

The disease incidence (D.I.) was calculated according to El-Morsi *et al.* (2009) as: $D.I. (\%) = (\sum (n \times v) / N \times V) \times 100$ Where, (n) = the number of diseased transplants per category, (v) = category number, N = total number of the transplants, (V) = maximum of category.

Re-isolation was carried out from infected transplants showing disease symptoms and the isolated fungi were compared with the original fungal cultures used

2.4. Bio-fungicides

In this study, 4 bio-fungicides and one chemical product, used as reference to be effective against young olive trees dieback, were tested. The composition, the concentration of the active ingredient and the recommended dose of the tested product are presented in the Table 1.

Table 1. Trade name, composition, active ingredient (%) and recommended doses of the tested fungicides.

Trade name	composition	Percentage of Active ingredient	Recommended concentration in the field
Dalgin active	seaweed extract (<i>Ascophyllum nodosum</i>) and amino acids	6.78%	3-5 L/hectare
Biobac	<i>Bacillus subtilis</i> Y1336	1×10^9 CFU/g	1,5kg/hectare
Sanbio Planta	Activated blend of natural seaweed, nutrients, trace elements, selectively and adapted highly effective natural bacterial cultures and mycorrhiza fungi	K2O: 10.4% MgO: 12.2% S: 11.4% Na: 3.04%	1,5kg/hectare in 500L of fresh water
Sanbio Epsomit	Activated Magnesium sulphate heptahydrate	Mg 16% - S 13%	1 kg/hectare in 500L of fresh water
Uniform	Azoxystrobin + Mefenoxam	322 g/l of Azoxystrobin + 124 g/l of Mefenoxam	1L/hectare

2.5. Effect of bio-fungicides on mycelial growth of the selected pathogens

The antagonistic activities of the four bio-fungicides on mycelial radial growth of the selected pathogens were determined by growing the fungus on a PDA containing the different bio-fungicides in Petri plates (85-mm diameter). Each bio-fungicide was added at recommended label rates to 100 ml sterilized PDA media at 60°C, and then poured equally into five Petri plates.

Control plates were made by replacing the quantity of bio-fungicides with the same quantity of sterile distilled water. A disc (6mm diameter) of 6-day-old pathogen mycelial culture was aseptically transferred to the center of the solidified PDA media in plates. The plates were subsequently incubated for 5-7 days at 25°C (Abdel-Monaim *et al.*, 2014).

Mycelial growth of the selected pathogens was measured on each plate, and the growth in PDA containing bio-fungicides was compared with the growth of the same pathogen in plates containing water (control). The experiment was replicated three times for each treatment and the mean values taken. Effect of bio-fungicides on mycelial growth of the selected was recorded in terms of percentage colony inhibition and calculated according to Hmouni *et al.* (1996). Percentage growth inhibition was determined as $(1 - C_n/C_o) \times 100$, where C_n is the average diameter increase of fungal colony with treatment, and C_o is the average diameter increase of fungal colony with control.

2.6. Effect of bio-fungicides on disease incidence

Four organic compounds and one fungicide (**Table 1**) were evaluated to control root rots and wilt diseases on olive transplants. This experiment was carried out on healthy-looking olive transplants (cv. Chamlali) under greenhouse conditions during 2014. To test preventive and curative effects of the tested bio-fungicides, three experiments were performed: (i) bio-fungicides were added to the media (peat+ perlite) one week before inoculation; (ii) bio-fungicides addition and inoculation were applied at the same time; and (iii) bio-fungicides were added to the media one week after inoculation.

Olive plants (5 months old) planted in plastic pots (12 cm diameter x 9 cm high, one plant per pot) containing 600 ml of previously autoclaved soil (perlite : peat, 1:1) were drenched with 100 ml of organic compound or fungicide and 50 ml of an inoculum suspension of a mixture of all the isolated pathogens, prepared as previously described.

