-3 days in order to create good conditions for infection by *Pythium*.

Data collection: Incidence of Damping off: The incidence of damping off on eggplants was monitored each two days for a period of two weeks and determined through dividing the number of symptomatic plants by the total number per plot.

Plant height: In each plot, 10 plants were randomly labelled and their height was measured using a ruler each 7 days for a period of one month.

Fresh and dry weight: One month after planting, three plants in each plot were uprooted from the greenhouse. The canopy was separated from the root system and the fresh weight was then measured. The dry weight was determined after placing them in the oven for 24 hours at a temperature of 70°C.

Statistical analysis

The significance of the difference between the treatments for damping off, plant height and fresh/dry weight was determined by the Analysis of Variance (ANOVA). Tests were performed using IBM SPSS Statistics software version 21.

Results

Effect of different treatments on damping off

Pot experiment: The percentage of damping off two weeks after planting is shown in **Figure 1**. The incidence was the highest (44.4%) in the positive control (inoculated control/no treatment), whereas the lowest percentage of disease (6.7%) was detected in seedlings treated with the synthetic chemical product Uniform (Mefenoxam+Azoxytrobine). Treatment with Fulzyme Plus-SP (*Bacillus subtilis*) was not capable of reducing the incidence of the disease (42.2%). Compared to the inoculated non-treated control, treatment with Polyversum (*P. oligandrum*) reduced the incidence of damping off by the half to 22.2%, although no significant difference between the means was observed. This value was also comparable to the incidence of damping off in the non-inoculated control (24.4%), which represents the infection by the inoculum naturally present in the potting soil. The evolution of the incidence of the disease during the first two weeks after planting was also studied in **Figure 2**. In general, the incidence of damping off increases mainly in the first week after planting. The disease continues to appear in plants treated with *B. subtilis* and untreated inoculated (positive) control even after two weeks. On the other hand, plants treated with *P. oligandrum* were mainly infected by damping off during the first week after planting. Treatment with the mixture Mefenoxam+Azoxytrobine was the most efficient in preventing the infection during the first week after planting. The plants, later on, seemed to acquire a resistance.

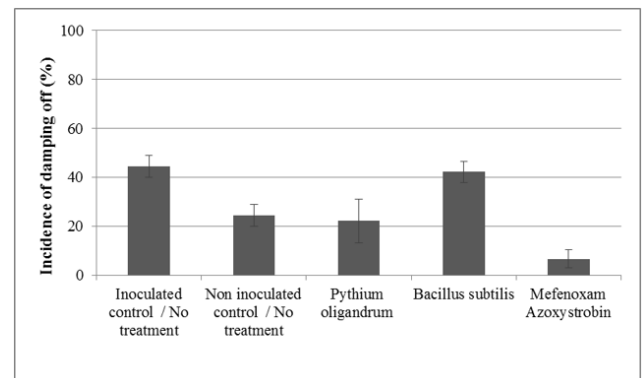


Figure 1: Effect of the different treatments on the incidence of damping off in pot experiment. Bars indicate standard error. Columns marked by different letters represent means that are significantly different at $p=0.05$.

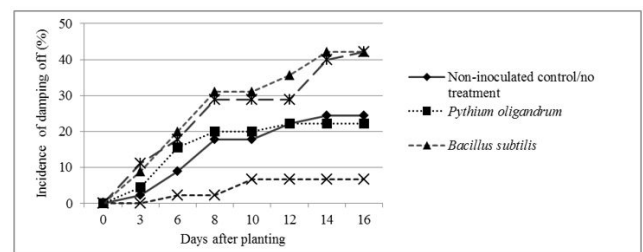


Figure 2: Evolution of the incidence of damping off in potted tomato seedlings subjected to different treatment.

In-soil experiment: In the in-soil experiment, the effect of the same treatments on the incidence of damping off was determined on eggplant seedlings planted in naturally infected soil. Eggplant seedlings were used because they are usually more sensitive to infections and they express more evidently the symptoms of the disease [16]. Two weeks after transplanting, we could notice that the average incidence of the disease was generally low independently from the treatment performed. The low percentage of damping off in the non-treated control plots (5.2%) indicates that the infection in the greenhouse was low during the year of study. The highest incidence of damping off (7.6%), surprisingly, was revealed on plants treated with *P. oligandrum* by dipping at the moment of transplanting.

Both *B. subtilis* and the mixture (Mefenoxam+Azoxytrobine) were able to reduce the incidence of damping off to 2.1% and 0.9%, respectively, compared to the non-treated control. Nevertheless, differences between the means were not significant at $p=0.05$, probably due to the low percentages found.