Five plants per elementary treatment plus five positive (un-inoculated and un-treated) and five negative (inoculated and un-treated) control ones were placed in the greenhouse (15-30 °C, 50-80% RH). Plants were watered once a week. Three months later, disease incidence (DI) and efficacy values were calculated according to the following formula:

$$\text{Product Efficacy (\%)} = \frac{\text{DI of control transplants} - \text{DI of treated transplants}}{\text{DI of control transplants}}$$

2.7. Statistical analyses

Variance analysis of the treatment effect on measured data was performed by using the general linear model procedure of SPSS (SPSS 20.0). Experiments were analyzed using standard analysis of variance (ANOVA) with factorial treatment structure and interactions. When F values were significant at $p > 0.05$, differences among the treatments were determined by S-N-K (Student-Newman-Keuls) test.

3. Results

3.1. Disease Survey

Surveillance of the disease in the three governorates (Sidi Bouzid, Kasserine and Kairouan) shows that in 2013 the percentage of infected plants varies not only according to the governorate but also depending on the soil type and the presence or absence of intercalary crops.

Indeed, the data presented in Tables 2 show that the governorate of Kairouan is the most affected by this disease with an infection percentage of about 20%. This value is measured in a field having a slightly clay soil, more in this field, a culture of melon was realized in 2013 and a green-pepper culture already installed in 2014 which will greatly increase the risk of olive plants contamination.

The analysis of the same table shows that the region Kasserine, characterized generally of sandy soil, is the less infected with a percentage not exceeding 5%.

In the following year (2014) surveillance of the same fields shows a slight increase in the percentage of infected plants. Indeed, obtained results (Table 2) show that the region of Kairouan is the most threatened by this disease with an infection percentage of 22.5% followed by the region of Sidi Bouzid (16.2%) and finally the Kasserine region, with 7% of infection.

In the same context and in a study performed in Egypt, El-Morsi and Mahdy (2013) showed that the highest means of disease incidence and severity were recorded on the transplants grown at El-Dakhla followed by El-Kharga while the least on those grown at El-Farafrah districts of New Valley governorate. These authors concluded that these results are in agreement with those reported by Mousa *et al.* (2006) and El-Morsi *et al.* (2009).

Table 2. Occurrence of root rot/wilt disease complex of young olive plants in the newly planted fields of Sidi Bouzid, Kairouan and Kasserine governorates during 2013 and 2014 successive seasons.

Location	Percentage of infected plants		Mean
	2013	2014	
Sidi Bouzid	15.4	16.2	15.8
Kairouan	20	22.5	21.25
Kasserine	5	7	6

3.2. Isolation, identification and frequency of the causal fungi

Isolations made from olive plants showing dieback symptoms in the three governorates show that there is a variation in the frequency isolation depending on the governorate and on isolated pathogens. Results presented in **Table 3** show that *Fusarium solani* and *F. oxysporum* are the most isolated in the governorates of Sidi Bouzid and Kasserine. However, in the region of Kairouan, the most isolated pathogens are *Verticillium dahliae* and *Rhizoctonia solani* with a frequency of 38% and 22% respectively.

Concerning *Pythium* sp., this pathogen was isolated almost at the same frequency in the three governorates and which is about 10%.

In Tunisia, a preliminary study conducted by Boulila and Mahjoub (1994) demonstrated that the dieback of young olive plants in the Sahel of Tunisia is caused by fungus such as *Armillariella mellea*, *Corticium rolfsii*, *Fusarium solani* and *Rhizoctonia bataticola*.

A similar study was carried out in samples of affected young trees collected during a seven year period (1989-1995), and in two field surveys in 1994-95 and 1996 demonstrated that besides some insect damage and agronomic problems, the young olive dieback was associated with Verticillium wilt, winter frost and root rot fungi especially *Fusarium acuminatum*, *F. eumartii*, *F. oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Cylindrocarpon destructans*, *Phytophthora megasperma*, and *Pythium irregulare* (Sanchez Hernandez *et al.*, 1998)

Table 3. Frequency of pathogens isolated from naturally infected olive plants collected from the three governorates.

Isolated pathogens	Isolation frequency (%)			Mean
	Sidi Bouzid	Kairouan	Kasserine	
<i>Fusarium solani</i>	28	13	33	24.66
<i>Fusarium oxysporum</i>	34	17	25	25.33
<i>Verticillium dahliae</i>	12	38	10	20
<i>Rhizoctonia solani</i>	14	22	15	17
<i>Pythium sp.</i>	10	9	11	10
Other fungi	2	1	6	3
Total	100	100	100	100

3.3. Pathogenicity tests

In this study, three isolates of each pathogen were subjected to pathogenicity test. Isolates of each pathogens and their origin are presented in Table 4.

Results presented in Figure 1 demonstrated that all the tested fungi have caused symptoms of wilting and / or root rot of olive plants. Analysis of the same figure shows that there is a variation in pathogenicity between the tested pathogens. Indeed, *Fusarium* (*F. solani* and *F. oxysporum*) are the most aggressive with a disease incidence always more than 75% and exceeded 85% in some cases.

For *R. solani* and whatever the tested isolate, the disease incidence ranged between 65 and 70% contrary to the *Pythium sp.* which disease incidence has not exceeded 30%. Concerning *V. dahliae*, this pathogen was moderately aggressive with values varied between 40 and 45% (Figure 1).

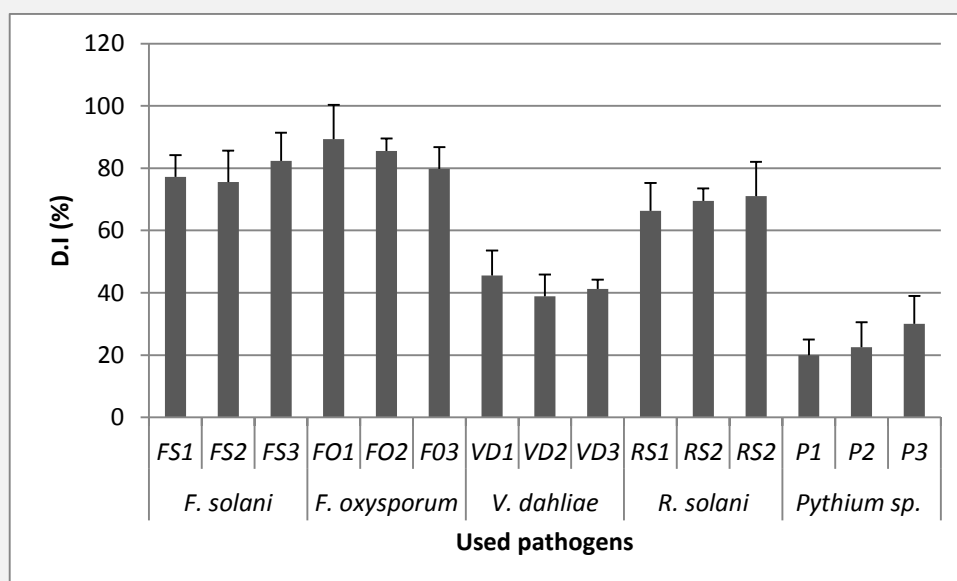


Figure 1. Pathogenicity of soil borne fungal isolates to olive tree plants under greenhouse conditions. Six months old plants were grown for three months in sterile soil infested with isolates of *Fusarium solani* (FS1, FS2, FS3), *Fusarium oxysporum* (FO1, FO2, FO3), *Verticillium dahliae* (VD1, VD2, VD3), *Rhizoctonia solani* (RS1, RS2, RS3), and *Pythium sp.* (P1, P2, P3).

In the same context, isolation made from rotted and/or wilted samples of young olive trees revealed the presence of several fungi essentially *F. oxysporum*, *F. solani*, *R. solani*, *V. dahliae* and *Pythium* sp. pathogenicity tests showed that these fungal species were pathogenic to olive plants and reproduced symptoms of dieback in rooted cuttings of cultivar Chemlali. All these pathogens were soil borne fungi causing root rot in many different hosts. Frequently, a mixture of these fungi has been associated with diseased olive tree roots (Boulila and Mahjoub, 1994, Boulila *et al.*, 1995, Triki *et al.*, 2006, Boughalleb M⁷Hamdi *et al.*, 2011).