Effect of treatments on agronomic performance

Plant height: The effect of the different treatments in the in-soil experiment on the growth of eggplant plants was evaluated through the height parameter in **Table 2**. In all the studied treatments, the height of the eggplant seedlings increased although with slight differences. One week after planting, all treatments except *P. oligandrum* had statistically similar effect on plant height since the average height of plants treated with *P. oligandrum* (10.43 cm) was significantly ($p<0.05$) the lowest.

This significant difference was maintained two weeks after planting but later, after three and four weeks, the average growth of plants subjected to different treatments was statistically similar, varying between 29.26 cm and 30.23 cm in **Table 2**. This result indicates that *P. oligandrum* can retard the growth of plants in the first two weeks after its application, but this effect will be lost soon later.

Treatments	Week 1*	Week 2	Week 3	Week 4
Non-treated control	12.80 ± 0.82a	15.56 ± 0.84a	21.73 ± 0.73a	29.90 ± 0.90a
Pythium oligandrum	10.43 ± 0.72b	12.76 ± 0.73c	21.06 ± 0.86a	30.23 ± 0.78a
Bacillus subtilis	12.20 ± 0.73a	14.16 ± 0.74b	21.56 ± 0.90a	29.26 ± 0.99a
Mefenoxam +Azoxystrobin	12.73 ± 0.77a	14.96 ± 0.82ab	21.36 ± 0.96a	30.06 ± 0.94a

* Means are followed by standard error. In each column, means followed by the same alphabet are not significantly different ($p>0.05$)

Table 2 : Effect of the different treatments on plant height (cm) at one, two, three and four weeks after planting.

Fresh and dry weight of roots and canopy: The effects of the different treatments on the fresh and dry weights of root system and canopy in the in-soil experiment in **Figures 3 and 4** show respectively. Eggplant seedlings treated with *B. subtilis* showed the highest fresh and dry root weight (73 g and 41.4 g, respectively), whereas those treated with Uniform showed the lowest (36.6 g and 19.3 g, respectively). The fresh weight of roots of the plants treated with *P. oligandrum* increased compared to the untreated control. These means of root weight, however, were not significantly different ($p>0.05$). Similar results were obtained for the canopy fresh and dry weight, although in this case, significant differences among means of dry weight were noticed. Both *B. subtilis* and *P. oligandrum* formulations were able to enhance the growth of plants compared to the untreated control. On the other hand, the fresh and dry weights of plant canopies treated with the mixture Mefenoxam +Azoxystrobin were the lowest in **Figure 4**.

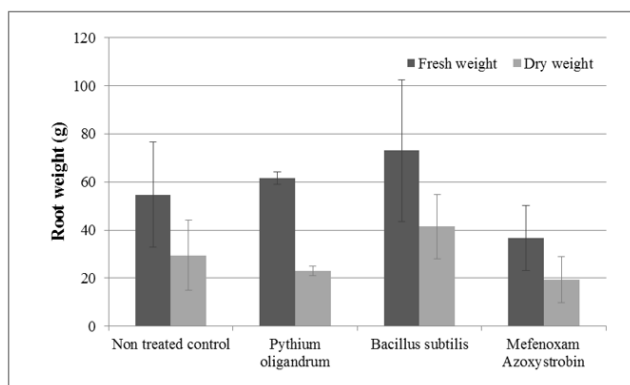


Figure 3: Effect of the different treatments on average fresh and dry root weights. NS: means are not significantly different at $p=0.05$.

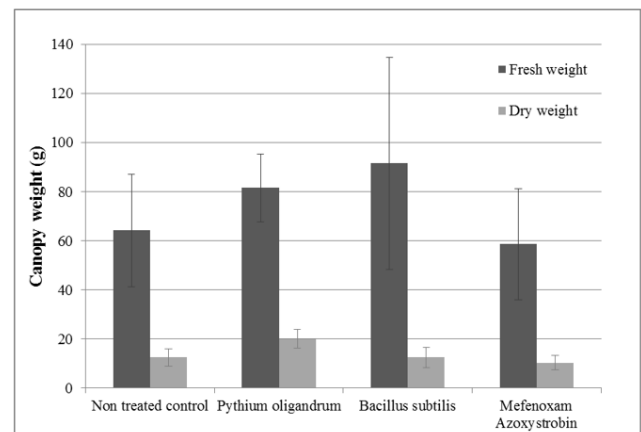


Figure 4: Effect of the different treatments on average fresh and dry canopy weights. For each variable (Fresh and Dry weight), columns marked by different letters represent means that are significantly different at $p=0.05$.