Table 4. Origin and identification of fungal isolates tested for pathogenicity on olive trees.

Species	Isolation frequency	Isolate	Origin	Date of Isolation
<i>Fusarium solani</i>	24.66	FS1	Sidi Bouzid	Apr 2013
		FS2	Kairouan	Mar 2013
		FS3	Kasserine	Apr 2013
<i>Fusarium oxysporum</i>	25.33	FO1	Sidi Bouzid	Feb 2013
		FO2	Kairouan	Mar 2013
		FO3	Kasserine	Mar 2013
<i>Verticillium dahlia</i>	20	VD1	Sidi Bouzid	Jun 2013
		VD2	Kairouan	May 2013
		VD3	Kasserine	May 2013
<i>Rhizotonia solani</i>	17	RS1	Sidi Bouzid	Apr 2013
		RS2	Kairouan	Mar 2013
		RS3	Kasserine	Apr 2013
<i>Pythium</i> sp.	10	P1	Sidi Bouzid	Feb 2014
		P2	Kairouan	Jan 2014
		P3	Kasserine	Mar 2014

3.4. Effect of the selected bio-fungicides on the in vitro development of the used pathogens

Based on pathogenicity tests, the most aggressive isolate of each pathogen was selected for testing the efficacy of the tested bio-fungicides both in vitro and in vivo.

Adding bio-fungicides to the PDA media has inhibited mycelial growth of the five used pathogens. Results obtained (**Figure 2**) show that the four bio-fungicides have inhibited the development of all pathogens by more than 50% and the highest values were obtained with the bio-fungicide (Biobac). Indeed, the use of this bio-fungicide inhibited mycelial growth of the five pathogens by more than 73%. This product has created an inhibition percentage which is not far from that obtained by the chemical fungicide (Uniform) which is ranging between 81 and 86%. Analysis of the same figure demonstrate that with the exception of Dalgin active which entailed the lowest growth inhibition (about 50%), the two other products (Sanbio Planta and Sanbio Epsomit) have significantly inhibited mycelial growth of all pathogens and the growth reduction recorded was more than 60%.

Similarly, an in vitro essay of biological control against pathogens causing dieback of the olive tree has been done by Ben Salem *et al.* (2011). These authors showed that isolates of *Trichoderma harzianum* and *Gliocladium virens* have inhibited the mycelial growth of *F. solani*, *F. oxysporum*, *R. solani* and *V. dahlia* by more than 50%.

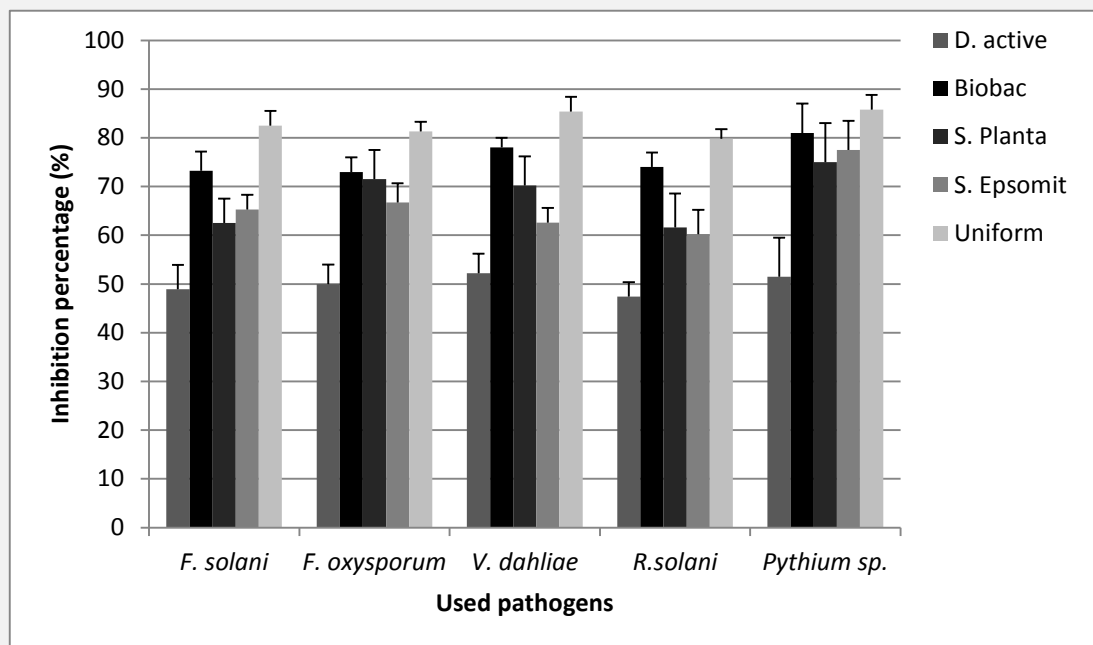


Figure 2. Effect of the tested products on the mycelial growth of the five used pathogens, after an incubation period of 6 days at 25°C.

3.5. Effect of bio-fungicides on disease incidence under growth chamber experiments

Although the application of fungicides is made one week after the inoculation of olive plants, results of mean comparison showed a significant difference ($p < 0.05$) between treated control and untreated plants whatever the used product. Indeed, the severity notation and the calculation of the disease incidence shows that values obtained with the treated plants ranged between 25 and 40% which are significantly lower than those of inoculated and untreated control (85%), generating thus a product efficacy not less than 50% (Table 5).

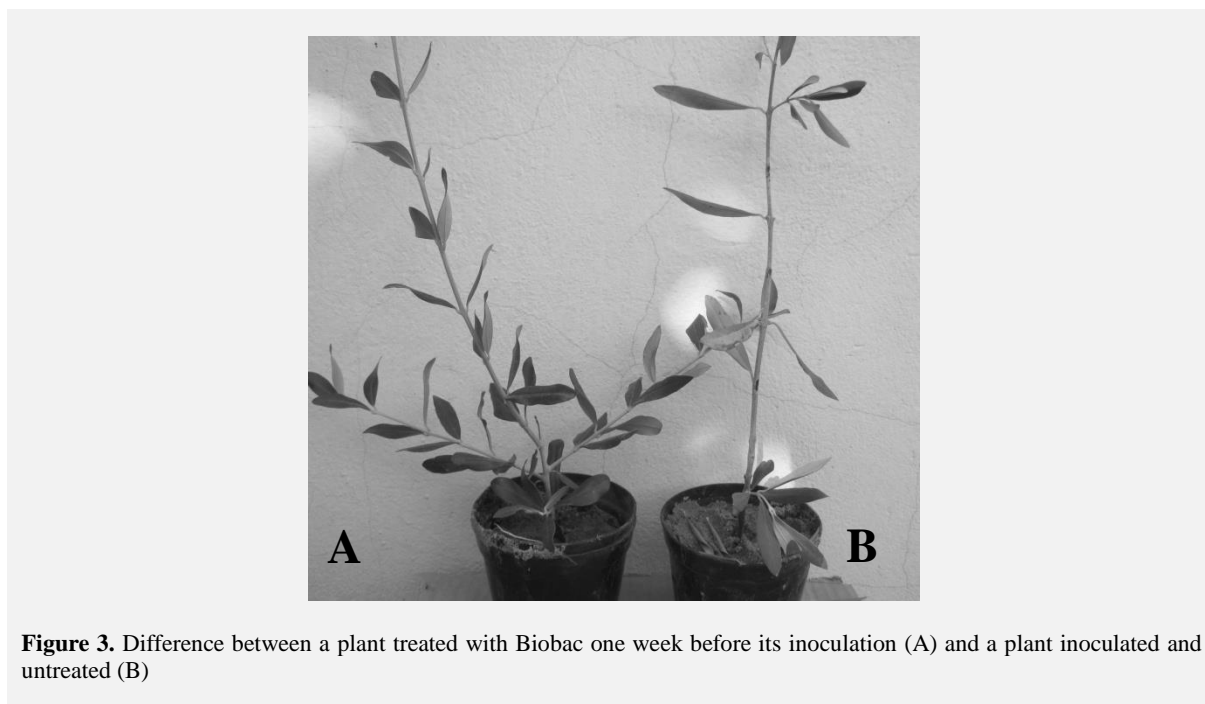
Table 5. Effect of the various treatments and the date of their application on the disease incidence and the efficacy of each product after an incubation period of three months under greenhouse conditions