Discussion

In this study, both pot and field experiments were carried out in order to find the best treatment against damping off to be applied on fruity vegetables, precisely of the *Solanaceae* family, grown in greenhouses. Results of the pot experiment confirmed that the chemical treatment was the most efficient in reducing the incidence of the disease [17]. Between the two commercial formulations of BCAs tested, *P. oligandrum* was more efficient than *B. subtilis*. *Pythium oligandrum* considered as a non-pathogenic microorganism has been reported to have antagonistic activity against a wide range of plant pathogenic oomycetes by mycoparasitism and antibiosis [18]. An impressive number of diseases of various plants can be in fact controlled by *P. oligandrum* however; the reduction of pathogenic attacks varies between 15 and 100 %, depending on the host plant, the target pathogen and the application method [19]. In the present study, treatments with *P. oligandrum* reduced the incidence by 50%. Our results are in accordance with previous researches carried out in different countries or on different host plants and targeting other pathogens [20-22].

On the other hand, results obtained in the second experiment, in which seedlings were planted in naturally infected soil of a greenhouse with a long history of damping off disease, might not be reliable. Indeed, the frequency of infected plants was too low to determine the efficiency of the applied treatments. Infection by pathogenic species of *Pythium* depends greatly on climate conditions, particularly temperature and water [3]. Regarding water requirements, the plants were given sufficient water each two days following planting. However, the temperature during the period of the experiment (26.8°C-23.6°C) was probably much lower than the optimum temperature for the *Pythium* growth 34°C [4]. In our study, particularly in the field experiment, the amount of applied inoculum of *P. oligandrum* might have led to shock symptoms of damping off following the rapid penetration. Moreover, studies have shown that the formulation of the mycoparasitic *P. oligandrum* can greatly influence the efficiency of treating damping off caused by *Pythium spp.* [23]. Therefore, different

formulations could be tried for different crops and under different application conditions in order to optimize the benefits from this microorganism.

In the present study, *Pythium oligandrum* effect appeared mainly one week after planting, which allowed the synthetic chemical product (Mefenoxam+Azoxystrobin) to perform better. For that, it is suggested to apply it as seed dressing or preventively on seedlings in nurseries before the transfer to the field in order to fasten the colonization of the roots and promote earlier the plant defence mechanisms. It is well established that during the host plant- *P. oligandrum* interaction, signalling molecules involved in the molecular dialogue are involved. Some of these act as auxin-like compounds such as tryptamine (TNH₂), an auxin-compound, which, upon root absorption, stimulates plant growth [24]. Our results confirmed that *P. oligandrum* could enhance the growth of the plants in delay compared to the non-treated control. These findings were similar to [20]. Who showed in their experiment in controlling sugar-beet damping off that after sugar-beet treatment with *P. oligandrum* an initial retardation in emergence and growth rates was observed until the cotyledons leaves were formed? After this stage a stimulation of plant growth was recorded indicating that the switch from depression to stimulation occurred at the stage 1-2 pairs of true leaves.

Stimulation of growth is expressed by a higher fresh weight of plants and deep green leaves. However, no significant difference between the treatments was noticed probably due to the time that *P. oligandrum* needs to enter the root tissue and enhance the plant growth without damaging the plant [25,26]. Moreover, *B. subtilis* had the greatest effect on fresh/dry weight of roots and canopy. This is probably because the microorganism is known as Plant Growth Promoting Rhizobacteria (PGPR) and commonly used as a BCAs to enhance the plant growth [27,28]. Despite the hope raised by the use of BCAs and the research currently being done, the application of microbiological control by farmers is still limited due to the lack of consistency when BCAs are applied in the field which also copes with the results obtained in our in-soil experiment [29-31]. In order to optimize the use of BCAs against damping off, several recommendations could be raised. For instance, field experiments could be carried out in different geographical areas and in sites where other *Pythium* spp. are causing damping off. It would be useful also to combine the application of *P. oligandrum* with good sanitation practices and adequate greenhouses ventilation to create unfavourable conditions for disease development.

Conclusion

In the present field experiment, the rate of *P. oligandrum* used was 15 g/20L of water and the time of dipping was 10 min. In the commercial formulation under study, the range of the time of application and the application rate recommended by the manufacturer is very wide. For that, different rates and time could be tried in the future experiments, in order to select the best conditions for reducing damping off on *Solanaceous* hosts. *Trichoderma* spp. are well-known efficient BCAs which have been used successfully for the control of numerous plant pathogens particularly damping off. In the present study, it was

difficult to find a commercial product containing *Trichoderma* spp. in the Lebanese market. It would be therefore interesting to insert also this treatment for the future experiments. Management strategies based on the integration of all available means for the control of damping off are necessary to decrease the losses. Activating plant defence responses by using biocontrol agents could be a promising strategy for reducing chemical applications, however, further studies are still necessary to improve their application.

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