	Traitement	Disease incidence (%) ^z	Product efficacy (%)
Inoculation with pathogens was achieved one week before treatment	Dalgin active	40 b	52.94
	Sanbio Planta	25 b	70.58
	Sanbio Epsomit	25 b	70.58
	Biobac	25 b	70.58
	Uniform	25 b	70.58
Treatment and inoculation were performed at the same time	Dalgin active	25 b	70.58
	Sanbio Planta	20 b	76.47
	Sanbio Epsomit	20 b	76.47
	Biobac	20 b	76.47
	Uniform	20 b	76.47
Treatment with the tested products was realized one week before inoculation	Dalgin active	15 ab	82.35
	Sanbio Planta	10 ab	88.23
	Sanbio Epsomit	0 a	100
	Biobac	0 a	100
	Uniform	0 a	100
	Inoculated and untreated plants	85 c	
	Healthy plants	0 a	

^z Within columns, means followed by the same letters are not significantly different ($P=0.05$) according to S.N.K. test

Simultaneous application of bio-fungicides and pathogen spores to olive plants has significantly reduced disease incidence. In fact, olive plants treated with Biobac, Sanbio Planta and Sambio Epsomit have had a disease incidence (20%) similar to which obtained on plants treated chemically with Uniform. Also, in this experiment, product efficacy exceeded 70% and it ranged from 70.58% for Dalgin active to 76.47% for the other products (**Table 5**)

By applying bio-fungicides one week before inoculation with the mixture of the used pathogens, disease incidence was low for all bio-fungicides treatments, and statistical analysis classified plants treated with Biobac or with Sanbio Epsomit as healthy plants (control) with a disease incidence equal to zero. Results obtained show that disease incidence has never exceeded 15% and it reached 0% with the bio-fungicides Biobac and Sanbio Epsomit (**Table 5**). The two other bio-fungicides (Dalgin active and Sanbio Planta) have also significantly reduced disease incidence when compared with control. Moreover, olive plants treated one week before inoculation showed healthy appearance and an optimal vegetative growth compared to inoculated plants. For example, **Figure 3** illustrates the comparison between a plant treated with Biobac and another plant, inoculated and untreated.



In in vivo control tests against *V. dahliae* isolated from olive trees, Triki *et al.* (2012) showed that the use of garlic extracts at the amount of 500 ml per plant, three days before or after inoculation of young olive trees (Var. Chemlali), led to a significant reduction of the disease impact in the two plots that are treated preventively and curatively by garlic extract compared to the untreated control. Indeed reductions of about 50% and 25% were recorded in the case of preventive and curative treatments respectively. However, severe unilateral wilting was observed in untreated plants.

Currently and after the development of bio-fungicides, some of them were the subject of a control test against pathogens causing dieback of olive trees in Egypt. Indeed, El-Morsi and Mahdy (2013) and after isolation of the main pathogens responsible of olive tree dieback in Egypt including *F. oxysporum*, *F. solani*, *R. solani*, *F. moniliforme*, *F. equiseti* and *Macrophomina phaseolina*, they tested 7 bio-fungicides to control these pathogens. The used bio-fungicides are composed of plant extracts, antagonistic bacteria or humus. The obtained results show that all the tested bio-fungicides have significantly reduced the disease incidence compared to the untreated control and the most encouraging results were obtained with humic acid, Inicium Fulvital and Bio-health where disease incidence has not exceeded 15% and the protection is always above 80%.

The efficacy of the use of bio-fungicides can be explained by their ability in enhancing natural resistance against plant diseases and pests, stimulated plant growth through increased cell division, as well as optimized uptake of nutrients and water. Also, soil microorganisms play a role in reducing root rot

diseases (Chen and Aviad 2004, Scheuerell and Mahaffee, 2004, Noble and Coventry 2005; El-Morsi *et al.*, 2009 and Kamble *et al.*, 2012).

In our study, the bio-fungicide Biobac has greatly reduced disease incidence and this positive action may be due to its composition (*Bacillus subtilis*) an antagonistic bacteria able to maintain contact with plants and promote their growth (Sivasakthi *et al.*, 2014).

4. Conclusion

After their successful establishment in the field, young olive trees are subject to attack by several soil-borne pathogens, causing severe losses in new orchards in the central regions of Tunisia. Common symptomatology observed includes a sudden wilting and death of young trees or a slower wilting, dieback and death that is usually accompanied by defoliation

Survey of root rot and wilt disease complex in the three governorates (Sidi Bouzid, Kairouan and Kasserine) during two successive seasons (2013 and 2014), indicated that these trees showed an extensive wilting of leaves and branches without defoliation, together with a dark brown xylem necrosis. Frequency of infected plants in the region of Kairouan exceeded 20%, this high value of disease occurrence may be attributed to warm and dry conditions in this governorate as well as the use of intercalary cultures without using the correct, strict sanitation methods and preventive therapeutic control measures. Isolation made from infected plants show that several pathogens are responsible of these symptoms especially *Fusarium oxysporum*, *F. solani*, *Verticillium dahlia*, *Rhizoctonia solani*, and *Pythium* sp. Results of control tests demonstrated that all the tested substances significantly reduced disease incidence. The efficacy of the tested bio-fungicides was important when these products were applied one week before inoculation with pathogens especially by using Sanbio Epsomit or Biobac where efficacy was 100%.

In conclusion, results of the present study could suggest that an earlier treatment (in the nursery) with one of the tested bio-fungicides can significantly reduce the occurrence of these symptoms to the field.

5. References

- Abdel-Monaim, M. F. EL-Morsi, M. E. A., Hassan, M. A. E. 2014.** Control of root rot and wilt disease complex of some evergreen fruit transplants by using plant growth promoting rhizobacteria in the New Valley Governorate, *Egypt. Journal of Phytopathology and Pest Management* 1: 23-33.
- FAO Stat, 2013.** <http://faostat.fao.org/>.
- Barnett, H.L. and Hunter, B.B. 1986.** Illustrated Genera of Imperfect Fungi. 4th ed. Macmillan Publishing Co., New York. 218 pp.
- Barreto, D., Babbitt, S., Gally, M. and Perez, B.A. 2003.** *Nectria haematococca* causing root rot in olive greenhouse plants. *INTA, Argentina, RIA* 23: 49-55.
- Ben Salem, I., Boughalleb, N., Souli, M., Selmi, S., and Romdhani, M. E. 2011.** Fungitoxicity of some fungicides against to pathogens responsible of olive trees decline in the Chebika's area in Tunisia. *Research in Plant Biology*, 1: 30-39
- Booth, C. 1977.** *Fusarium Laboratory Guide to the Identification of the Major Species.* Commonwealth Mycological Institute, Kew, Surrey, England. 58 pp.
- Boughalleb-M'Hamdi, N., Souli, M., Ben Salem, I., Selmi, S. and Romdhani, M.E. 2011.** Screening of fungi implicated in the dieback of olive trees (*Olea europea*) in Chebika's area *Research in Plant Biology*, 1:33-38.
- Boulila, M. and Mahjoub, M. 1994.** Inventaire des maladies de l'olivier en Tunisie. *Bulletin OEPP* 24 : 817-823.
- Boulila, M., Mahjoub, M. and Chaieb, M. 1995.** Synthèse de quatre années de recherches sur le dépérissement de l'olivier en Tunisie. 9ème consultation du réseau coopératif inter-régional de recherche sur l'olivier (20-23 septembre 1995) Hammamet (Tunisie). *Olea* 23, pp: 112. (Abstract).
- Boulila, M. 2001.** Maladies de l'Olivier en Tunisie : connaissances actuelles. *Olivae*, 85 :22-25.
- Chen, Y., De Nobili, M. and Aviad, T. 2004.** Stimulatory effect of humic substances on plant growth. In (Magdoff F., Weil R.R. Eds) "Soil organic matter in sustainable agriculture". pp: 103-130. Press: Boca Raton, FL.
- El-Morsi, M. E. A. and Mahdy, H. E. 2013.** Efficacy of some organic compounds against root rot wilt diseases of olive transplants and their growth response in New Valley Governorate, *Egypt. J. Agric. Res.* 91: 389-401.
- El-Morsi, M.E.A., Hassan M.A.E., Abo-Rehab, M.E.A and Radwan, F.M. 2009.** Incidence of root-rot and wilt disease complex of olive trees in New Valley Governorate in Egypt and its control. *Assiut J. Agric. Sci.* 40: 105-123.

- Hmouni, A., Hajlaoui, M. R., and Mlaiki, A. 1996.** Résistance de *Botrytis cinerea* aux benzimidazoles et aux dicarboximides dans les cultures abritées de tomate en Tunisie. *Bulletin OEPP* 26: 697-705.
- Kamble, S.M, Rokde, A.U. and Chavan, A. M. 2012.** Antifungal activity of algal extracts against plant pathogenic fungi. *International Multidisciplinary Research Journal*, 2:23-24.
- Leslie, J.F. and Summerell, B.A. 2006.** *Fusarium laboratory manual*. Blackwell Publishing. www.blackwellprofessional.com. SB741.F9L47 2006, 388p.
- Mousa, M.S., Ali, M.K. Mousa, A.A. and Elewa, I.S. 2006.** Root rot disease of olive transplants and its biological control. *Arab Universities Journal of Agricultural Sciences* 14: 395-409.
- Noble, R. and Coventry. E. 2005.** Suppression of soil-borne plant diseases with composts. *Biocontrol Science and Technology* 15: 3-20.
- Radwan, F. M., Hilal, A.A. and El-Said, M.E. 1995.** Basal stem and root rots of olive cuttings in rooting medium under mist propagation and their chemical and biological control. *Zagazig J. Agric. Res. (Egypt)* 22: 975-989.
- Sanchez Hernandez, M.E., Ruiz Davila, A., Perez de Algaba, A., Blanco Lopez, M.A. and Trapero Casas, A. 1998.** Occurrence and etiology of death of young olive trees in southern Spain. *European Journal of Plant Pathology* 104: 347-357.
- Scheuerell, S.J. and Mahaffee, W.H. 2004.** Compost tea as a container medium drench for suppressing seedling damping-off caused by *Pythium ultimum*. *Phytopathology* 94: 1156-1163.
- Sergeeva, V., Tesoriero, L., Spooner-Hart, R. and Nair, N. 2005.** First report of *Macrophomina phaseolina* on olives (*Olea europaea*) in Australia. *Australasian Plant Pathol.* 34: 273-274.
- Sivasakthi, S., Usharani, G. and Saranraj, P. 2014.** Biocontrol Potentiality of Plant Growth Promoting Bacteria (PGPR) *Pseudomonas fluorescens* and *Bacillus subtilis*: A review. *African Journal of Agricultural Research* 9: 1265-1277.
- Triki, M. A., Krichen, W., Hammemi Mallouli, I., Samira, K., Cheffi, M., Aouissaoui, H., Ikram, J., Drira, N. and Hassaïri, A. 2012.** Activité antifongique de l'extrait d'ail vis-à-vis de quelques champignons isolés d'oliviers en déperissement. *Revue Ezzaitouna* 13:1-11.
- Triki, M. A., Hassaïri, A. and Mahjoub, M.. 2006.** Premières observations de *Verticillium dahliae* sur olivier en Tunisie. *Bulletin OEPP* 36: 69-71.
- Sanei, S. J. and Razavi, S. E. 2012.** Survey of Olive Fungal Disease in North of Iran. *Annual Review & Research in Biology* 2: 27-36.